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Surface Plasmon Resonance (SPR) Analysis of Binding Interactions of Proteins in Inner-Ear Sensory Epithelia

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Abstract

Surface plasmon resonance is an optical technique utilized for detecting molecular interactions. Binding of a mobile molecule (analyte) to a molecule immobilized on a thin metal film (ligand) changes the refractive index of the film. The angle of extinction of light, reflected after polarized light impinges upon the film, is altered, monitored as a change in detector position for the dip in reflected intensity (the surface plasmon resonance phenomenon). Because the method strictly detects mass, there is no need to label the interacting components, thus eliminating possible changes of their molecular properties. We have utilized surface plasmon resonance to study the interaction of proteins of hair cells.

Keywords

Surface plasmon resonance (SPR); hair cell; molecular cloning; protein-protein interaction; fusion polypeptides; interaction kinetics; sensor chip CM5; Biacore 3000

1. Introduction

Surface plasmon resonance (SPR) binding analysis methodology is used to study molecular interactions (1,2). SPR is an optical technique for detecting the interaction of two different molecules in which one is mobile and one is fixed on a thin gold film (1). In the work described here, affinity-purified fusion polypeptides are immobilized by an amine-coupling reaction on a sensor chip (Biacore, Piscataway, NJ, USA) inserted into the flow chamber of a Biacore 3000 instrument (Biacore, Uppsala, Sweden). Addition of a second polypeptide, the flow-through *analyte*, to the chamber, results in binding to the immobilized polypeptide *ligand*, producing a small change in refractive index at the gold surface (3), which can be quantified with precision (4). Binding affinities can be obtained from the ratio of rate constants, yielding a straightforward characterization of protein-protein interaction. SPR directly detects mass (concentration) with no need for special radioactive or fluorescent labeling of polypeptides (5) before measurement, presenting a great advantage in minimizing time and complexity of the studies.

2. Materials

2.1. Selection of Sensor Chip

1. A CM5 chip, research grade (cat. no. BR-1000-14, Biacore-GE Healthcare, Piscataway, NJ), is commonly used (*see* Note 1).

2.2. Production of Ligands and Analytes

1. All reagents are prepared with Milli-Q water. The protein/polypeptide, used as immobilized ligand or mobile analyte, is purified either directly from a tissue source or by recombinant technology with overexpression in bacterial cells (*see Section 3.2.4*). Ligand or analyte can be a fusion protein tagged with hexahistidine or glutathione-S-transferase (GST) (as an aid to purification), or can be a synthetic oligopeptide, 20–30 amino acids in length.
2. Mouse brain QUICK-clone cDNA (cat. no. 637301, BD Biosciences-Clontech, Palo Alto, CA, USA) or custom cDNA.
3. Expression vectors pRSET (Invitrogen, Carlsbad, CA, USA) or pGEX (GE Healthcare-Amersham, Piscataway, NJ, USA), for producing fusion proteins/polypeptides by in-frame cloning.
4. Restriction enzymes *Bam*H1, *Eco*R1, *Hind*3, *Bgl*2, *Not*I (Invitrogen).
5. QIAEX-II gel extraction kit (cat. no. 20021, Qiagen, Valencia, CA, USA).
6. *E. coli* Subcloning Efficiency™ DH5α™ cells (cat. no. 18265-017, Invitrogen).
7. One Shot Top 10 cells (cat. no. C4040-03, Invitrogen) for cloning and plasmid isolation.
8. *E. coli* BL 21 Star™ (DE3) cells (cat. no. C6010-03, Invitrogen) for protein expression.
9. LB liquid medium: 1% bacto-tryptone, 0.5% yeast extract, 1% NaCl, adjusted to pH 7.0 with 5 N NaOH (cat. no. 244620, BD Biosciences-Difco, San Jose, CA).
10. LB solid medium: 12 g/L agar in LB liquid medium. Autoclave.
11. S.O.C medium: 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose (cat. no. 15544-034, Invitrogen).
12. Wizard plus SV Miniprep Kit (cat. no. A1330, Promega, Madison, WI).
13. IPTG (isopropylthio-β-D-galactoside) stock solution: 100 mM in water. Sterilize by filtration and store at –20 °C.
14. Ampicillin stock solution: 100 mg/mL in water. Sterilize by filtration and store at –20 °C.
15. Protease Inhibitor Cocktail for use in polyhistidine tagged protein (cat. no. P8849, Sigma-Aldrich, St. Louis, MO).
16. Protease Inhibitor Cocktail for general use (cat. no. P2714, Sigma-Aldrich).
17. Pre-cast 4–12% gradient SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) gels (NuPAGE, cat. no. NP0321BOX, Invitrogen).
18. Electrophoresis running buffer (20X NuPAGE SDS Running Buffer, cat. no. NP0001, Invitrogen). Dilute buffer appropriately.

¹The Biacore CM5 chip is widely employed for SPR interaction studies. Carboxymethylated dextran molecules are attached to a gold-coated surface. The CM5 chip can detect nucleic acids, carbohydrates, and small molecules, in addition to proteins. The chip surface is prepared for binding studies by coupling ligand molecules to the carboxymethyl group via NH₂, -SH, -CHO, -OH or -COOH linkages. As alternatives to the CM5 chip, CM4, CM3 and C1 chips can be used, which have a lower matrix density of functional groups. Another chip, SA, can be used for immobilization of biotinylated peptides, proteins, nucleic acids, and carbohydrates. HPA and L1 chips are employed for lipid or liposome immobilization. NTA chips are utilized for capturing histidine-tagged molecules (7).

