Assay Designs for Immobilization of His-tagged Proteins

Summary

An alternative to direct covalent immobilization of a ligand is its capture via an affinity tag. Three methods for immobilization of a His-tagged protein were evaluated using the SKi Pro-X10 instrument: binding to the NTA-Ni sensor surface, capture with an anti-His-Tag antibody, and capture with the same antibody followed by covalent cross-linking of the captured protein. The results show that all three approaches resulted in binding surfaces suitable for kinetic characterization of protein-protein interaction.

Introduction

A number of options exist for immobilization of affinity tagged proteins on a SKi Sensor surface for the purpose of monitoring binding interactions. Immobilization may be carried out by using succinimide ester chemistry to create linkages between the lysine residues of the protein and the carboxyl sensor surface. This is a straightforward and often effective technique. For some applications, however, it may be better to avoid modifying the protein's lysine groups in order to retain activity. In other cases an oriented immobilization may be preferred.

The polyhistidine epitope is a useful affinity tag due to its high affinity interaction with nickel ion, allowing for purification of the His-tagged protein in chromatography applications. A similar technique may be adapted using a nickel-charged nitrilotriacetic acid (NTA-Ni) SKi Sensor chip to capture a His-tagged protein for binding tests. Another alternative is to immobilize an anti-affinity tag antibody by which to capture the tagged protein.

With these non-covalent capture routes, the stability of the protein surface will depend on the strength of the specific interaction between the protein and the capture molecule (*i.e.* nickel ion, or antibody), and this must be verified on a case-by-case basis. In some cases, a covalent stabilization of the captured species may increase the stability of the protein surface without adversely affecting its binding activity.

In this work, hexahistidine tagged green fluorescent protein (6xHis-GFP) was captured via three routes: the NTA-Ni sensor surface, an anti-6xHis antibody, and the same antibody with cross-linking of the captured 6xHis-GFP. Each GFP surface was evaluated for specific binding with an anti-GFP antibody.

Method

All experiments were carried out using a Silicon Kinetics SKi Pro-X10 flow cell instrument. Carboxyl and NTA flow cartridges as well as carboxyl coupling reagents 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and sulfo N-hydroxy succinimide (sulfo-NHS) were from Silicon Kinetics. In each of the three cases described below, a hexahistidine-tagged green fluorescent protein surface (6xHis-GFP, Biovision, 4999-100) was generated and binding interactions with an anti-green fluorescent protein antibody (anti-GFP, Roche, 11814460001) was monitored. An antihexahistidine tag antibody was obtained from Rockland Immunochemicals (anti-6xHis epitope tag rabbit antibody, 600-401-382). Other buffers and reagents were from Sigma-Aldrich, J.T. Baker, and Thermo Scientific.

NTA-Ni Sensor Surface

An NTA sensor chip was equilibrated for 10 minutes in HBS buffer (20 mM HEPES, 150 mM NaCl, pH 7.0) with 10 mM imidizole, then treated with 250 mM

