Chapter 23

Surface Plasmon Resonance Method to Evaluate Anti-citrullinated Protein/Peptide Antibody Affinity to Citrullinated Peptides

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Abstract

Surface plasmon resonance (SPR) technique is extremely interesting in immunology because it has the potential to directly visualize biomolecular interactions in real-time monitoring antibody affinity, one of the parameters affecting pathogenicity in autoimmune diseases. Herein we describe the affinity evaluation of anti-citrullinated peptide antibodies (ACPA) to a peptide-based biosensor by SPR. The method describes the purification of ACPA isolated from rheumatoid arthritis (RA) patients using affinity columns, the strategy employed for the immobilization of citrullinated peptides onto a sensor chip, and the evaluation of the specific binding of purified ACPA to immobilized peptides.

Key words Antibody affinity, Kinetic, Citrullinated peptides, Biacore, Surface plasmon resonance (SPR)

1 Introduction

Biosensor technology based on surface plasmon resonance (SPR) has become increasingly popular for monitoring binding interactions. SPR technique has been largely applied to characterize analyte-ligand affinity in different fields of biology and medicine including immunology [1–4]. The technique allows both the screening for candidates and a detailed kinetic and mechanistic analysis of molecular interactions [5]. Comparison of the association and dissociation rates for anti-citrullinated protein/peptide antibodies (ACPA) recognizing diagnostic peptides was performed in the particular case or rheumatoid arthritis (RA), suggesting possible cross-reactivity with potential clinical relevance [6].

Herein, we present a detailed description of an SPR method to characterize and evaluate affinity of purified RA antibodies to diagnostic citrullinated peptides. A similar procedure has been recently used to study ACPA profiles directly on sera of early arthritis

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patients without providing, however, an evaluation of affinity [7]. The affinity of autoantibodies for their target has a relevant role in determining their pathogenicity. In fact, previous studies on ACPA affinity and avidity, employing the classical immunoenzymatic ELISA, indicate that high-avidity ACPA are produced in RA prior to disease onset and are detectable in a symptomatic patients [8, 9].

The protocol herein described includes three steps: (a) purification of ACPA from RA patients' sera by affinity chromatography, (b) immobilization of diagnostic citrullinated peptides onto gold sensor chip, and (c) peptide-antibody binding experiments and affinity evaluation. The first step provides purified ACPA to be further used in SPR avoiding nonspecific interactions due to the presence of other serum components including immunoglobulins of other specificity. Thus, we obtain clearer experiments/signals decreasing bulk effects. Thanks to the second step we can assess the orientation of peptides not only in the case of linear sequences but also when immobilizing citrullinated multiple antigenic peptides (MAPs) in the sensor chip. MAPs are synthetic peptides built upon a core resulting in three-dimensional molecules with highly localized peptide density, and classic amine coupling protocol did not guarantee a well-known orientation. These steps supply the optimal conditions to further evaluate the interaction of ACPA with citrullinated MAPs and the calculation described in the third step.

2 Materials

Conduce experiments using a SPR instrument (e.g., Biacore T100) and prepare all solutions and buffers with Milli-Q water or similar quality.

Commercially available sensor chips CM5, amine coupling kit, thiol-coupling reagent, and running buffer (e.g., HBS–EP+10×) were used.

Eject sensor chip from the instrument, place it into the appropriate plastic case, and store at +4 °C; gently wash chip surface with Milli-Q water and dry under a nitrogen stream before inserting it into the instrument. Filter buffers daily with a 0.22 μm system. Store enriched IgG fractions from RA patients' sera and purified antibody samples at −20 °C avoiding thawing and freezing cycles. Follow the waste disposal regulation for biological and chemical waste.

2.1 Affinity Chromatography

1. Enriched IgG fraction: Purify IgG antibodies using the protein G-sepharose column as reported [10].
2. Phosphate buffer: 20 mM Na_2HPO_4, 150 mM NaCl, pH 7.2. Weigh 567.84 mg of Na_2HPO_4 and 1.77 g of NaCl, add 200 mL of water, and adjust pH at 7.2. Store at room temperature.

3. Elution buffer: 100 mM glycine, pH 2.8. Weigh 750.7 mg glycine, add 100 mL water, and adjust pH at 2.8. Store at +4 °C.
4. Tris buffer: 1 M Tris, pH 8.0. Weigh 12.11 g of Tris, dissolve in 100 mL water, and adjust pH at 8.0. Store at +4 °C.
5. D-PBS buffer 10×: Place 50 mL of buffer in a graduate cylinder, add 450 mL water, and adjust pH at 7.2. Store at room temperature.