19. Coomassie Blue electrophoresis stain (Simply Blue™ Safe Stain, cat. no. LC6060, Invitrogen).
20. BenchMark™ pre-stained protein ladder (cat. no. 10748-010, Invitrogen).
21. Cell lysis buffer for hexahistidine fusion polypeptide purification: 8 *M* urea in 100 *mM* NaH₂PO₄, 10 *mM* Tris-HCl, pH 8. The wash buffer and elution buffers are the same as the cell lysis buffer, but with the pH adjusted to 6.3 and 4.5, respectively.
22. Nickel-nitrilotriacetic acid (Ni-NTA) spin columns for purification of hexahistidine-tagged proteins (Qiagen).
23. Sonication lysis buffer for protein purification: 1X PBS, 100 *mM* EDTA, 0.5 *mM* DTT, 1X Protease Inhibitor Cocktail for general use, pH 7.4 (Sigma-Aldrich).
24. Glutathione-Sepharose 4B beads for GST fusion tags (cat. no. 17-0756-01, GE-Amersham).
25. L-Glutathione, reduced (cat. no. G4251, Sigma-Aldrich).
26. DispoDialyzer (cat. no. Z368296-10EA, Sigma-Aldrich) or Slide-A-Lyzer Dialysis Cassettes (cat. no. 66107, Pierce, Rockford, IL).
27. Anti-X-Press HRP antibody (cat. no. R911-25, Invitrogen).
28. Anti-GST antibody (cat. no. G7781, Sigma-Aldrich).
29. Coomassie Plus-200 Protein Assay Reagent (cat. no. 23238, Pierce).
30. Quant-iT™ Protein Assay Kit for use with Qubit fluorometer (Invitrogen).
31. Phosphate-buffered saline (PBS): 0.01 *M* PO₄, 0.138 *M* NaCl, 0.0027 *M* KCl, pH 7.4 (cat. no. P3813, Sigma-Aldrich).

2.3. Ligand Immobilization

1. HEPES buffered saline-NaCl (HBS-N buffer): 0.15 *M* NaCl, 0.01 *M* HEPES, pH 7.4 (cat. no. BR-1003-69, Biacore).
2. HBS-P buffer: HBS-N with 0.005% v/v surfactant P20 (cat. no. BR-1003-68, Biacore).
3. HBS-EP buffer: HBS-N with 3 *mM* EDTA, 0.005% v/v P20 (cat. no. BR-1001-88, Biacore).
4. EDC amine coupling reagent: 0.4 *M* 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride in water (part of cat. no. BR-1000-50, Biacore).
5. Ethanolamine solution: 1.0 *M* ethanolamine-HCl, pH 8.5 (part of cat. no. BR-1000-50, Biacore).
6. NHS amine coupling reagent: 0.1 *M* N-hydroxysuccinimide in water (part of cat. no. BR-1000-50, Biacore).
7. Sodium acetate buffers for immobilization in the acidic range: 10 *mM* sodium acetate, pH 4.0, 4.5, 5.0, 5.5 (cat. no. BR-1003-49 through -52, Biacore).
8. Sodium tetraborate buffer for immobilization in the basic range: 10 *mM* sodium tetraborate, pH 8.5 (cat. no. BR-1003-53, Biacore).
9. 1.0 *M* NaCl solution.
10. Glycine-HCl solutions: 10 *mM*, pH 1.5, 2.0, 2.5, 3.0. Use for regeneration (cat. no. BR-1003-54 through -57, Biacore).

11. 50 mM NaOH solution. Use for regeneration (cat. no. BR-1003-58, Biacore).
12. BIAdesorb Solution 1: 0.5% SDS (cat. no. BR-1008-23, Biacore).
13. BIAdesorb Solution 2: 50 mM glycine, adjusted to pH 9.5 with 5 N NaOH (cat. no. BR-1008-23, Biacore).

2.4. Experimental Binding Measurements

1. HBS-N buffer.
2. HBS-P buffer.
3. HBS-EP buffer.
4. Phosphate -buffered saline with Tween (PBST): 10 mM PO₄, pH 7.5, 2.4 mM KCl, 138 mM NaCl, 0.05% Tween-20.
5. Nonspecific binding reducer (NSB): 10 mg/mL of carboxymethyl dextran sodium salt in 0.15 M NaCl, containing 0.02% sodium azide (cat. no. BR- 1006-91, Biacore).
6. Bovine serum albumin (BSA): 2 mg/mL (Pierce).

3. Methods

We perform surface plasmon resonance experiments with a Biacore 3000 instrument. The immobilization involves activation of carboxymethyl groups on a dextran-coated chip by reaction with N-hydroxysuccinimide, followed by covalent bonding of the ligands to the chip surface via amide linkages and blockage of excess activated carboxyls with ethanolamine (6). Reference surfaces are prepared in the same manner, except that all carboxyls are blocked and no ligand is added. During analysis, each cell with an immobilized fusion polypeptide is paired with an adjacent cell on the chip, the latter serving as a reference. The final concentration of bound ligand, expressed in response units (RU), is calculated by subtracting the reference RU from the ligand RU. HBS-N, HBS-P, HBS-EP, or PBST buffer may be used as both running and analyte-binding buffer. Purified fusion polypeptide or protein (analyte), typically at 100 nM, is allowed to flow over the immobilized-ligand surface and the binding response of analyte to ligand is recorded. The chip surface is regenerated by removal of analyte with a regeneration buffer. The maximum RU with each analyte indicates the level of interaction, and reflects comparative binding affinity.

