Published in final edited form as:

Methods Mol Biol. 2010 ; 627: 91–100. doi:10.1007/978-1-60761-670-2_5.

A capture coupling method for the covalent immobilization of hexahistidine tagged proteins for surface plasmon resonance

Adam J. Kimple^{a,*}, Robin E. Muller^a, David P. Siderovski^{a,b}, and Francis S. Willard^a

^a Department of Pharmacology, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7365 USA

^b UNC Neuroscience Center and Lineberger Comprehensive Cancer Center, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7365 USA

i. Summary

Surface Plasmon Resonance (SPR) is a robust method to detect and quantify macromolecular interactions; however, to measure binding interactions, one component must be immobilized on a sensor surface. This is typically achieved using covalent immobilization via free amines or thiols, or noncovalent immobilization using high affinity interactions such as biotin/streptavidin or antibody/antigen. In this Chapter we describe a robust method to covalently immobilize His₆ fusion proteins on the sensor surface for SPR analysis.

Keywords

Biacore; SPR; Capture Coupling; Immobilization; Hexahistidine; His-tag

1. Introduction

Detection of protein-protein or protein-ligand interactions using surface plasmon resonance (SPR) is possible because the refractive index of a gold/fluid interface fluctuates as molecules interact with the surface and change the apparent molecular mass of the surface (1,2). This change in the refractive index is directly related to the total mass of interacting molecules at the surface; thus, by immobilizing a protein analyte on the chip surface using high affinity interactions (*i.e.* antibody capture or Streptavidin/Biotin (1–3)) or covalently using amine coupling (4,5), one can directly observe the binding of a ligand (protein or small molecule) to the immobilized component. The kinetic nature of these measurements allows for real-time observation and quantification of molecular interactions. This data can be used to determine on and off rates and affinity constants. Once protocols are established for immobilizing a particular analyte on a sensor surface, determination of binding affinities and screening for interaction partners can be performed in an automated manner for tens to hundreds of ligands per day (6); however, in our experience, identifying an optimal way to

*Corresponding author: Adam Kimple, University of North Carolina Chapel Hill, Department of Pharmacology, Chapel Hill, NC 27599, kimplead@med.unc.edu, Phone: 919-843-9364, Fax: 919-966-5640.

⁷An analogous method to the one presented in this chapter has been described in (15) although, in this particular method, amine coupling reagents are added after NTA immobilization has occurred. We have tested this method with His₆-Gβγ subunits and found it ineffective in generating a functional surface.

⁸To increase the probability of experimental success, it is critical to use a homogenous protein sample. We highly recommend a multiple step chromatographic approach starting with affinity purification and ending with gel exclusion chromatography to ensure that the protein samples are homogenous and non-aggregated. For more information regarding protein purification, we recommend the Protein Purification Handbook which can be downloaded for free from the GE Healthcare website under product number 18-1142-75.

immobilize protein analytes resulting in a stable surface with high binding capacity can be tedious.

The availability of commercial nitrilotriacetic acid (NTA) sensor chips allows for repeated immobilization, stripping, and regeneration of His₆-tagged proteins; however, this method of immobilization has multiple deficiencies that prevent its wide-spread use. Firstly, while the NTA/His₆ interaction is robust, slow and continuous dissociation of immobilized components is common (7–9). The decaying surface can be corrected *post-hoc* using computational methods (10); however, this solution is less than ideal and would prove daunting even to aficionados of SPR. The second commonly encountered problem with NTA sensor chips is the idiosyncratic drift of flow cells (*see* Note 1). If equivalent drifts occur across all flow cells, double referencing can be used to correct for systematic deviations across all flow cells (11); however, we have frequently encountered deviations that only occur on individual flow cells which cannot be corrected for using this method. While this problem is often attributed to contaminating cation interaction with the sensor surface, in our hands it is often found to be random and apparently independent of routine maintenance or buffer composition and can sometimes be resolved by simply redocking a chip or using a new sensor chip. We have developed a capture coupling method for His₆ fusion proteins that results in the capture of a His₆-fusion protein in a non-random orientation by the His₆ epitope prior to direct amine coupling. This method, referred to as “capture coupling”, is superior to Ni²⁺-NTA/His₆ or amine coupling alone because there is no loss of surface over time and the protein is immobilized initially by the His₆-tag. This method more reproducibly generates a high specific activity, functional SPR surface. We have used this approach to investigate protein-protein interactions in heterotrimeric G-protein biology (2, 9) (*see* Note 2) and other groups have also used our method to look at a diverse array of protein-protein interactions. (12–14).