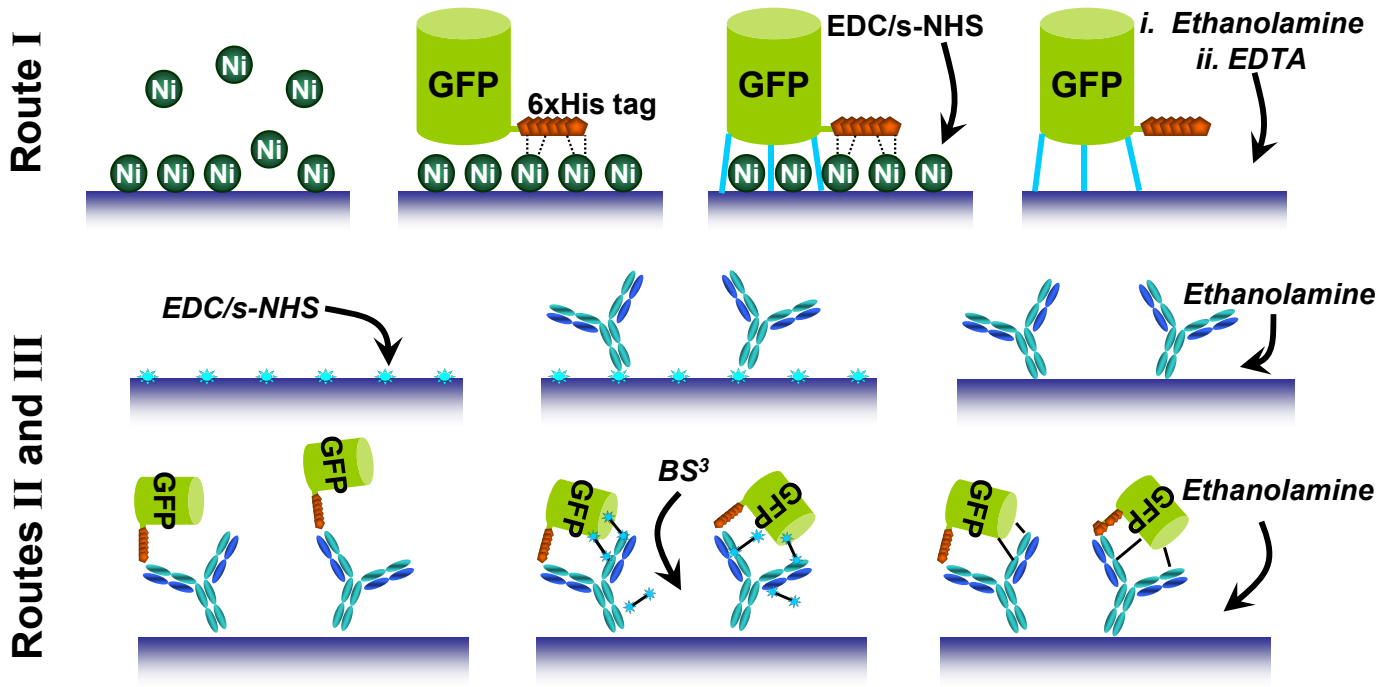

Figure 1: Capture of 6xHis-GFP on NTA-Ni surface (**Route 1**) and anti-6xHis surface (**Routes 2 and 3**). **Route 1**: NTA sensor surface is loaded with Ni^{2+}. GFP is captured via interaction between its hexahistidine tag and the nickel surface. Then surface carboxyl groups are activated using EDC/sulfo-NHS mixture to form succinimide esters; GFP is stabilized via covalent linking between succinimide esters and primary amines on the protein. Unreacted succinimide esters are blocked by reacting with ethanolamine (*i*). Nickel is eluted with EDTA. Any non-covalently linked protein is also eluted (*ii*). Surface is now ready for monitoring specific binding with green fluorescent protein antibody. **Route 2**: Carboxyl sensor surface is activated using EDC/sulfo-NHS mixture to form succinimide esters. Anti-His tag antibody is immobilized via covalent linking between activated succinimide esters and primary amine groups on the antibody. Unreacted succinimide esters are blocked by reacting with ethanolamine. Green fluorescent protein is captured via interaction between the His-tag and the anti- His-tag antibody. Surface is now ready for monitoring specific binding with green fluorescent protein antibody. Applying acidic regeneration conditions after binding elutes the GFP, necessitating repetition of capture step prior to another binding cycle. **Route 3**: GFP is captured on the anti-His tag antibody surface as described above. *bis*(sulfosuccinimidyl) suberate (BS^3) is used to cross-link primary amines between the antibody and protein. Unreacted BS^3 cross-linker is quenched with ethanolamine. The GFP surface is now ready for monitoring specific binding with green fluorescent protein antibody. Subsequent regeneration under acidic conditions elutes bound anti-GFP without removing the GFP.

$NiSO_4$ for 10 minutes. A solution of 5 μM 6xHis-GFP in HBS was applied to the sample channel only of the NTA-Ni chip for 7.5 minutes. Covalent stabilization of the 6xHis-GFP surface was carried out by applying 100 mM EDC and 25 mM sulfo-NHS for 3 minutes, followed by 1 M ethanolamine, pH 8.5, for 10 minutes. Noncovalently bound 6xHis-GFP was eluted with 280 mM EDTA. Two 3-minute injections of 20 mM glycine, pH 2.0, were done to further verify stability of the surface. Binding of anti-GFP was carried out at concentrations from 0.4 nM to 300 nM in HBS buffer with 0.05 % bovine serum albumin (BSA). Anti-GFP was applied for 3 minutes, followed by an 8.5-minute dissociation period in buffer and a 3-minute regeneration period in 20 mM Glycine, pH 2.0.