3.1. Selection of Sensor Chip

1. Store the CM5 sensor chip at 4 °C. Bring the chip to room temperature just before the experiment.
2. At the analysis step, dock chip in the instrument. Prime with immobilization running buffer (HBS-N or HBS-EP). The priming process flushes the pumps, the integrated fluidic cartridge, autosampler, and flow cells of the sensor chip with the chosen running buffer. In the *Biacore 3000 Control* program, click the TOOLS tab and then select WORKING TOOLS. Place the running buffer in the buffer shelf, select PRIME, and click START

3.2. Production of Ligands and Analytes

3.2.1. Design of PCR Primers for Production of Insert

1. Design primers so that the target insert will remain in frame when ligated (by adding additional nucleotides to the primer sequence, if necessary).

2. Use a different restriction site for each member of the primer pair to support directional cloning.
3. Amplify desired cDNA message, e.g., from mouse brain cDNA, with the primer pair.
4. Purify the PCR product containing the desired restriction sites by electrophoresis in agarose gels, followed by band excision and sequencing (*see* Note 2).

3.2.2. Ligation of Insert into Plasmid Expression Vector

1. Digest the PCR product at 37 °C overnight with appropriate restriction enzymes corresponding to the restriction sites of the PCR product. If the buffer supplied for both restriction enzymes is the same, the two enzymes can be incubated simultaneously with the PCR product. Otherwise, perform the two incubations, as well as **step 2, Section 3.2.2**, for each enzyme consecutively.
2. Extract the digested product (insert) with phenol-chloroform and precipitate it in ethanol (*see* Note 2).
3. Digest, extract, and precipitate the expression vector (e.g., pRSET) in a manner similar to that used for the PCR product (*see* Note 3).
4. Measure the concentration of the vector and insert by gel analysis using a molecular mass standard of known quantity, by spectrophotometry at 260 nm, or by fluorescence with a Qubit fluorometer (*see* Note 4).
5. Perform a standard ligation reaction using T4 DNA ligase (Invitrogen Kit 15224-017). Use a 3:1 insert:vector ratio (*see* Note 5).

3.2.3. Transformation of Bacteria by Plasmid Expression Vector

1. Mix 1–2 µL of the vector ligation product with *E. coli* DH5α competent bacterial cells and incubate on ice for 30 min (2).
2. Heat-shock the cell mixture at 42 °C for 45 s.
3. Add 1 mL of S.O.C. medium and mix vigorously in an orbital shaker at 37 °C for 90 min.
4. Spread a 50- to 200-µL aliquot onto an LB agar plate (1.2% agar in LB liquid medium), containing a 75 µg/mL final concentration of ampicillin, and incubate at 37 °C overnight.

²If no non-specific PCR bands are present, the product can be purified by phenol-chloroform extraction and alcohol precipitation. Adjust the sample volume to 200 µL by adding deionized water. Add an equal volume of phenol-chloroform (50% phenol saturated with 0.1 M Tris-HCl, pH 7.6, 48% chloroform, 2% isoamyl alcohol) to the sample and vortex to mix. Centrifuge at 14,000 g for 5 min. Carefully pipette out the upper phase into a fresh tube and add an equal volume of chloroform, vortex, and centrifuge as before. To the supernatant, add 1/10 volume of 3 M sodium acetate, pH 4.5, and 2.5 volumes of ethanol. Incubate at –20 °C for 1 h. Centrifuge at 14,000 g for 10 min at room temperature. Remove the supernatant. Wash pellet in excess 70% ethanol, centrifuge as before, decant the supernatant, and dry pellet in air.

³The pRSET vector contains sequences for six contiguous histidine residues upstream of the multiple cloning site, and thus the his-tagged fusion protein expressed in bacteria can be purified using a nickel affinity column (Qiagen). Immediately adjacent and upstream to the multiple cloning site is an eight-amino-acid epitope against which antibodies are commercially available (Invitrogen), facilitating antibody-based detection. The pGEX expression vector can also be used (*see* Note 10).

⁴The Qubit fluorescence spectrometer (Invitrogen) is a convenient instrument for measurement of nucleic acid and protein concentration by fluorescence. In a 0.5 mL tube, pipette 190 µL of the Quant-iT working solution (1/200 dye in buffer; buffer is double-stranded DNA Broad-Range reagent). Add DNA sample (1–10 µL) and bring to a total volume of 200 µL with water. Vortex 2–3 s, incubate for 2 min, and read the fluorescence.

⁵Add, to an autoclaved 0.5-mL microcentrifuge tube, 4 µL of 5X ligase reaction buffer, 15–60 fmol vector DNA, 45–180 fmol insert DNA (0.1–1.0 µg total DNA), and 1 unit of T4 DNA ligase in 1 µL (Invitrogen). Bring the total volume to 20 µL, mix gently, and centrifuge briefly to collect the contents at the bottom of the tube. Incubate at 4 °C overnight.

5. Select 10 or more colonies from the plate and culture them in 3–10 mL of liquid LB medium containing 75 µg/mL of ampicillin overnight.
6. Pellet the cells by centrifugation at 4,000 g for 5 min.
7. Isolate plasmids with the Wizard plus SV Miniprep Kit.
8. Verify presence of insert by digestion with the two restriction enzymes that were used for cloning (*see* Note 6).

3.2.4. Protein Expression from Plasmid Expression Vector

1. Transform *E. coli* BL 21 cells (Invitrogen) with the expression vector construct as in **steps 1–3, Section 3.2.3**.
2. Spread cells onto LB agar plates, containing 75 µg/mL of ampicillin, and allow them to grow overnight.
3. Culture resulting colonies in LB liquid medium, containing 75 µg/mL of ampicillin for 8 h, or until the absorbance at 600 nm reaches an optical density (OD) reading of 0.3.
4. Add IPTG stock solution to yield a final concentration of 1 mM in autoclaved LB liquid medium.
5. Culture cells for another 3–4 h in the LB liquid medium.
6. Pellet cells at 4,000 g, add lysis buffer, vortex, and incubate at room temperature for 1 h.
7. Centrifuge at 18,000 g for 25 min and collect the clear supernatant.
8. Electrophoretically analyze 5 µL of the supernatant with a molecular mass standard in a pre-cast SDS-PAGE gel. Stain with Coomassie Blue.
9. Repeat **steps 1–8, Section 3.2.4**, using the expression vector not containing an insert, as a negative control.
10. As a further control, repeat **steps 1–8, Section 3.2.4**, with the omission of the inducer IPTG (*see* Note 7).