2.2 PH Scouting Buffers (*See* Note 1)

1. Running buffer HBS–EP+10× (0.1 M Hepes, 1.5 M NaCl, 30 mM EDTA, and 0.5 % [v/v] P20): Place 50 mL of buffer in a graduate cylinder, dilute ten times with water, and adjust pH at 7.4. Store at +4 °C.
2. D-PBS buffer 10×: *See* Subheading 2.1.
3. Sodium acetate buffers: 5 mM $CH_3COO^-Na^+$, pH 4.5, 5.0, and 6.0. Weigh 68.04 mg of $CH_3COO^-Na^+$, prepare 100 mL solution, and adjust pH at 4.5; repeat the same procedure for pH 5.0 and 6.0. 0.5 mM $CH_3COO^-Na^+$ pH 4.5 and 5.0, measure 10 mL of the previously prepared buffers at the desired pH, and add water up to 100 mL. 0.1 mM $CH_3COO^-Na^+$ pH 4.5 and 6.0. Measure 2 mL of the 5 mM buffers at the desired pH and add water up to 100 mL.
4. Regeneration solution: 0.1 M NaOH. Weigh 2 g of NaOH and dissolve in 500 mL water.
5. Peptide stock solutions: Dissolve each peptide in water to obtain a final concentration of 1 mg/ml (weigh 500 μg of peptide and dilute it in 500 μL of water).

2.3 Citrullinated Peptide Immobilization Reagents

1. Amine coupling kit: 0.1 M *N*-hydroxysuccinimide (NHS) stock solution, dissolve 115 mg in 10 mL water. 0.4 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) stock solution, dissolve 750 mg in 10 mL water. Dispense stock solutions separately in aliquots and store frozen at −18 °C. 1 M Ethanolamine-HCl pH 8.5, store at +4 °C.
2. Thiol-coupling reagent: 120 mM 2-(2-pyridinyldithio)ethaneamine (PDEA) stock solution, dissolve 100 mg of PDEA in 3.74 mL water, store stock solution in aliquots at −18 °C.
3. Sodium borate buffer: 0.1 M $Na_2B_4O_7 \cdot 10\ H_2O$ pH 8.5. Weigh 381.37 mg of $Na_2B_4O_7 \cdot 10\ H_2O$, dissolve in 10 mL water, and adjust pH at 8.5.
4. Disulfide blocking buffer: 50 mM cysteine—HCl, 1 M NaCl, 0.1 M $CH_3COO^-Na^+$ pH 4.3. Weigh 394.05 mg of cysteine-HCl, 2.92 g of NaCl, and 680.45 mg of $CH_3COO^-Na^+$, add 50 mL of water, adjust pH at 4.3, and store at room temperature.

2.4 Solutions for Kinetic and Affinity Studies

1. Running buffer (RB) (*see* Subheading 2.2, **item 1**).
2. Anti-citrullinated peptide antibody fractions: Use different final concentrations of each fraction (1000, 500, 250, 125, 62.5, 31.25, and 0 nM). Prepare 120 μL of every concentrations, by serial dilution in running buffer (*see* **Note 2**): take the appropriate initial volume from each fraction according to the calculated initial concentration (*see* Subheading 3.1, **step 3**) (*see* **Note 3**).
3. Regeneration solutions: 0.1 M NaOH (*see* Subheading 2.2, **item 4**). 10 mM glycine pH 2.5. weigh 75.07 mg, dissolve in 100 mL water, adjust pH at 2.5.

3 Methods

Conduce all the experimental work at room temperature.

3.1 Anti-peptide Antibody Purification

1. Conjugate 1 mg of the citrullinated peptide to 100 mg of CNBr-activated sepharose according to the standard manufacturer instructions. Insert the functionalized resin into a chromatography column of 5 mL volume. Repeat this procedure for each single peptide separately.
2. Mix 1 mL of the enriched IgG fraction with 4 mL of phosphate buffer pH 7.2, apply the solution to the column three times, wash with 10 mL of phosphate buffer pH 7.2, elute anti-citrullinated peptide antibodies with 5 mL of elution buffer, and immediately neutralize with 50 μL of Tris buffer (*see* **Note 4**). Dialyze overnight against D-PBS buffer (*see* **Note 5**).
3. Calculate the concentration of each fraction using a quartz cuvette to measure the sample UV absorbance: C[mg/ml] = UV absorbance at 280_{nm}/1.4.

3.2 pH Scouting: Selection of Immobilization Buffer

1. Mix 1 μL of peptide stock solution and 99 μL of each immobilization buffer.
2. Inject the first sample onto the chip surface for 120 s at a flow rate of 10 μL/min.
3. Regenerate chip surface with a pulse of 0.1 M NaOH for 30 s at a flow rate of 10 μL/min.
4. Repeat **steps 2** and **3** for each ligand in each immobilization buffer (*see* **Note 6**).
5. Select the buffer with the highest sensorgram slope as immobilization buffer.