2. Materials

2.1 Buffers

1. Running Buffer: 10 mM HEPES pH 7.4, 150 mM NaCl, 50 μ M EDTA, 0.005% (v/v) NP-40. NP-40 is no longer commercially available; thus, we use “NP-40 Alternative” available from Calbiochem (La Jolla, CA) or Surfactant P20 available from GE Healthcare.
2. Dispenser Buffer: 10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% (v/v) NP-40.
3. Regeneration Buffer: Running Buffer containing 350 mM EDTA
4. Nickel Sulfate Solution: Running buffer containing 500 μ M NiSO₄.

N.B.: The addition of guanine nucleotides, MgCl₂, NaF, and AlCl₃ are specific to the application of this method to studying heterotrimeric G α subunits and are not needed for the study of other protein families.

¹The capture coupling method of immobilizing fusion proteins eliminates the possibility of the immobilized component dissociating from the sensor chip surface; however, it is not uncommon to encounter drifting baselines that are not consistent between flow cells. These are idiosyncratic and are difficult to correct. One method we use to account for buffer shifts is to use multiple negative controls for background subtraction. We often use a flow cell with no protein attached that has been treated with a 20 μ l injection of regeneration buffer at 20 μ l/min. If experimental conditions allow, it is also helpful to have an additional negative control flow cell with a His₆-fusion protein that will not interact with the analyte (*see* Fig. 3 for representative data).

²In this chapter, we describe the use of this method for the immobilization of a novel RGS protein (Fig. 2 and Fig. 3). Similarly, we have used this method to immobilize G α_q (2) and G $\beta\gamma$ subunits (2, 9). We anticipate that this method will be applicable to the analysis a wide variety of molecular interactions. Other representative examples of the use of this methodology from other groups include refs. (13,14)

5. Ground State Running Buffer: Running Buffer with an additional 100 μ M GDP
6. Active State Running Buffer: Running Buffer with 5 mM MgCl_2 and 100 μ M $\text{GTP}\gamma\text{S}$
7. Transition State Running Buffer: Running Buffer with 5 mM MgCl_2 , 100 μ M GDP, 20 mM NaF, 30 μ M AlCl_3 .

2.2 Biacore 2000 Supplies

1. NTA Sensor Chip (GE Healthcare)
2. Amine Coupling Kit (GE Healthcare) (*see* Note 3)

3. Methods

1. Twenty-four hours prior to use, run a DESORB and “Super Clean” (*see* Note 4) and inspect pumps for corrosion (*see* Note 5).
2. Prepare all buffers fresh and filter using bottle top or syringe filters (0.22 μ m or smaller pore size). If using syringe filters, be sure to degas all buffers as microbubbles accumulate along the fluidics and can be deleterious to the sensor chip surface.
3. Undock maintenance chip and dock new NTA Sensor Chip.
4. Prime with the right pump in dispenser buffer and the left pump in running buffer. To verify which tube is connected to each pump, the panel that separates the rack area from the buffer storage area can be removed by undoing 2 thumb screws. Follow each tube to the originating pump (*see* figure 1 for details).
5. Start a new sensorgram.
6. Direct flow path to a single flow cell with a flow rate of 20 μ l/min.
7. Inject 20 μ l of Regeneration Buffer using the Extraclean feature.
8. Wash Needle (in addition to the Extraclean injection in step #7)
9. Inject 40 μ l of Nickel Sulfate Solution using the Extraclean feature
10. Wash Needle
11. Set flow rate to 5 μ l/min

³We typically purchase an Amine Coupling Kit from GE Healthcare (GE Healthcare, BR-1000-50) and make separate 100 μ l aliquots of NHS and EDC. These aliquots must be stored at -20°C . If poor covalent coupling is observed, it is likely that the NHS/EDC reagents have lost activity.