3.2.5. Purification of Protein (Ligand or Analyte) (*see* Note 8)

3.2.5.1. Ni-NTA Affinity Purification

1. This purification step is preceded by scaling up the culture (if required), following **steps 1–7, Section 3.2.4**. All procedures are performed at room temperature.
2. Equilibrate a Ni-NTA spin column with 600 µL of cell lysis buffer and centrifuge for 2 min at 700 g at room temperature.
3. Load up to 600 µL of the cleared lysate supernatant from **step 7, Section 3.2.4** onto the Ni-NTA spin column. Centrifuge at 700 g for 2 min and collect the flow-through. Add another 600 µL, if required, and centrifuge again.

⁶Further confirmation of the correct insert sequence is accomplished by nucleotide sequencing of the insert before carrying out the protein expression.

⁷In verifying the expression of protein, there should be a heavily-stained protein band at the estimated molecular size of the fusion product for the IPTG-induced sample. There may be a low-to-moderately stained band in the non-induced samples. There should be no comparable band in the empty vector sample. Once expression is verified, proceed to the purification step.

⁸After establishing protein overexpression for the clonal sequence, the bacterial cells are grown in cultures for scaled-up protein purification. For purification of the fusion protein, generally 50–250 mL of culture is sufficient for small-to-medium-scale protein yield. From a 50 mL culture, one can purify up to 250 µg of protein.

4. Wash the column with 600 μ L of wash buffer, centrifuge for 2 min at 700 g. Repeat the wash step 3–5 times.
5. Elute the bound fusion polypeptide by adding 200 μ L of elution buffer to the spin column and centrifuge at 700 g for 2 min. Repeat the elution two more times.
6. Analyze the protein sample for purity as in **step 8, Section 3.2.4**, on an SDS-PAGE gel, and stain with Coomassie Blue (*see* Note 9).

3.2.5.2. Purification by Glutathione-Agarose Beads

1. This purification step is preceded by verification of overexpression and scaling up of the culture (to 50 mL or more), following **steps 1–5, Section 3.2.4** (*see* Note 10).
2. Pellet the cells at 4,000 g, wash the pellet by resuspending in sonication lysis buffer, and pellet again. Repeat this wash procedure three times.
3. For subsequent steps, keep the cells on ice.
4. Sonicate cells on ice for 1 min three times, with 30 s pauses between exposures. Add Triton X-100 or Tween-20, to yield a final concentration of 1%, and mix well.
5. Centrifuge at 18,000 g for 25 min at 4 °C and collect the supernatant.
6. Prepare the GST-sepharose 4B beads by washing four times in PBST buffer and preparing a 50% slurry in PBST (*see* also the GE-Amersham manual).
7. Add 100 μ L of GST-sepharose slurry for each 1 mL of the supernatant.
8. Incubate at 4 °C overnight with gentle mixing on a rotator. Spin the mix at 600 g, remove the supernatant, and wash the beads 3–5 times with 1X PBST.
9. Elute the bound fusion protein, by mixing the beads with 10 mM reduced glutathione, centrifuge at 600 g, and collect the supernatant. Repeat three times and pool the eluted protein.
10. Check the purity of the protein, with SDS-PAGE gels, for the aliquots of lysate, sample flow-through fluid, and buffer washes.

3.2.6. Assay of Purity of Protein Samples (Ligands and Analytes) Used for Binding Studies

1. Dialyze the purified protein extensively (at least 16 h, with 6–7 changes of buffer) at 4 °C, against the running buffer to be used in the SPR binding experiment (*see* Notes 11, 12).
2. To check purity, run protein on SDS-PAGE and stain with Coomassie Blue.

⁹For Ni-NTA spin-column (Qiagen) purification, as much as 100 μ g of pure protein can be obtained from 1.2 mL of the lysate solution, depending on the level of expression. In some cases, minor bands are visible in the purified sample, and a re-purification step with a fresh spin column is required.

¹⁰For purification using glutathione-agarose beads, PCR primers for domains of interest, containing desired restriction sites, are used in PCR reactions to clone the domains into the pGEX-6P-1 vector. This vector contains a GST coding region followed by a multiple cloning site. The fusion polypeptide product will contain an N-terminal, 26-kDa GST tag, which allows affinity purification. The pGEX-6P-1 vectors are utilized to transform *E. coli* BL21 (DE3) as described in **Section 3.2.3**, and the protein is purified by the glutathioneagarose beads.

¹¹In general, both ligand and analyte should be as pure as possible, particularly for kinetic analysis of interactions. However, if a well-purified ligand is used, it is possible to determine the concentration of the analyte even if the latter is present in a mixture (9).

¹²It is critical that there be no glycerol in the protein samples and buffers.