3.3 Peptide Immobilization: Ligand Thiol Coupling Strategy

1. Chip surface activation: Mix 80 μL of NHS stock solution and 80 μL of EDC stock solution, and inject for 420 and 60 s at a flow rate of 10 μL/min (*see* **Note 7**).
2. Active disulfide group introduction: Mix 67 μL of PDEA stock solution and 33 μL of sodium borate buffer, and inject for 420 s at a flow rate of 10 μL/min (*see* **Note 8**).
3. Ligand injection: Mix 4 μL of each peptide stock solution with 396 μL of the previously selected immobilization buffer, and inject for 420 and 60 s (*see* **Note 9**) at a flow rate of 10 μL/min (*see* **Note 10**).
4. Unreacted disulfide group block: Inject 200 μL of disulfide blocking buffer for 420 s at a flow rate of 10 μL/min.
5. Unreacted succinimide group block: Inject 200 μL of 1 M ethanolamine–HCl pH 8.5 for 420 s at a flow rate of 10 μL/min.
6. Reference channel: Repeat steps from Subheading 3.3, **steps 1–5**, except step in Subheading 3.3, **step 3** (*see* **Note 11**).

3.4 SPR Kinetic and Affinity Studies

1. Sensor chip surface start-up: Take 3 mL of running buffer and inject for 120 s, take 1 mL of 10 mM glycine pH 2.5 and inject for 30 s, take 1 mL of 0.1 M NaOH and inject for 60 s, and use a flow rate of 30 μL/min. Repeat this step at least four times.
2. Cycle of analysis: Take 120 μL of each concentration of the first anti-citrullinated peptide antibody fraction and contemporary inject over up to the three different immobilized peptides for 120 s; flush running buffer for 200 s; regenerate chip surface by injecting 10 mM glycine pH 2.5 for 30 s and 0.1 M NaOH for 60 s; use a flow rate of 30 μL/min. Repeat this cycle for each purified antibody fraction (*see* **Note 12**).
3. Result elaboration: Use the evaluation software (e.g., Biacore evaluation software version 2.0) to fit experimental data to mathematic models (Fig. 1) and to calculate the kinetic parameters of the association k_a (M^{-1} s^{-1}) and the dissociation rate k_d (s^{-1}), and the affinity constants K_D (M).
4. Fitting validation: Verify that the residual values between the experimental points and the theoretical ones are closely distributed along the zero, the chi-square value is as lower as possible, and the standard errors are less than 10 % of the referred parameter value (*see* **Note 13**).
5. Results comparison: Use the Biacore T100 kinetic summary application to obtain the k_a/k_d plot (Fig. 2) showing all kinetic and affinity parameters (*see* **Note 14**).

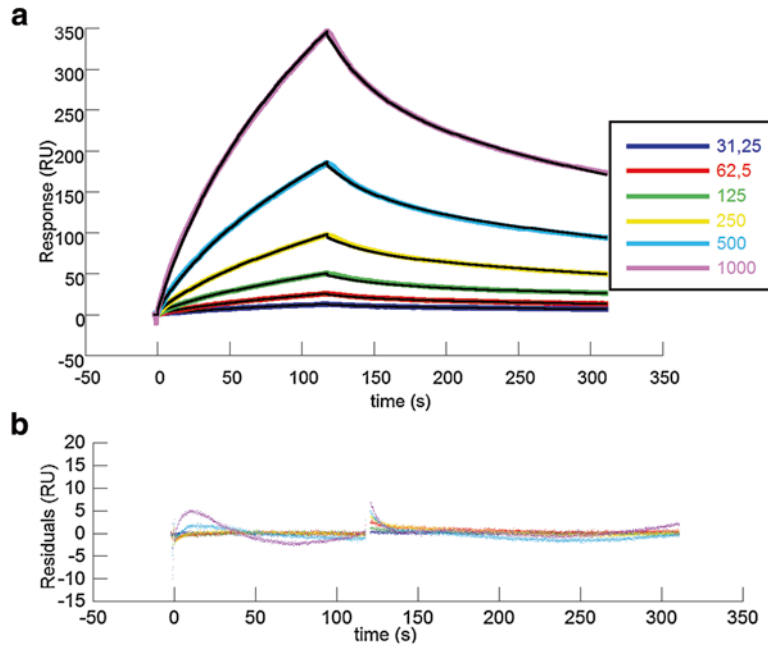

Fig. 1 (**a**) Sensorgrams resulting from the kinetic and affinity study of purified antibodies from one RA patient and one immobilized citrullinated peptide. Each colored curve corresponds to a different sample concentration (nM); the fitted binding 1:1 model is reported in *black lines*. (**b**) Residual values indicating the difference between experimental and mathematical points