⁴In addition to regularly performing the DESORB procedure, we also recommend performing a “Super Clean” procedure before performing capture coupling. This automated procedure can be accessed under the “Tools” menu, “Service Tools” option and then selecting “Super Clean.” This procedure will require 4 ml aliquots of H_2O , 1% (v/v) Acetic Acid, 0.2 M NaHCO_3 , 6M Guanidine HCl, and 10 mM HCl. It is essential to filter and degas all solutions.

⁵In the past several years, we have observed a corrosive build-up on the shaft of the Hamilton syringe pumps. We recommend an extensive visual inspection before performing any experiment but this is of special importance when conducting covalent capture techniques employing NTA-based immobilization. If rust/oxidation is observed, we recommend replacing the syringe (GE Healthcare, BR-1000-84). The new syringe can be easily replaced by the user by following the instruction found under the “Tools” menu, “Service Tools” option and then selecting “Syringe/Tip (Right/Left).” Standard cleaning procedures such as Desorb and Super Clean do not vigorously clean the syringes, so we recommend performing a Sanitize procedure several times a year using the Sanitize command in the “Working Tools” menu. The Sanitize procedure will require BIADisinfectant solution (1% sodium hypochlorite) which can be purchased as part of a Biacore Maintenance Kit (GE Healthcare, BR-1006-66, BR-1006-67, BR-1006-51).

12. Make up 100 μ l of coupling solution (a 1:1 (v/v) mixture of NHS/EDC N-hydroxysuccinimide/N-ethyl-N'-(3-diethylaminopropyl)carbodiimide (included in the Amine Coupling Kit)).
13. Inject 30 μ l of coupling solution
14. Dilute His₆-Fusion protein in running buffer (*see* Note 6)
15. Inject 66 μ l of the His₆-Fusion protein.
16. Inject 35 μ l of 1M ethanolamine (included in the Amine Coupling Kit).
17. Set flow rate to 20 μ l/min
18. Inject 20 μ l of Regeneration Buffer using the Extraclean feature
19. Repeat 6–16 to load as many flow cells as desired.
20. Start a new sensogram over all flow cells to be used with both inlets in the appropriate running buffer.
21. Once the immobilized protein surface has been generated, multiple rounds of analyte injection can be automated as follows:

N.B.: comments located after “//” should not be included

```
! ----
! This method was generated from... //stamp indicating this program was
! Timestamp: 03-Nov-08 14:28 //created from the command queue
! Software: BIACORE 3000 ...
! Version: 3.2
! Configuration: IFC6
! ----
DEFINE APROG Command Queue //defining program name
FLOW 20 //setting flow to 20  $\mu$ l/min
INJECT R2A1 20 //injecting 20  $\mu$ l of regeneration buffer
EXTRACLEAN //with Extraclean (from position R2A1)
WASH n //washing needle
INJECT R2A2 40 //injecting 40  $\mu$ l of NiSO4 with
EXTRACLEAN //Extraclean from position R2A2
WASH n //washing needle
FLOW 5 //set flow to 5  $\mu$ l/min
INJECT R2A3 30 //injecting 30  $\mu$ l of NHS/EDC
INJECT R2A4 66 //injecting 66  $\mu$ l of His-fusion protein
INJECT R2A5 35 //injecting 35  $\mu$ l of ethanolamine
FLOW 20 //set flow to 20  $\mu$ l/min
INJECT R2A1 20 //inject regeneration buffer with
```