3. Measure spectrophotometric absorbance of the sample at 595 nm (for Coomassie Blue stain) and calculate the concentration from a standard Coomassie Blue absorbance curve constructed from proteins at known concentrations.
4. One may also determine protein concentration by fluorescence methods, such as with the Qubit fluorometer, which is sensitive enough to detect sub-nanogram concentrations of protein (*see* Note 4).
5. Store purified protein solutions at -20°C in separate aliquots to avoid repeated freeze-thaw cycles that may inactivate proteins.
6. Prior to SPR studies (**Section 3.4**), thaw the protein on ice and centrifuge at 4°C for 30 min at 18,000 g to pellet any precipitated protein.
7. Carefully collect the supernatant.
8. De-gas the sample solution before use (*see* Note 13).

3.2.7. Preparation of Cell Lysate Containing Analyte for Binding Studies

1. Pellet bacterial cells that express the analyte protein by centrifuging at 4,000 g.
2. Instead of placing cells in lysis buffer (as in **step 6, Section 3.2.4**), sonicate the cells as described in **step 4, Section 3.2.5.2**. Centrifuge samples at 18,000 g for 25 min at 4°C .
3. Collect the clear supernatant. As prepared here, non-purified analyte can sometimes be used if the ligand partner is pure, as determined by detection of the ligand as a single band by polyacrylamide gel electrophoresis (*see* Note 11).
4. Estimate total protein in the filtered sample by the Coomassie Blue spectrophotometric method or the fluorescent method, as described previously.
5. Dilute sample in SPR running buffer (HBS-N or HBS-EP) to the desired concentration, starting with $10\text{ }\mu\text{g/mL}$ of total protein. Typical analyte concentrations are $10\text{--}250\text{ }\mu\text{g/mL}$, depending on the analyte affinity.

3.3. Ligand Immobilization

3.3.1. Preparation for Pre-Concentration: Determining the Ligand Concentration for Optimal Immobilization

1. Utilize 10 mM buffer solutions at three to four pH values, e.g., pH 4.0, 5.5 and 6.0, for “pH scouting” (Fig. 20.1). Sodium acetate is used at lower pH values, whereas sodium tetraborate is used at pH values greater than 6.0. The pH range is chosen to be below the estimated pI of the fusion protein/polypeptide (*see* Note 14).
2. Dilute protein sample in the 10 mM buffer solutions prepared at several pH values. The concentration of ligand should be in the range of $2\text{--}200\text{ }\mu\text{g/mL}$ (*see* Note 15).
3. Using a sample volume of $100\text{ }\mu\text{L}$, determine the pH value that gives maximum surface retention for immobilization (Fig. 20.2). The Biacore 3000 instrument is

¹³De-gas solutions by fitting a rubber stopper, with inlet tube attached to an aspirator and trap, to the top of a vessel containing the solution and reduce the pressure until air bubbles are expelled.

¹⁴The pH should be adjusted so that the protein has a net positive charge. The pI and molecular mass of the protein can be estimated using the COMPUTE pI/MW tool found at http://au.expasy.org/tools/pi_tool.html.

¹⁵The ligand should be at least 95% pure. Generally, a ligand polypeptide of smaller molecular mass should be paired with a polypeptide/protein of higher molecular mass as analyte, because the SPR response will be higher, due to the more advantageous (higher) density that can be achieved for the smaller molecular-mass molecule bound to the surface.

automated, so once the program is loaded and samples are placed, the entire reaction proceeds.

4. Open *Biacore 3000 Control* software, click on RUN tab, and then click on APPLICATION WIZARD, then select SURFACE PREPARATION and click on START. In the next panel select pH SCOUTING and click NEXT.
5. In the opening panel, select BUFFERS or add a NEW BUFFER and then click on NEXT, type the LIGAND NAME, specify the INJECTION TIME, FLOW RATE, and FLOW CELL to use.
6. In the next panel, select WASH METHOD, WASH FLOW RATE, SOLUTION TO INJECT (see Note 16), and INJECTION TIME.
7. In the next panel, assign RACK POSITIONS to samples and solutions, click on NEXT and click START. The result of a pH scouting run is shown in Fig. 20.1.

3.3.2. Preparation for Immobilization Via the Amine Coupling Reaction (see Note 17)

1. Run a DESORB step with a maintenance chip docked (see Note 18).
2. Prepare two vials each containing 115 μ L of EDC.
3. Prepare two vials each containing 115 μ L of NHS.
4. Prepare two empty vials for mixing the reagents in **steps 2 and 3, Section 3.3.2.** (The vials are inserted in the instrument racks for automated mixing of reagents.)
5. Prepare sample (typically 200 μ L) by mixing the required amount of ligand with sodium acetate solution at a pH determined by the pH scouting step.
6. Prepare two vials each containing 80 μ L of ethanolamine solution, for blocking the unreacted active sites on the chip.
7. Prepare a vial containing 100 μ L of 50 mM NaOH, for surface regeneration after pre-concentration.
8. Dock the CM5 sensor chip, using the *Biacore 3000 Control* software, and prime the chip with the running buffer (see Note 19).

3.3.3. Setting Up the Pre-Concentration and Immobilization Steps

1. Open the *Biacore 3000 Control* software (Fig. 20.2).

¹⁶A single injection of 50 mM NaOH at a flow rate of 20 μ L/min usually removes all the ligand from the surface (inspect the baseline to see if it returns to the same level as before the run). If the baseline remains elevated, a second wash with the same or another solution is performed to remove the ligand completely.

¹⁷The amine coupling reaction is the reaction generally used for proteins because of readily-available amine groups in proteins. Fig. 20.2 shows a sensorgram of a typical immobilization experiment using an amine coupling reaction. However, amine coupling is not suitable if the ligand is too acidic (pH < 5), if the amine groups are present in the active site, or if there are too many amine groups. In such situations, if thiol groups are present in the ligand, thiol coupling is preferred as an alternative (7). If both are not suitable, capture techniques such as streptavidin-biotin or His-tag nickel affinity methods can be selected. If a well-characterized antibody is available against the ligand, the antibody is immobilized and the ligand captured for binding studies. Non-amine-coupling protocols are not covered here.

