Chapter 9

SPR Biosensor as a Tool for Screening Prion Protein Binders as Potential Antiprion Leads

Beining Chen

Abstract

Prion diseases, also called transmissible spongiform encephalopathies (TSEs), are a group of neurodegenerative disorders affecting animals and humans. No effective treatments are currently available for the diseases, vCJD in particular. It is believed that the formation of protease-resistant insoluble prion protein (PrP^{Sc}), which is the main component of amyloidal deposits, from the cellular prion protein (PrP^{C}), is essential for the progression of the disease. Therefore, both PrP^{Sc} and PrP^{C} are currently being used as potential drug targets.

This protocol details an optimised experimental protocol to conduct an affinity screening of compound libraries by the immobilisation of PrP^{C} using an SPR-based instrument, Biacore 3000.

Key words: Prion protein, surface plasmon resonance, Biacore, affinity binding, drug screening, dissociation constant.

1. Introduction

Prion diseases, also called transmissible spongiform encephalopathies (TSEs), are a group of neurodegenerative disorders affecting animals and humans that include scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in deer and elk, Gerstmann–Sträussler–Scheinker syndrome and Creutzfeldt–Jakob disease (CJD) in humans (1). Formation of amyloidal deposits in an affected brain is a hallmark of these diseases and the deposits are constituted mainly of aggregated prion protein in a misfolded state, hence the name of the disease. Prion diseases are also infective

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and can be transmitted via transplants, contaminated biological products from cadavers, blood transfusions, contaminated surgical instruments and ingestion of infected materials. The latter has been observed in humans after consumption of beef affected by BSE and has been termed variant CJD (vCJD). No effective treatments for CJDs, vCJD in particular, are currently available. It is believed that the formation of protease-resistant insoluble prion protein (PrP^{Sc}), which is the main component of amyloidal deposits, from the cellular prion protein (PrP^{C}), is essential for the progression of the disease. Therefore, both PrP^{Sc} and PrP^{C} are currently being studied as potential drug targets (2). However, PrP^{Sc} is insoluble and its structure is not known in detail, and it is technically impossible to produce sufficient quantities for in vitro screening. PrP^{Sc} is therefore mainly used as a biomarker in cellular assays to assess antiprion efficacy of compounds. Most of the affinity-screening assays use more widely available recombinant PrP^{C} as a target protein. The biological efficacy of the affinity binders is normally confirmed in cellular assays using PrP^{Sc} as a biomarker.

PrP^{C} is a membrane protein of unknown physiological function (3–5). It consists of an unstructured, flexible N-terminal domain (AA23–110) which contains five octarepeats (AA51–91); a globular C-terminal domain (AA111–230) which contains two glycosylation sites (Asn181, Asn197) (6); and a glycosylphosphatidylinositol (GPI) anchor (Ser230) (7). The SPR technique is easy to implement and it has medium sample throughput with automated sample handling. SPR has been reported as a binding assay for small drug-like molecules against other drug targets (8). Several groups have reported using SPR as an assay platform to screen compound libraries in order to discover leads which can bind to PrP^{C}, stabilise it and prevent its conformational change to PrP^{Sc} (9–11). For convenient and optimal throughput in drug screening for antiprion lead discovery, immobilising PrP^{C} on the surface and injecting a compound of interest to study their interactions would be ideal. This protocol details an optimised experimental method to conduct affinity screening of compound libraries through immobilised PrP^{C} using an SPR instrument, Biacore 3000. The recombinant full-length human PrP^{C} ($flhPrP^{C}$) is used to highlight the procedures involved.

2. Materials

2.1. Immobilisation of Recombinant Prion Protein PrP^{C}

1. An SPR instrument, Biacore 3000 (Biacore, Uppsala, Sweden) equipped with a CM5 sensor chip (*see* **Note 1**).
2. Immobilisation buffer: HBS-EP buffer consisting of 0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20 at pH 7.4 (*see* **Note 2**).