Anti-His-tag antibody sensor surface

A carboxyl sensor chip surface was equilibrated in deionized water, activated with a 10-minute injection of EDC/sulfo-NHS mixture at 100/25 mM concentration. Anti-6xHis antibody was diluted to 0.67 μM in 20 mM sodium acetate, pH 5.0. This solution was applied to both sample and reference channels for 12 minutes. 1 M ethanolamine (pH 8.5) was injected for 10 minutes in order to quench remaining active sites. Chip was re-equilibrated in PBS (100 mM phosphate, 150 mM NaCl, pH 7.2) with 0.5% BSA. 6xHis-GFP was diluted to 1.33 μM in PBS with 0.5% BSA and applied to the sample channel for 15 minutes. The resulting 6xHis-GFP surface was monitored for 1 hour to verify stability

and allow dissociation of loosely-bound species.

Binding of anti-GFP was carried out at concentrations from 0.3 nM to 300 nM in PBS buffer with 0.5% BSA. Anti-GFP was applied for 3 minutes, followed by a 10 minute dissociation and a 4-minute regeneration with in 20 mM glycine, pH 2.0. After each regeneration cycle, 6xHis-GFP was recaptured as described and monitored for 20 minutes to verify stability.

Anti-His-tag antibody surface with cross-linking

Anti-6xHis was immobilized on a carboxyl sensor chip as described above. 6xHis-GFP was applied to the sample channel for 15 minutes at 1.0 μM in PBS.

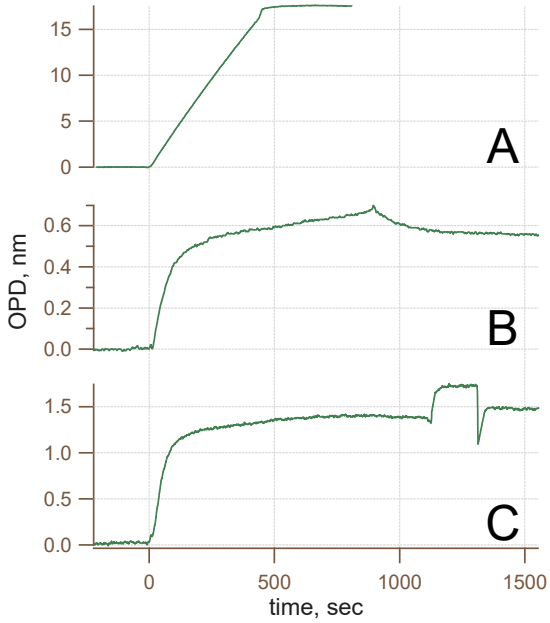

Figure 2: **A**: 6xHis-GFP is captured on NTA-Ni surface. Binding surface was further stabilized by EDC/sulfo-NHS injection as described in Methods (data not shown). **B**: 6xHis-GFP is captured on anti-His-tag surface (capture was repeated following each binding/regeneration cycle). **C**: 6xHis-GFP is captured on anti-His-tag surface with subsequent BS^3 cross-linking.

To cross-link the 6xHis-GFP to the anti-6xHis thus stabilizing the surface, a 5 mM solution of Bis(sulfosuccinimidyl) suberate (BS3) in 25 mM PBS was applied for 3 minutes. This was followed by a 10-minute injection of 1 M ethanolamine to quench unreacted BS^3. Binding of anti-GFP was carried out at concentrations from 0.6 nM to 300 nM in PBS buffer with 0.05% BSA. Anti-GFP was applied for 3 minutes, followed by an 8.5-minute dissociation period in buffer and a 4-minute regeneration with 20 mM glycine, pH 2.0. No recapture of 6xHis-GFP was required for subsequent binding steps, as the acidic regeneration conditions

All data were analyzed with SKi Report software. Referenced and blanked binding-dissociation curves were fit globally with a two-state binding kinetics model.

Results

Sensorgrams in Figure 2 illustrate different methods utilized to immobilize 6xHis-GFP. Capture on an NTANi surface (panel A) leads to high immobilization level.