¹⁸It is important to perform DESORB before each run in order to clean the micro fluidic tubes, chamber, and injection port. Such recommended instrument maintenance should be carried out on a regular basis. In the *Biacore 3000 Control* program, click the TOOLS tab and then WORKING TOOLS. Place one vial each of BIADesorb Solution 1 and BIADesorb Solution 2 in the rack designated by the program. Click DESORB and then START. Use a maintenance chip (chip lacking gold or other surface coatings) during the DESORB; do not use the CM5 chip.

¹⁹As noted in **step 2, Section 3.1**, the sensor chip should be primed before each run. The total duration for the priming is 6.3 min.

2. Click on the RUN tab, then click on to RUN APPLICATION WIZARD, select SURFACE PREPARATION, and START.
3. When the SURFACE PREPARATION STEPS window opens, select IMMOBILIZATION pH SCOUTING, and then select BUFFER (a list of buffers will appear). Click NEXT after selecting the buffer or buffers.
4. Type the NAME of the ligand to be tested, select INJECTION TIME, FLOW RATE, and FLOW CELL (FC; any of the four flow cells, FC 1, 2, 3, or 4 can be used for testing). An injection of 2-min duration, which is the default time, is usually sufficient to test the surface retention of the ligand for the selected pH. The flow rate is set to 5 $\mu\text{L}/\text{min}$ (*see* Note 20). Click NEXT to continue.
5. For the immobilization reaction, click RUN APPLICATION WIZARD, select IMMOBILIZATION, and click NEXT to select the type of SENSOR CHIP (CM5).
6. Select the IMMOBILIZATION METHOD, then in the next box select AIM FOR IMMOBILIZED LEVEL and click NEXT (*see* Note 21).
7. Select the WASH SOLUTION. Use the same wash solution(s) that were used for the pH scouting wash.
8. Select the SAMPLE POSITIONS for the vials to be placed in the instrument rack. Pipette out the required amount of each reagent, including the ligand solution. Manually insert the vials in the rack (*see* Note 22).
9. Click NEXT to start the run. At the completion of the run, a table is displayed showing the immobilization achieved (*see* Note 23).

3.3.4. Surface Preparation: Regeneration Scouting and Surface Performance Test

1. Open the control software. Perform DESORB, dock the CM5 chip containing the ligand, and prime with running buffer.
2. Click RUN tab, select RUN APPLICATION WIZARD, select SURFACE PREPARATION, and click START when the panel opens.
3. Select REGENERATION SCOUTING and click NEXT. In the panel, enter the ANALYTE NAME, INJECTION TIME, FLOW RATE, and FLOW CELL that contains the ligand. Click NEXT and select the rack positions. Click START and save the file to begin the run.
4. Select SURFACE PERFORMANCE TEST and click NEXT to enter the analyte name. Specify INJECTION TIME, FLOW RATE, AND NUMBER OF CYCLES (2–5 cycles). Select the FLOW CELL (1, 2, 3, 4, 2-1, or 4-3) and click NEXT to set the regeneration conditions.
5. In the panel, select a REGENERATION METHOD (dissociation in buffer, single injection, or two injections), FLOW RATE, SOLUTION/INJECTION (e.g., 10 mM glycine HCl, pH 3.0), and INJECTION TIME. Click on the box for STABILIZATION TIME, set the time (2 min or more), and click NEXT.

²⁰A slow flow rate (5 $\mu\text{L}/\text{min}$) is best, since the chance for the ligand to adsorb to the surface increases as the flow rate decreases.

²¹All the buffers, solutions, and sensor chips are brought to room temperature before the run. De-gas all the solutions. The buffers and regeneration solutions must be filtered through standard 0. 2- μm or 0. 4- μm filters.

²²When analyzing the samples, make sure that there are no air bubbles trapped in the sample solution. Even with prior de-gassing, bubbles can form during the sample dilution, and sometimes the bubbles may not be clearly visible. Eliminate these bubbles by spinning the tube at top speed (18,000 g) in a benchtop centrifuge for 10–15 s.

²³To minimize the mass transport effect (8), a low level of ligand immobilization is preferred.

6. Select the RACK POSITIONS and click START to save and run the experiment (*see* Note 24).

3.4. Experimental Binding Measurements

3.4.1. Experimental Binding Run

1. Run DESORB with the maintenance chip docked. Remove the maintenance chip, dock the CM5 ligand chip, and prime.
2. Click RUN, then select APPLICATION WIZARD. Select BINDING ANALYSIS and click START.
3. Choose DIRECT BINDING, click NEXT to select the FLOW CELL, FLOW RATE and the NUMBER OF SAMPLE INJECTIONS. Set the SAMPLE INJECTION TIME and WAIT AFTER INJECTION time.
4. Click NEXT to enter the ANALYTE NAME, NUMBER OF REPLICATIONS, and the ORDER in which the sample is to be analyzed (as entered or random).
5. Select the regeneration (wash) method. Choose SINGLE INJECTION or TWO INJECTIONS.
6. Select FLOW RATE for the regeneration run.
7. Select the TYPE OF SOLUTION (*see* Note 24).
8. Click NEXT to reach the next panel to assign the sample positions in the rack. Drag and drop each sample to whichever position is wanted, but make sure the programmed placement corresponds exactly to the subsequent actual placement. This panel also tells one how much is required of each solution.
9. Pipette out the required amount of the solutions and keep in the assigned racks.
10. Press NEXT, and then once again verify if all the samples are in the correct position and in the correct rack.
11. Click START to run the experiment. Begin with a low analyte concentration with a flow rate of 30 $\mu\text{L}/\text{min}$, and choose a reference cell and the ligand cell.