3. Coupling reagents: 100 mM *N*-hydroxysuccinimide (NHS), 400 mM *N*-ethyl-*N*′-(3-diethylaminopropyl) carbodiimide hydrochloride (EDC) (*see* **Note 3**).
4. Recombinant PrP^Cs (human full length) are available from Prionics (Switzerland) or expressed in-house. It is diluted in 10 mM sodium acetate buffer pH 5.5 to the concentration required.
5. Blocking reagent: 1 M ethanolamine (*see* **Note 4**).
6. Regeneration solution 1 consisting of 25 mM NaOH, 1 M NaCl, 0.0005% SDS at pH 8.0.
7. Regeneration solution 2 consisting of 10 mM glycine–HCl at pH 3.0.
8. Running buffer: PBS buffer consisting of 10 mM Na_2HPO_4 – NaH_2PO_4, 0.15 M NaCl, 3 mM EDTA and 0.005% surfactant P20 at pH 7.4. The buffer needs to be degassed and filtered through nitrocellulose membrane with a pore size of 0.22 μm prior to use.

2.2. Screening of Small Molecule Compound Libraries

1. DMSO (Sigma-Aldrich).
2. Running buffer: the same as in 2.1.
3. Regeneration solutions 1 and 2: the same as in 2.1.
4. BIAdesorb and BIAdisinfectant solutions (Biacore).

3. Methods

It is well known that prion protein binds strongly to metal surfaces (12, 13). It may also bind to the carboxymethylated dextran (CM-dextran) on the gold surface because, structurally, CM-dextran resembles heparin, which is known to bind to prion proteins (14–16). It is important that an appropriate immobilisation protocol is developed and optimised so that the required immobilisation can be achieved.

On the other hand, drug-like compounds normally have a low molecular weight between 300 and 800 Da. To observe binding between a prion protein and those compounds it is essential to be able to immobilise prion proteins at a level between 3,000 and 10,000 RU and keep the baseline as stable as possible.

3.1. Immobilisation of Recombinant Prion Protein PrP^C

1. Two of the four flow cells are used in this assay (*see* **Note 5**). The CM-dextran on flow cells 1 and 2 of a CM5 sensor chip is washed and equilibrated with the running buffer. It is then activated by mixing equal volumes of 100 mM NHS and 400 mM EDC followed by injection of the mixture over

the sensor chip surface for 7 min at a flow rate of 5 μL/min (*see* **Note 6**).

2. The flhPrP^{c} to be immobilised is diluted in 10 mM sodium acetate buffer at pH 5.5 to a concentration of 2 μg/mL and is injected over the flow cell 2 (the test channel) for 7 min (*see* **Note 7**). The flow cell 1 (reference channel) is activated as described above but no protein is injected over the surface. The final immobilisation level of the PrP^{C} is around 4,000 RU on flow cell 2 (*see* **Fig. 9.1** and **Note 8**).
3. The unreacted sites on both flow cells of the sensor chip surface are blocked by the injection of 1 M ethanolamine pH 8.5 for 7 min (*see* **Note 9**).
4. The prepared flow cells on the sensor surface are washed thoroughly, immediately after the ethanolamine blocking step, by three consecutive injections of regeneration solution 1 at an interval of 8 s.
5. The surface is then equilibrated with the running buffer for 30 min prior to the injection of sample solutions.

