APPLICATION NOTE

Multimode Detection

Authors:

Saioa R. Elezgarai[1,3]

Emiliano Biasini[2,3]

Valentina Bonetto[1]

[1] Department of Molecular Biochemistry and Pharmacology

[2] Department of Neuroscience

IRCCS – Istituto di Ricerche Farmacologiche Mario Negri, 20156
Milan, Italy

[3] Dulbecco Telethon Laboratory of Prions and Amyloids

Centre for Integrative Biology (CIBIO), University of Trento, 38126
Trento, Italy

Identifying Pharmacological Chaperones for the Cellular Prion Protein using a Label-free Biochemical Assay on the EnSight Multimode Plate Reader

Introduction

Transmissible spongiform encephalopathies (TSE), also called prion diseases, are a group of fatal neurodegenerative disorders that affect both humans and animals[1]. The key pathogenic event underlying all forms of prion diseases is the conversion of the cellular prion protein (PrP^{C}) into an aggregated form (PrP^{Sc}) that self-propagates by imposing its abnormal conformation onto PrP^{C} molecules.

Previous attempts to identify anti-prion compounds have been aimed at reducing the load of PrP aggregates by decreasing their stability or increasing their clearance. Some of these compounds showed activity *in vitro*, but little or no efficacy *in vivo*. Multiple pieces of evidence support the notion that PrP^{C} loses its native fold in the initial steps of the aggregation process. This concept provides a rationale for tackling PrP^{C} aggregation by stabilizing the monomeric protein precursors, instead of disrupting pre-formed PrP^{Sc} species[2]. The underlying idea is to block aggregation by increasing the Gibbs free energy barrier (ΔG) required for the initial misfolding events. This goal could be achieved with small, high affinity ligands of PrP^{C}, capable of acting as pharmacological chaperones. In order to identify such compounds, we developed the label-free biochemical (LFB) assay as a novel screening method to identify high affinity ligands for PrP^{C}.

PerkinElmer®
For the Better

The LFB assay was performed on the EnSight™ Multimode Plate Reader equipped with Corning® Epic® optical label-free technology, which is a resonant waveguide grating based method, capable of detecting molecular interactions at the equilibrium. This assay opens up the possibility of performing highly sensitive label-free biochemical binding assays which avoid the use of tagged biomolecules. The assay measures changes in the index of refraction upon a binding event and this change is indicated by a shift in wavelength. The initial step involves the immobilization of the target protein onto the amine-coupling surface of the label-free biochemical microplate biosensor.

Next, the unbound target is washed away followed by further equilibration of the biosensor and the initial baseline read. Finally, the analyte is added to the immobilized target, enabling the final read (Figure 1). The EnSight software records the baseline and the final data, and the resulting change in wavelength, which is named the binding response, is calculated by subtracting the baseline signal from the final signal.

When LFB technology is applied to study a new target protein, several steps are necessary to optimize the new binding assay, including selecting the right pH and buffer conditions, optimizing the concentration of bound target, and evaluating assay robustness with respect to buffer conditions. In this application note all the necessary assay optimization steps performed for studying interactions between PrP^C and small ligands are described.

Material and Methods

Recombinant PrP Protein

Mouse PrP recombinant protein (rec-PrP) was expressed and purified in the laboratory of Alessandro Negro (Department of Biomedical Science, University of Padua, Italy). Human recombinant PrP was expressed and purified in the laboratory of Jesus R. Requena (CIMUS, University of Santiago de Compostela, Spain). Methods and protocols for both proteins were performed as described previously[3].

Compounds

The compound FeTMPyP (Fe(III) tetrakis (N-methyl-4-pyridyl) porphyrin pentachloride, Cayman Chemical, Ann Arbor, MI) was dissolved in H_2O, stored at -20 °C and sonicated before use. FeTMPyP was used for assay development and also as positive control in subsequent experiments. The small molecules (SM) were synthesized by many collaborators and are included in our own library. The small molecules were dissolved in dimethyl sulfoxide (DMSO) and stored at -20 °C. They were also sonicated before use in binding experiments.

Label-free Biochemical Assay

The EnSight Multimode Plate Reader was used to carry out the LFB experiments and LFB high sensitivity microplates (PerkinElmer) were selected for performing the binding experiments between PrP protein and small molecules. These plates allow increased immobilization of target protein and also higher accessibility to the immobilized protein leading to higher binding signals. Kaleido™ Data Acquisition and Analysis Software (PerkinElmer) was used to acquire and process the data.