⁶We currently have not identified a quantitative method for determining the amount/concentration of protein to covalently couple to the sensor surface. To empirically determine a useful protein concentration, we start with a 50 μ l injection at a flow rate of 5 μ l/min using 1 μ M of the His₆ fusion protein. In general with His₆ capture coupling, we saturate the flow surface in the shortest amount of time possible; however, if excess protein is injected, a significant loss of protein from the surface will be observed when the injection is stopped and protein may bind through conventional amine coupling, thereby immobilizing protein in a random orientation. If too little protein is injected, a linear increase in protein loading will be occurring even when the injection ends. An additional consideration that must be noted is the likelihood of observing mass transport effects if excess protein loaded on the surface or flow rates are too slow (15–17).

```

EXTRACLEAN           //Extraclean
END
MAIN
  RACK 1 THERMO_C      // setting up racks
  RACK 2 THERMO_A
  RACK R REAG_A
  DETECTION 2          // Change 2 to any flow cell (1–4) to
  APROG Command Queue // perform this method on a different flow
  APPEND Standby       // cell
END                   // machine will be left in standby

```

After you have generated one flow cell using the command queue, go to the “File” menu in the command queue window and select “Generate Method.” This will save a *.blm file that generates a surface exactly as indicated in the command queue. This script can easily be edited to generate a surface on a different flow cell by manually changing the “DETECTION 2” command (in our example) to DETECTION X, where X is any flow cell you want to generate (flow cell 1–4). The reagents must be in the same positions that were used while using the command queue initially.

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Figure 1. Identification of Left and Right pump inlets on Biacore 3000

Loosen the two screws (white circles/arrows) on the panel to remove the cover. This allows the user to trace each inlet back to the right and left pump. The Biacore Running Buffer should be placed in the inlet corresponding to the left pump while the right pumps inlet should be submerged in Dispenser Buffer.

Figure 2. Loading – A typical Sensogram generated during the production of a single flow cell using the capture coupling method

The flow cell surface was stabilized by the injection of regeneration buffer (20 μ l Regeneration Buffer at 20 μ l/min) followed by binding of nickel to the surface (40 μ l Nickel Sulfate Solution at 20 μ l/min). The surface was then activated for primary amine coupling by the injection of coupling solution (30 μ l EDC/NHS at 5 μ l/min). His₆-G α fusion protein in pH 7.4 Running Buffer was then injected over the surface (66 μ l of 1 μ M His₆-G α at 5 μ l/min). To block uncoupled primary amines on the sensor surface, ethanolamine was injected over the surface (35 μ l ethanolamine at 5 μ l/min). To prevent further immobilization of His₆-fusion proteins and to remove non-covalently coupled His₆-G α , EDTA-containing Regeneration buffer was injected (20 μ l at 20 μ l/min).

Figure 3. Curves – Capture coupling was used to generate a sensor surface with a novel His₆-RGS protein

Four flow cells were prepared: FC1 (“REGEN FC”) - a blank surface that had 20 μ l of Regeneration Buffer injected over it; FC2 (“BLANK FC”) - a non-treated NTA surface that only had experimental injections; FC3 (“His₆-G α FC”) - ~6000 RUs of His₆-G α were immobilized; FC4- ~6000 RUs of a novel His₆-RGS protein. Varying concentrations of His₆-G α were injected over all four flow cells, in the presence of Transition State Running Buffer, using the KINJECT command (300 μ l injections with a 200 second dissociation phase at 20 μ l/min). Specific binding of the injected G α was only seen to His₆-RGS as would be expected (2); however, the qualitative appearance of the curves changed depending on the flow cell used to subtract non-specific binding and changes in the refractive index upon injection of buffers. **(A)** Specific binding was determined by subtracting non-specific binding to flow cell 1 (REGEN FC) that had 20 μ l of regeneration buffer injected over it prior to injections. **(B)** Non-specific binding was subtracted by using a flow cell loaded with a non-interacting protein immobilized on the surface (*i.e.*, G α does not dimerize). **(C)** An otherwise untreated flow cell (BLANK FC) was used to subtract non-specific binding and changes in refractive index. **(D)** Using equilibrium saturation analysis, dissociation constants (K_d values) for the G α /RGS protein-protein interaction were determined to be 469 (95% *C.I.* 426–512) nM, 218 (95% *C.I.* 149–288) nM, and 651 (95% *C.I.* 389–917) nM using background subtraction to a regenerated blank flow cell, an immobilized His₆-G α flow cell, and a blank flow cell, respectively.