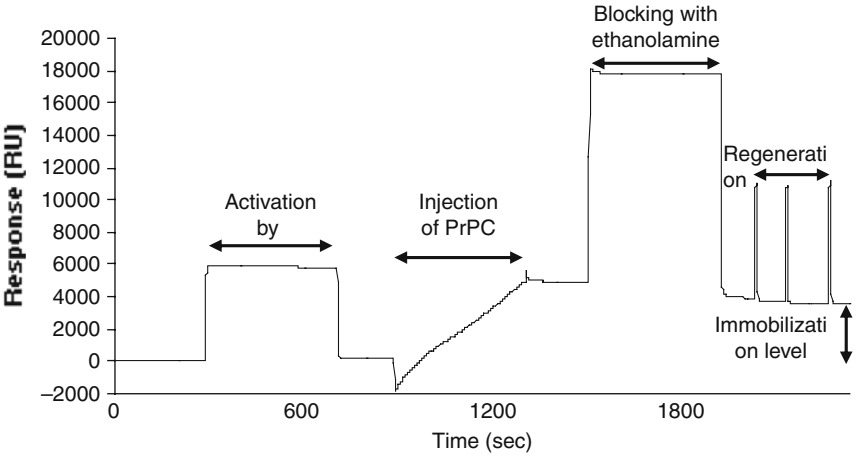

Fig. 9.1. Typical PrP^{C} immobilisation sensorgram (10). The difference between baseline at the start and end indicates the amount of immobilised protein in RU.

3.2. Screening of Small Molecule Compound Libraries

3.2.1. Sample Preparation

The compounds tested can be divided into two groups: water soluble and water insoluble.

1. 6.1 mM stock solutions of all water-soluble compounds are made using running buffer. Stock solutions are diluted to the required 40 μM concentration using the same running buffer prior to the screening.
2. Similarly, 6.1 mM stock solutions of all water-insoluble compounds are made in 100% DMSO. 10 μL of the stock solution is then added to 90 μL of DMSO to give a concentration of 610 μM. A final dilution is carried out by

mixing 65 μL of the second DMSO dilution solution with 935 μL of the running buffer to give a final compound concentration at 40 μM containing 6.5% (v/v) of DMSO. 6.5% DMSO is added to the running buffer.

3.2.2. Compound Screening

1. All assays are carried out at 25°C with a flow rate of 30 μL/min and a compound concentration of 40 μM, using phosphate buffer as the running buffer.
2. For water-soluble compounds, each analytical cycle consisted of running buffer for 1 min (stabilisation phase), a sample injection at 40 μM in running buffer for 1 min (association phase) and running buffer for 3 min (dissociation phase). Subsequent surface regeneration at a flow rate of 35 μL/min was carried out using the two regeneration solutions 1 and 2 sequentially at an interval of 8s. After regeneration the surface was allowed to stabilise for 1 min. The total run time was approximately 8 min/cycle. The sensor chip was usually discarded after 5 days.
3. For compounds using DMSO as a co-solvent, 6.5% DMSO is added to the running buffer and a DMSO calibration using buffer samples containing DMSO at concentrations of 5.5, 6, 6.5, 7 and 7.5% respectively was carried out at the beginning and the end of each block of 10 compounds to correct for solvent effects (*see* **Note 10**). Each screening cycle consists of one 10-injection buffer block, one 5-concentration DMSO calibration block, one 10-compound screening block and another 5-concentration DMSO calibration block (*see* **Note 11**).
4. The data is analysed and the binding is expressed as $\%RU_{max}$ (*see* **Note 12**). The binding strength of compounds can be ranked and compared.

3.2.3. Binding Affinity Study

The binding affinity of positive binders from initial screening in 3.2.2 can be further studied and quantified by the following protocols.

1. For aqueous soluble compounds the PrP^C surface is prepared using the optimal immobilisation procedure (*see* **Section 3.1**). A series of concentrations are injected at a flow rate of 30 μL/min over immobilised PrP^C, and the reference surface at 25°C. A single injection of each solution was carried out from the lowest to the highest concentration and sensorgrams were recorded for each injection (*see* **Fig. 9.2a**). The sensor surface is regenerated with a pulse of 25 mM NaOH/1 M NaCl between each injection. The detailed binding kinetics can be analysed using BIAevaluation software 4.1 (*see* **Note 13**).