First, LFB high sensitivity microplates were activated using a freshly prepared activation buffer (15 µL in each well) that contains 50 mM Sulfo-NHS (Thermo Fisher Scientific, Waltham, MA) and 200 mM EDC hydrochloride (Sigma-Aldrich, St. Louis, MO). The plate was incubated for 30 min at room temperature and washed three times using deionized H_2O. In each washing step 25 µL of H_2O was added to each well, then the plate was centrifuged for 2 min at 700 rpm (Eppendorf 5810R) and 25 µL of liquid was removed from the wells. After the final wash, the plate was centrifuged upside down with lid to remove all liquid from the wells.

For the protein immobilization, mouse or human PrP recombinant proteins were diluted using 10 mM sodium acetate buffer. The concentration of the protein differed depending on the experiment performed. 15 µL of protein solution was added to each well and after centrifuging the microplate for 2 min at 700 rpm, the microplate was sealed and incubated overnight at 4 °C for an optimal immobilization of the protein.

The following day the microplate was incubated for 30 min at room temperature and then equilibrated by performing 4 washing steps using assay buffer (10 mM PO_4, pH 7.4, 2.4 mM KCl, 138 mM NaCl, 0.005% Tween-20 and variable concentrations of DMSO). In each washing step 25 µL of assay buffer was added to each well, the plate was centrifuged for 3 min at 700 rpm and 25 µL of liquid was removed from the wells. The plate was incubated for two hours at room temperature and for the following hour, inside the reader. Subsequently, the baseline read was performed. Finally, 15 µL of freshly prepared dilutions of the small molecules were

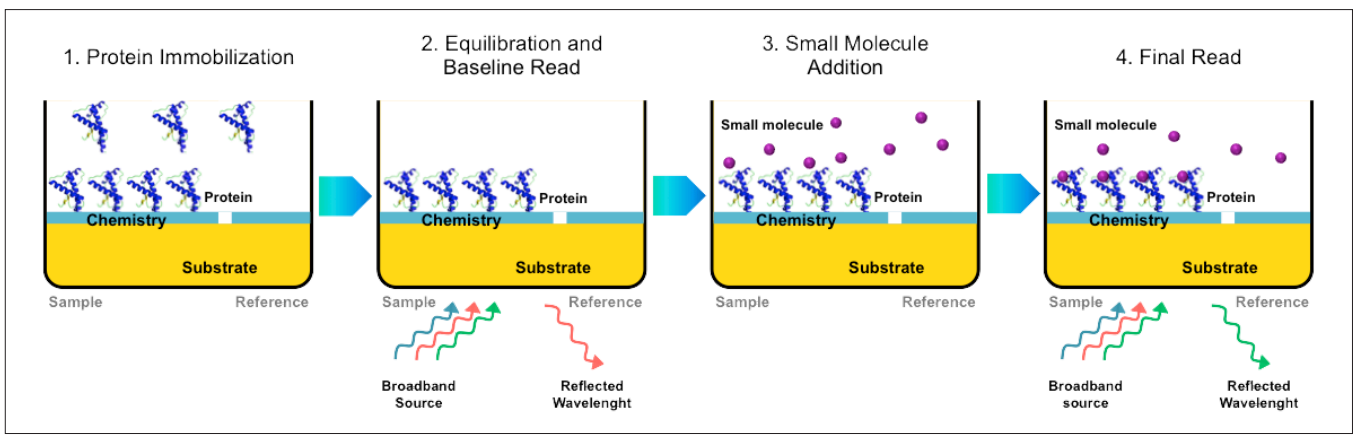

Figure 1. Adapted Label-free biochemical assay workflow for testing protein: small-molecule interactions[4].

added to each well, the plate was centrifuged for 2 min and after 30 min of incubation inside the plate reader, the final read was performed. The small molecules were diluted in assay buffer. The DMSO concentration was matched in all wells of the microplate, using the same concentration during the plate equilibration and during the binding assay.

Assay Automation

Zephyr® Compact Liquid Handling Workstation (PerkinElmer) was used for all liquid handling steps described above (Figure 2). Optimizing the assay using automated liquid handling decreased variability and increased reproducibility and speed. The Maestro software (PerkinElmer) was used to set up different applications corresponding to each assay step. Thus, different parameters were adjusted in each application for liquid aspiration and dispense steps: volume, speed, depth of the tip in the plate and number of mixing cycles.