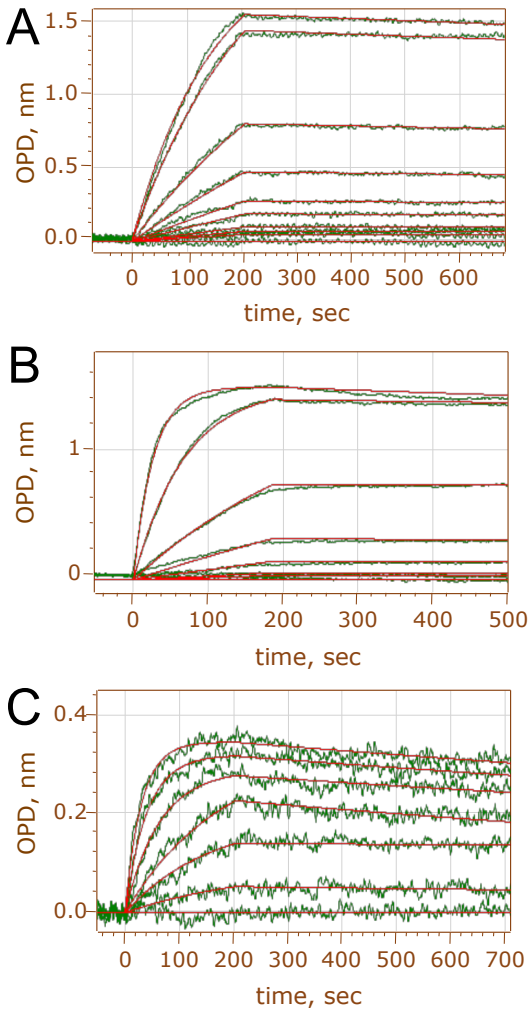

Figure 3: **A**: Anti-GFP antibody binding to captured 6xHis-GFP. **A**: 6xHis-GFP was captured on NTA-Ni surface; anti-GFP binding was carried out in 0.4âĂŞ300 nM range. **B**: 6xHis-GFP was captured on anti-6xHis surface; anti-GFP binding was at carried out in 0.3âĂŞ300 nM range. **C**: 6xHis-GFP was captured on anti-6xHis surface with subsequent crosslinking; anti-GFP binding was carried out in the 0.6–300 nM range.

Table 1: Results of 6xHis-GFP immobilization and anti-GFP antibody binding experiments.

Capture method	6xHis-GFP, nm	Max, anti-GFP, nm	k_{on}, $M^{-1}s^{-1}$	k_{off}, s^{-1}	K_D nM
NTA-Ni	17.5	1.56	2.23×10^4	$9.33 \times 10^{-}5$	4.2
Anti-6x His	0.65	1.52	1.20×10^5	$1.50 \times 10^{-}4$	1.3
NTA-Ni	1.49	0.36	1.31×10^5	$3.08 \times 10^{-}4$	2.34

Preliminary experiments indicated that 6xHis-GFP dissociates too quickly from NTA-Ni surface for accurate downstream measurements of its interaction with target molecules. Therefore, 6xHis-GFP capture on the NTA-Ni chip was followed by covalent cross-linking of the captured protein to the residual carboxyl groups (data not shown).

Alternatively, the protein was captured with an anti-His-tag antibody (panel **B**). The binding-dissociation curve is typical for a polyclonal antibody and shows considerable heterogeneity of interaction.

To prevent dissociation of the target protein from antibody and its complete loss at regeneration steps, the antibody-protein complex was stabilized by chemical cross-linking (panel **C**). In this case the binding surface is stable and can be regenerated several times.

The binding surfaces prepared as described above were used to characterize interaction of captured GFP with an anti-GFP antibody. The results of automated multi-concentration runs are shown in Figure 3 and kinetic data is summarized in the Table 1.

Discussion

All three methods of target protein immobilization yielded binding surfaces suitable for interaction analysis. The measured binding parameters are similar and show binding affinity in low nanomolar range that is typical for an analytical monoclonal antibody.

Capture of 6xHis-GFP on NTA-Ni chip was fast but the resulting binding surface was not stable enough for further interaction analysis. Additional covalent crosslinking of the captured protein to the residual carboxyl groups did affect the binding capacity of GFP to the anti-GFP antibody. Antibody binding curves to the large 17.5 nm GFP surface were far from saturation and obtained kinetic parameters could have been affected by mass transfer limitation.

6xHis-GFP capture with an anti-His-tag antibody provided an optimal surface for the anti-GFP antibody binding. However, it was necessary to recapture 6xHis-GFP after every binding-dissociation cycle. Cross-linking 6xHis-GFP to the anti-His-tag antibody allowed regeneration of the binding surface without stripping of the target protein. The number of binding sites available for the anti-GFP antibody significantly diminished after cross-linking. This indicates that BS3 treatment could modify amino acid residues involved in antibody binding. Nevertheless, the resulting stable binding surface was sufficient for kinetic characterization of GFP interaction with the antibody.

Taken together, these results show that His-tagged proteins can be immobilized on SKi Biosensor surfaces in a number of different ways for subsequent interaction analysis. The choice of immobilization method provides additional means for assay optimization depending on characteristics of the binding pair and project goals.

Silicon Kinetics, Inc.
9853 Pacific Center Ct. Suite O
San Diego, CA 92121-4339
www.siliconkinetics.com
+1[858]275-2842 (tel)
+1[858]630-4976 (fax)
info@siliconkinetics.com

August 7, 2016 13.02.011512