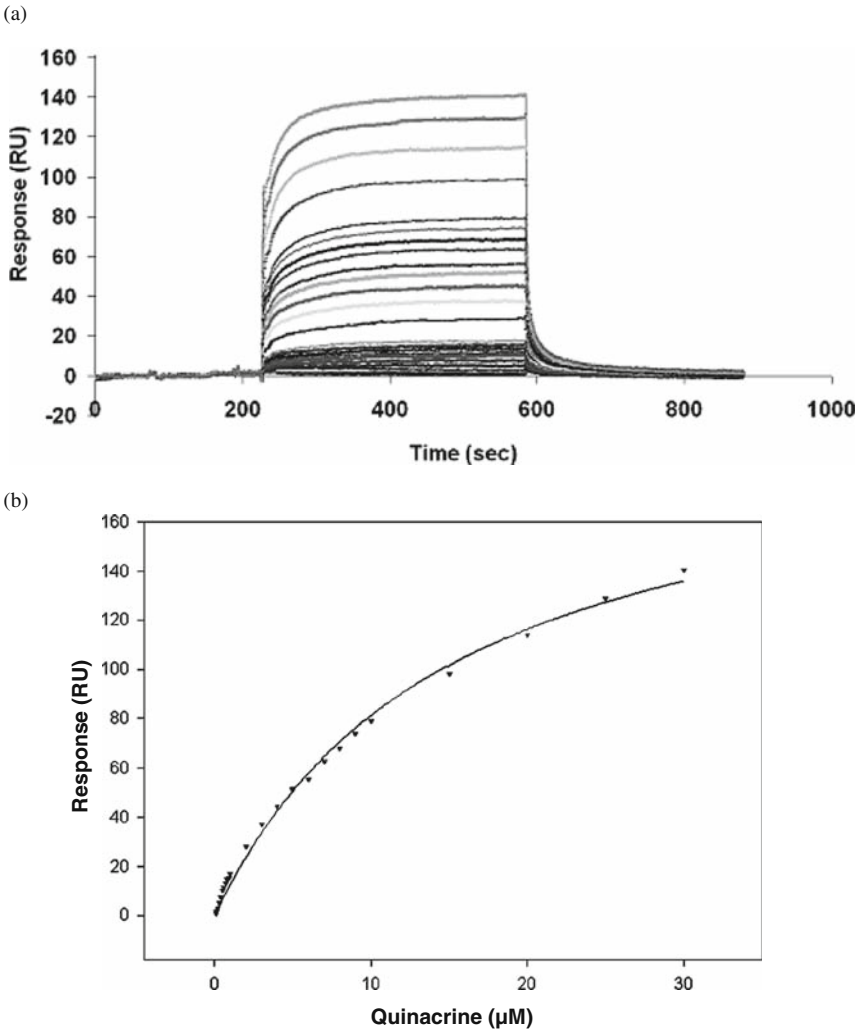

Fig. 9.2. Sensorgrams of quinacrine binding to huPrP^C (concentrations are from 0 to 30 μM) (**a**) and non-linear regression of kinetics data using Langmuir binding isotherm for 1:1 interaction (**b**) (K_d is estimated as 15 μM) (10).

2. For compounds requiring DMSO as a co-solvent, a series of concentrations were injected in triplicate, in ascending order, each containing 6.5% DMSO. DMSO calibration was performed over 2 min, instead of 1 min in the compound-screening protocol, and the equilibrium %RU_{max} calculated (*see* **Sections 3.2.2** and **3.2.3** for more details and **Note 11**). The sensorgrams were then overlaid, and the RU response obtained at the end of the association time was plotted against the concentration and analysed using Excel by non-linear regression and Langmuir 1:1 isotherm binding mode to give the affinity constant (K_d) for each compound (*see* **Fig. 9.2b**),