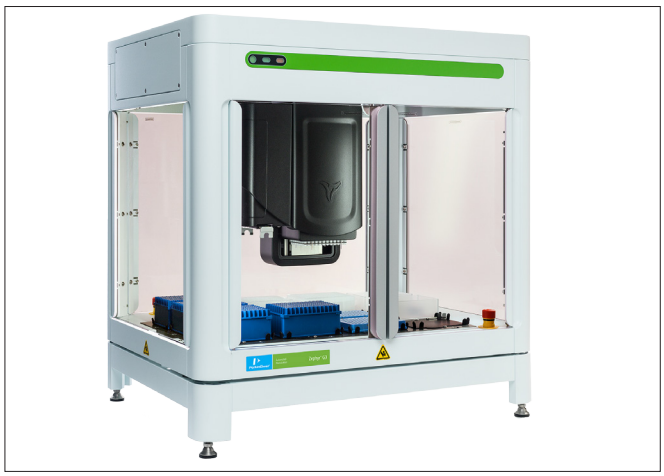

Figure 2. The Zephyr G3 Automated Workstation is the next generation benchtop liquid handler.

Results

PrP Immobilization Assay

The first objective in the development of the LFB assay was to choose optimal conditions for immobilization of the mouse or human recombinant PrP proteins. 10 mM sodium acetate buffer was used as immobilization buffer according to the previous experience of the laboratory using Surface Plasmon Resonance (SPR) technique with rec-PrP protein[6]. Eight different rec-PrP concentrations (20 µM, 10 µM, 5 µM, 2.5 µM, 1.25 µM, 0.62 µM, 0.31 µM, 0.16 µM) together with five different pH values (pH 4, pH 4.5, pH 5, pH 5.5, pH 6) were tested in order to select optimal conditions for the immobilization of the protein. Thus, immobilization buffers at different pH values were prepared and serial dilutions of the mouse rec-PrP were performed. All rec-PrP solutions were used for the immobilization of the protein in a LFB high sensitivity microplate by amine-coupling chemistry and using the data from the baseline read, the immobilization level for each condition was calculated. The immobilization level was not optimal (< 2000 pm) when the concentration of the protein was less than 1.25 µM. By contrast, between 2.5 µM and 20 µM of rec-PrP concentration, no significant differences were observed and more basic pH values (5-6) worked better (Figure 3A).

The same experiment was used for testing whether the selected assay buffer (PBS 1X pH 7.4, Tween 0.005%) was suitable for detecting the interactions between rec-PrP and small molecules. The binding of FeTMPyP compound, which binds to the unstructured region of PrP (determined using isothermal titration calorimetry, ITC)[5], was tested at 10 µM final concentration. The response was calculated subtracting the baseline from the final raw signals, and the raw signal of the reference area from the signal of sample area for each well. Based on this, the optimum detection of the binding (response) could be achieved for protein immobilization at 2.5 µM observing a clear decrease at lower and higher concentrations of rec-PrP (Figure 3B).

Taking these results into account, the following conditions were selected for the next binding experiments: 2.5 µM mouse rec-PrP in 10 mM sodium acetate buffer, pH 5 for protein immobilization and PBS 1X pH 7.4, Tween 0.005% assay buffer for the binding assays. Similar results were obtained with human rec-PrP (not shown).