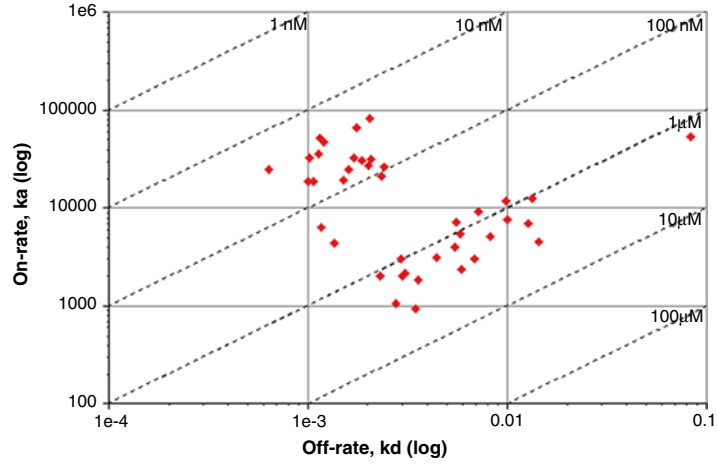

Fig. 2 k_a/k_d plot for the 1:1 binding model summarizing interactions among purified ACPA and the citrullinated peptides. Each point summarizes k_a, k_d, and K_D calculated values for each ACPA-citrullinated peptide kinetic study performed. Interactions characterized by same affinity and different kinetics are indicated by points lying on the same *diagonal line*

4 Notes

1. To achieve the electrostatic pre-concentration of ligands in the dextran matrix of CM5 chip, use immobilization buffers at pH between 3.5 and the isoelectric point of the ligands; pre-concentration is favored by low ionic strength in the buffer.
2. Dilution series play an important role in kinetics and affinity studies, preparing the samples by serial dilution where each dilution is used as the stock solution for the next step is useful to reduce the experimental error. Avoid bubble formation when diluting samples, or spin them to remove bubbles.
3. To prepare 120 μL of each sample concentration follow this scheme: prepare 240 μL of the 1000 nM concentration, take 117 μL and mix with 117 μL of RB for the 500 nM concentration, take 113 μL and mix with 113 μL of RB for the 250 nM concentration, take 105 μL and mix with 105 μL of RB for the 125 nM, take 90 μL and mix with 90 μL of RB for the 62.5 nM concentration, take 60 μL and mix with 60 μL of RB for the 31.25 nM concentration, and use 120 μL of RB for the zero concentration.
4. Collect the flow through (not retained fraction) and test it and anti-peptide antibody content in SP-ELISA, to confirm the success of the purification procedure.
5. This dialysis step is needed to match the refractive index of sample solution with RB in order to perform SPR studies avoiding the bulk effect which could affect the first part of the sensorgram of the higher injected concentration and that could be responsible for a wrong fitting to kinetic models during the association phase.
6. Discard buffers that give irregular sensorgrams or signals with irregular slopes, probably due to ligand aggregation/precipitation or chip saturation.
7. Mix equal volumes of EDC and NHS immediately before use.
8. The most appropriate covalent immobilization strategy has to be selected according to the reactive groups present in the ligand. Each citrullinated peptide herein used contains only one cysteine residue; thus the ligand thiol coupling strategy was chosen to obtain a well-defined and reproducible orientation of the ligands on the chip.
9. The best concentration will vary according to the nature of the ligand; higher concentrations and/or additional injections may be needed for ligands that do not pre-concentrate efficiently, probably due to their low isoelectric point.
10. A lower flow rate could be chosen for the immobilization of higher molecular weight ligands.

11. One channel without any immobilized ligand is used as reference, to remove the nonspecific signal depending on interactions between molecules present in the biological samples and gold on sensor chip surface.
12. A replicate concentration can be inserted to assess the surface regeneration performance and the reproducibility of the assay; start the analysis cycle by injecting the lower sample concentration at first.
13. A high-quality fitting could be obtained with more than one kinetic model. Consider that SPR signal is directly dependent on change in reflective index and thus in mass over the chip surface and consequently interactions between multivalent molecules do not produce an instrumental signal; if possible choose the more reliable binding 1:1 model.
14. Plot the logarithm of calculated k_d and k_a on X- and Y-axis, respectively, for each purified peptide-antibody interaction to summarize results if kinetic summary software Biacore 2.0 is not available.

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