4. Notes

1. Biacore X-100 and Biacore S51 are a newer generation of SPR instruments reported to have higher sensitivities and are specially designed for small molecule drug screening with an automated DMSO calibration function and more advanced software package for kinetic analysis. The protocol detailed here can be readily applied to either instrument.
2. Ready-to-use HBS-EP buffer (sterile, filtered and degassed) can be purchased from Biacore.
3. Ready made EDC and NHS solutions of the required concentrations are readily available from Biacore.
4. Blocking reagent, ethanolamine, at a concentration of 1 M can be purchased from Biacore.
5. Flow cells 3 and 4 can be used to immobilise different PrP^Cs such as truncated $hPrP^C$ or mouse PrP^C by repeat manual injection, to ensure that the same immobilisation level as for $flhPrP^c$ is achieved, so that comparisons of a compound binding to different species of PrP^C can be easily made.
6. CM5 chip has been modified with carboxylated methyldextran. The carboxyl group is activated by EDC followed by reaction with NHS to form an active ester, which can then react with the amine groups on the PrP^C surface to yield stable amide bonds between the dextran surface and the protein. More details can be found in **Chapter 3**.
7. The immobilisation capacity varies from one protein to the other; optimisation of immobilisation conditions including protein concentrations is required. Here, 2 μg/mL is the optimal concentration to achieve the immobilisation level of around 4,000 RU for all prion proteins.
8. The required prion protein immobilisation level of 4,000 RU is calculated using the equation in **Note 12**. To account for the detection limits of the machine and baseline noise (5–10 RU), 40–100 RU for a small molecular weight compound is generally required at a given immobilisation level of the protein. For a compound like quinacrine (MW 400 Da), if a binding of 100 RU is required to be observed on the chip surface, the level of prion protein (MW 17,000 Da) required to be immobilised is around 4,000 RU, calculated by the manipulation of the RU_{max} equation in **Note 12**. A typical immobilisation sensorgram can be seen in **Fig. 9.1**.

9. Chemical principle of ethanolamine blocking can be found in **Chapter 3**.
10. Biacore 3000 does not have an auto solvent correction function; correction of solvent effect is carried out by two programmed DMSO calibration blocks each containing triple injections of five different DMSO concentrations. The data is exported to Excel to generate a calibration curve, and a correction factor can be extrapolated and used to adjust the RU obtained from any DMSO sample injections (Excel macro can be obtained from the author). Newer models of Biacore such as T100 and S51 can auto-correct DMSO solvent effects in both screening and kinetic analysis.
11. Detailed programmed procedures and macros can be obtained by contacting the author via e-mail: b.chen@sheffield.ac.uk.
12. $\%RU_{max}$ can be calculated in two steps. In Step 1, for a given immobilisation level of a protein (it can be extrapolated from immobilisation sensogram **Fig. 9.1**), the theoretical maximum binding resonance units (RU_{max}) for a ligand can be calculated using the following formula:

$$\text{Theoretical } RU_{max} = \left(\frac{\text{RU immobilised protein}}{\text{MW protein}}\right) \times \text{MW of the compound} \quad [1]$$

The above equation can be manipulated to calculate the immobilisation of a protein (RU-immobilised protein) if the theoretical RU_{max} is reset. As with quinacrine, a theoretical RU_{max} of 100 is deemed to be reasonable to take the baseline noise and its small molecular weight (MW = 400), the level of protein required to be immobilised (RU-immobilised protein) = 100 × 17,000 ÷ 400 = 4,250, hence the value of ~4,000 RU is proposed. When a specific immobilisation level is achieved, RU_{max} for the positive control, quinacrine, and every compound to be tested should be recalculated accordingly before $\%RU_{max}$ is calculated and any ranking of initial binding based on $\%RU_{max}$ takes place.

In Step 2, assuming 1:1 binding stoichiometry, $\%RU_{max}$ can be calculated by the following formula:

$$\%RU_{max} = \left(\frac{\text{RU of compound}}{\text{Theoretical } RU_{max} \text{ of the compound}}\right) \times 100 \quad [2]$$

13. For water-soluble compounds, rates of association and dissociation, k_a and k_d, as well as equilibrium kinetic constant K_D can be obtained by analysing using BIAevaluation software 4.1 equipped with Biacore 3000.

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