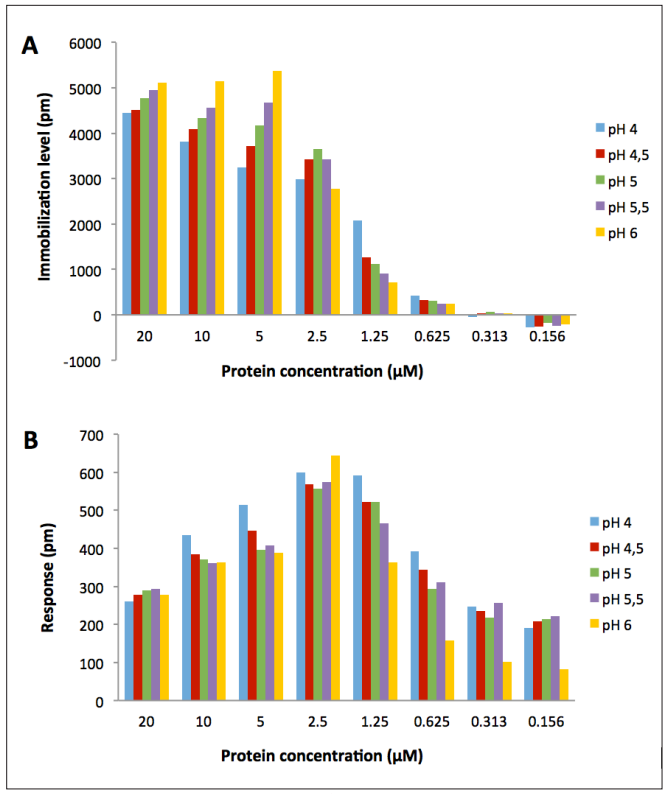

Figure 3. **Mouse PrP Immobilization Assay.** A) Graphical representation of the immobilization level obtained after immobilizing mouse PrP protein at different concentrations and different pH. The immobilization level was calculated comparing the raw signal of the wells containing the protein with the signal of buffer wells. B) Graphical representation of the binding response obtained with 10 µM FeTMPyP at different immobilization conditions.

LFB Assay Confirms Binding of Fe(III)-TMPyP to PrP^C

The next objective during the optimization of the assay was to test different concentrations of a predefined PrP^C ligand in order to obtain a dose-response curve of the binding and estimate the affinity constant (K_d). FeTMPyP compound was selected for this experiment. Full-length mouse rec-PrP protein was immobilized on the surface of an LFB high sensitivity microplate (15 µL of 2.5 µM rec-PrP) and FeTMPyP at different concentrations (0.1-300 µM) was added after the plate equilibration. Experimental controls, used to normalize the signals, included empty surfaces (reference area from each microplate well) and buffer injections.

Dose-dependent binding of FeTMPyP to PrP was observed in the concentration range of 0.1-1 µM and after which binding appeared to saturate (Figure 4). The affinity constant of the interaction was calculated (K_d = 0.63 µM) which was consistent with previously reported values confirming the reliability of the assay[6].

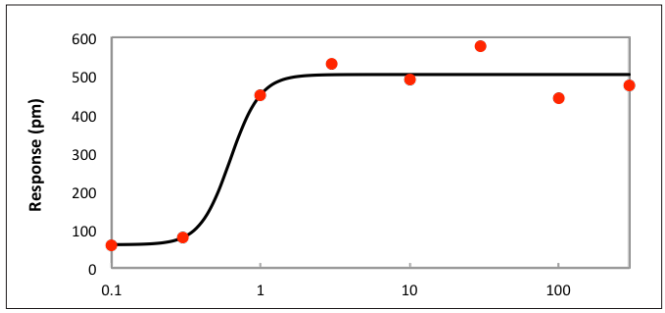

Figure 4. **FeTMPyP dose-response curve.** Different concentrations of FeTMPyP were tested by LFB assay after immobilizing full-length mouse PrP on the surface of an LFB microplate. The data (red dots) were fitted (black line) to a sigmoidal function using a 4 parameter logistic (4PL) nonlinear regression model. R_2 = 0.96; p = 4.4 x 10^{-3}.

Estimation of Assay Specificity

In order to evaluate the ability of the LFB assay to discriminate between true PrP^C ligands and non-relevant compounds, we tested several small molecules with low or no affinity for PrP^C (two of these are shown in Figure 5). These molecules were identified in previous in silico screening campaigns, and their binding to PrP^C evaluated by SPR. Once again, full-length mouse rec-PrP protein was immobilized on the surface of the LFB microplate and selected small molecules at different concentrations (5-1000 µM) were added after plate equilibration in order to monitor their binding to the protein.

We confirmed dose-dependent binding for small molecules predicted to interact with PrP^C (an example is shown in Figure 5A). By contrast, no binding was detected in the case of non-interacting small molecules obtaining response values close to 0 pm (Figure 5B). These results confirm the high specificity of the LFB assay. In a second independent experiment performed using the same molecules, previous data were confirmed showing the high reproducibility of the assay (data not shown).