3.4.2. Experimental Kinetic Run (Fig. 20.3)

1. Run DESORB and then dock the CM5 chip with ligand and prime in running buffer.
2. On the *Biacore 3000 Control* Software, click RUN, and select RUN APPLICATION WIZARD.
3. Select KINETIC ANALYSIS and click START.
4. Tick the box for CONCENTRATION SERIES and select as a control experiment MASS TRANSFER.
5. Next, select DIRECT BINDING and click NEXT.
6. In the panel, select the flow cell (2-1, 4-3, 1, 2, 3, or 4), set the FLOW RATE, STABILIZATION TIME, INJECTION TIME and DISSOCIATION TIME. Select the RUN ORDER (random or as entered). Enter ANALYTE NAME, MOLECULAR

²⁴Regeneration is achieved with the optimal pH and ionic concentration that keep the ligand active. A series of regeneration tests should be performed with regeneration buffers, from mild to more stringent conditions of pH and ionic strength. In most cases where the binding affinity is low or moderate, begin with a pH close to neutral (pH 4.5–8.5) and an ionic strength in the range of 0.5–1.0 *M*. 1.0 *M* NaCl should be sufficient to dissociate most of the bound protein (Figs. 20.5, 20.6).

MASS in Daltons, NUMBER OF REPLICATIONS, and required CONCENTRATIONS. Click NEXT. Select at least five different concentrations and include a buffer blank.

7. In the MASS TRANSFER control panel, select a CONCENTRATION to be analyzed. If using a range of 0–200 nM, use a concentration that is in the range of 100 nM. Click NEXT.
8. Select the REGENERATION METHOD, FLOW RATE, REGENERATION SOLUTION, and the INJECTION TIME. Set the STABILIZATION TIME after regeneration.
9. Select RACK POSITIONS, click NEXT, and click START to begin the run and save the file (*see* Note 25).

3.4.3. Data Analysis and Determination of Kinetic Constants

1. Using *BIAevaluation* software (*see* Note 26), select the data to be analyzed and create an overlay file by selecting the relevant curves. Align all the curves to the start of the injection. Remove the portions of the plots that lie outside the relevant regions of the association and dissociation curves, since the measurements require only the latter portions.
2. Choose FIT: KINETICS SIMULTANEOUS k_a/k_d to start the kinetic evaluation wizard and perform the curve-fitting process. Set the baseline to zero at a point at the start of the injection in the most stable region. Performing a Y-TRANSFORM command in the wizard panel enables alignment of all the desired curves.
3. Select START TIME and STOP TIME for the injection and select the DATA RANGE, for association and dissociation, to be used in the fitting process. Make necessary adjustments by zooming out and inspecting the selected region visually (*see* Notes 27, 28).
4. In the next panel, select the BINDING MODEL. Depending on the binding data, choose the model which best fits the ligand-analyte interaction. (a) 1:1 Langmuir binding, (b) 1:1 binding with shifting baseline, (c) 1:1 binding with mass transfer, (d) bivalent analyte, (e) heterogeneous analyte, (f) heterogeneous ligand, (g) two-state reactions.
5. Fit the data and observe the progress. For simple 1:1 reactions, the computation process is almost instantaneous, but a delay of several seconds to minutes may indicate complex binding or heterogeneity in the data set.
6. After the fit, the curves will appear as an overlay of the experimental and fitted data (Fig. 20.3). Close overlap, observed visually, is a good first indication of the validity of the fit.

²⁵When undocking the chip from the instrument, it is often advantageous to select *not* to remove the buffer from the flow cells by *un-checking* EMPTY FLOW CELL. The chip can then be wrapped in soft tissue paper or a piece of paper towel and stored in a tight container at 4 °C. The sensor chips with immobilized ligands can be stored for a few days to more than a month.

²⁶SPR binding measurements (Fig. 20.7) are generally performed in duplicate or triplicate. Experimentally-obtained kinetic data can be evaluated with the SPR kinetic evaluation software, *BIAevaluation*. In general, analysis of kinetic data involves the following steps: (a) make overlay plots of several interaction curves, (b) select analysis region, (c) select interaction model and curve fitting, (d) store results of the fitting procedure into an analysis results file.

²⁷In selecting the data range for the analysis of kinetic plots with *BIAevaluation* software, a short period before the injection start and before the stop (the beginning and end of the association phase of the kinetic plot) should be excluded in the selection to avoid the dispersion effect (the complex response in the refractive index at the beginning and end of injection, not clearly defined mechanistically).

²⁸Perform separate fits for different regions of the association and dissociation phases to ascertain if the calculated constants are consistent.

7. The result is displayed as a report, with all the constants in numerical form. Examine the “residual plot,” which is the calculated difference between the experimental and fitted data (Fig. 20.4), by clicking on the RESIDUALS tab. If there are systematic deviations between the experimental and fitted curves, the plot will indicate them by displacement from the zero line. Ideally, the noise level in the plot should be on the order of ± 2 RU (also *see* Note 29).

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²⁹Although not detailed here, association and dissociation constants can also be obtained directly by determining the concentrations of analyte and ligand under steady-state conditions (9).

Fig. 20.1.

The “pH scouting” of mouse syntaxin 1A hexahistidine fusion polypeptide (model polypeptide of 36 kDa molecular mass and pI 5.67) as ligand. The polypeptide was diluted in 10 mM sodium acetate to a final concentration of 50 $\mu\text{g/mL}$, at three different pH values (4.0, 5.5, and 6.0), prior to the “pre-concentration” testing (*see* Fig. 20.2). For pH 4.0 (red curve), the surface concentration of ligand on the chip was much higher than that obtained for pH 5.5 and 6.0 (pink and blue curves, respectively). Thus, for immobilization of the syntaxin 1A fusion polypeptide via the amine coupling reaction (Fig. 20.2), pH 4.0 was chosen (a value less than the pI).

Fig. 20.2.

Steps in the immobilization of purified syntaxin 1A fusion polypeptide ligand on a CM5 research-grade sensor chip via the amine coupling reaction. **(A)** “Pre-concentration” test to determine how much of the ligand to inject to reach a targeted (aimed) level of response (RU, response units), here, 1,500 RU. The injection period ends after attainment and overshoot of this criterion value above the baseline of approximately 19,000. The pre-concentration test reflects the proclivity of the ligand for the carboxymethylated dextran surface in its *non-activated* state, maximizing compatibility during the actual covalent binding step, E, and thus providing an estimate of the mass necessary for optimizing the association. **(B)** Washing of the non-covalently bound ligand with 50 mM NaOH to remove the ligand completely and obtain baseline. **(C)** Injection of the reaction mix (*see Section 3.3.2*) onto the surface, activating the carboxymethyl group by forming a highly-reactive succinimide ester (7), followed by termination of the injection. **(D)** Slight increase in the RU reflecting the activation, compared to the baseline between B and C. **(E)** After surface activation, injection of ligand sample diluted in buffer of the appropriate, pre-determined pH, with continuation of injection resulting in covalent binding of ligand to the reactive surface to yield the targeted RU value of 1,500. This value is the difference between the achieved constant level just before F and the baseline at D. **(F)** Blockage of remaining non-reacted-activated carboxymethyl groups by injection of ethanolamine, followed by cessation of injection. **(G)** Point of attained immobilization of ligand. Small triangles on time line below designate events such as injection, end of injection, and re-filling.

Fig. 20.3.

Plots illustrating the experimental curve-fitting methodology for a simple binding model (1:1 Langmuir). Association and dissociation phases can be seen in each plot. Pairs of red traces at each concentration indicate duplicate experimental determinations and blue traces show the corresponding binding model curves. In these studies, a syntaxin 1A fusion polypeptide ligand was immobilized on a CM5 sensor chip by the amine coupling method to a final targeted RU of 707 (immobilization targeting is illustrated in Fig. 20.2). A range of concentrations (0, 10, 20, 40, and 80 nM) of the analyte, a model hexahistidine fusion polypeptide of the calcium channel $\text{Ca}_v1.3$ II–III loop with molecular mass 18 kDa, was used for the kinetic evaluation.

Fig. 20.4.

“Residual” plots (9) reflecting minimal deviation between experimental and fitted data for a kinetic study (not shown) in which the data match the model. Left and right data sets correspond to chosen association and dissociation portions, respectively, of the kinetic curves. Colors represent different concentrations of analyte (top to bottom, *see* dissociation portion): light blue, 2 nM ; magenta, 0 nM; black, 4 nM; blue-green, 8 nM ; dark blue, 32 nM; green, 16 nM. Note that the scatter in the residual plots is within or around the criterion 2 RU, indicating that the experimental and model kinetic curves, in this case for a 1:1 Langmuir interaction (*see* **step 4**, *±* **Section 3.4.3**), show a good fit. Larger deviations from the zero line would indicate that the data should be refitted using another kinetic model.

Fig. 20.5.

Illustration of unstable vs. stable SPR baselines. Baseline stability is critical to obtaining accurate kinetic data. Plot (A) shows a decreasing baseline after each cycle, commonly due to loss of the bound ligand. Plot (B) indicates an increase in the baseline after each cycle, reflecting partial dissociation of analyte and poor regeneration. Plot (C) shows a stable baseline, indicating minimum loss of bound ligand as well as maximum regeneration. The latter reaction is most likely to yield accurate kinetic data. (Fig. 20.3 illustrates a data set where the baseline was stable and duplicate determinations were similar.)

Fig. 20.6.

Effect of flow rate on mass transfer. If the association rate constant of an interaction of interest is high, the measured binding rate may reflect the rate of *transfer* of analyte into the matrix (as the limiting rate) rather than the rate of the binding itself (8), and such mass transfer limitations may yield erroneous rate constants. Mass transfer problems, if present, are commonly detected by injecting a certain concentration of analyte sample at different flow rates, as shown. **(A)** The same analyte concentration gives considerably different RUs with different flow rates, indicating the presence of a mass transfer limitation. **(B)** The RUs at different flow rates are more similar, with almost identical dissociation phases, suggesting a minimal mass transfer limitation. If mass transfer limitation is present, as in A, choosing a relatively high flow rate,

low ligand density, and experimentally-optimal analyte concentration can considerably reduce the limitation.

Fig. 20.7.

Example SPR data: qualitative analysis of binding between ligand syntaxin 1A fusion polypeptide and otoferlin hexahistidine fusion polypeptide (a model analyte polypeptide of molecular mass 34 kDa), in the presence and absence of calcium. Bar 1 indicates essentially no binding (the slight elevation is a bulk response due to a small increase in the refractive index) when the binding buffer contains 3 mM EGTA. Bar 2 shows a higher response with omission of EGTA and addition of calcium (100 μ M) to the buffer, indicating a requirement of calcium for the interaction. Bars 3, 4, and 5 show a reduction of the interaction with increasing concentrations of calcium (0.2, 0.5, and 1.0 mM, respectively). Bars 6 and 7 are buffer blanks (negative controls; buffer plus ligand with no analyte). Bar values represent the maximum binding as determined by the RU at the end of the injection of the analyte, relative to baseline just before injection.