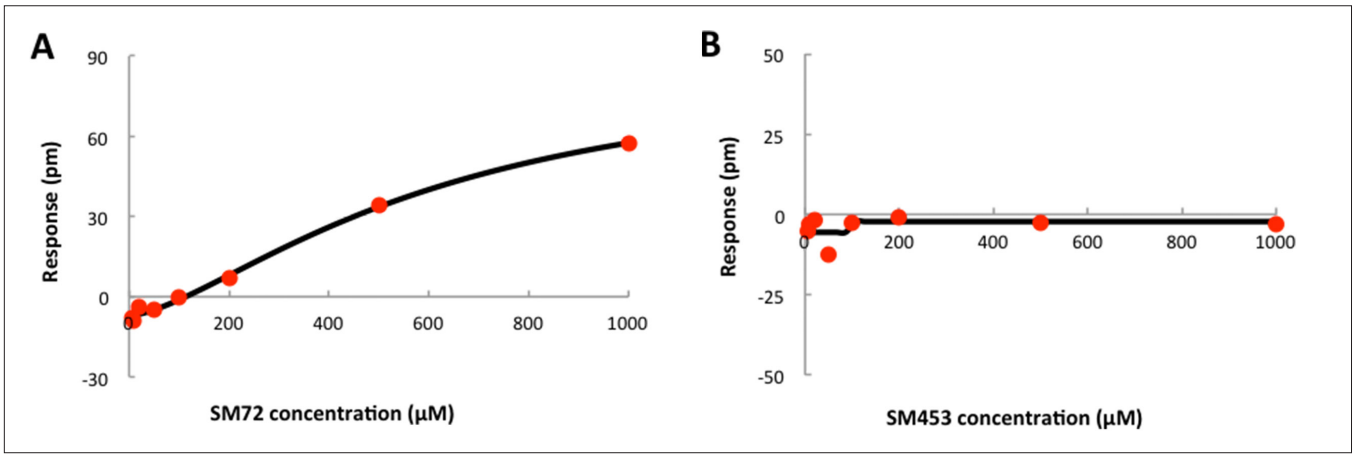

Figure 5. **Dose-response curves of previously characterized small molecules.** Different concentrations of small molecules were tested upon immobilization of full-length mouse PrP onto the surface of LFB microplates. The data (red dots) were fitted (black line) to a sigmoidal function using a 4 parameter logistic (4PL) nonlinear regression model. A) Example of PrP^C ligand (SM72); R^2 = 0.99, p = 4.5 x 10^{-5}; B) Example of a non-relevant compound (SM453), R^2 = 0.24, p = 0.86.

Conclusions

- We defined a novel screening method to rapidly identify PrP^C ligands, using the resonant waveguide grating based label-free technology on the EnSight Multimode Plate Reader.
- The assay was employed to confirm binding of a previously described PrP^C ligand [Fe(III)-TMPyP].
- Our analyses demonstrated that the LFB assay is highly specific, sensitive and reproducible.
- Using automated liquid handling reduced assay variability while increasing reproducibility and speed.
- Label-free binding results on the EnSight Multimode Plate Reader for the PrP^C ligand [Fe(III)-TMPyP] and two control compounds are in line with previously performed interaction studies using SPR and ITC.
- The new LFB assay is a reliable platform for the identification of novel PrP^C ligands, providing a unique opportunity to carry out HTS campaigns (ongoing at the time of publication).

References

1. Prusiner (1998). *Prions. Proc Natl Acad Sci USA* 95(23), 13363-83.
2. Biasini & Harris (2012). Targeting the cellular prion protein to treat neurodegeneration. *Future Med Chem* 4 (13), 1655-1658.
3. Negro *et al.* (2000). Susceptibility of the prion protein to enzymatic phosphorylation. *Biochemical and biophysical research communications* 271, 337-341.
4. Morgan *et al.* (2011). Sensitive protein:ligand biochemical assays using Corning® Epic® label-free technology on the EnSpire Multimode Plate Reader. *PerkinElmer Application Note.*
5. Nicoll *et al.* (2010). Pharmacological chaperone for the structured domain of human prion protein. Proc Natl Acad Sci USA 107, 17610-17615.
6. Massignan *et al.* (2016). A cationic tetrapyrrole inhibit toxic activities of the cellular prion protein. *Scientific Reports* 6, 23180.

For Research Only. Not for use in diagnostic procedures.

PerkinElmer, Inc.
940 Winter Street
Waltham, MA 02451 USA
P: (800) 762-4000 or
(+1) 203-925-4602
www.perkinelmer.com

PerkinElmer®

013038_01 PKI