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Quantitative monitoring of two simultaneously binding species using Label-Enhanced surface plasmon resonance

Lars Eng [a], Brandon L. Garcia [b], Brian V. Geisbrecht [b], Anders Hanning [a, *]

[a] Episentum, Karins väg 5, 194 61 Upplands Väsby, Sweden
[b] Department of Biochemistry and Molecular Biophysics, Kansas State University, Manhattan, KS 66506, USA

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Surface plasmon resonance (SPR) is a well-established method for biomolecular interaction studies. SPR monitors the binding of molecules to a solid surface, embodied as refractive index changes close to the surface. One limitation of conventional SPR is the universal nature of the detection that results in an inability to qualitatively discriminate between different binding species. Furthermore, it is impossible to directly discriminate two species simultaneously binding to different sites on a protein, which limits the utility of SPR, for example, in the study of allosteric binders or bi-specific molecules. It is also impossible in principle to discriminate protein conformation changes from actual binding events. Here we demonstrate how Label-Enhanced SPR can be utilized to discriminate and quantitatively monitor the simultaneous binding of two different species – one dye-labeled and one unlabeled - on a standard, single-wavelength SPR instrument. This new technique increases the versatility of SPR technology by opening up application areas where the usefulness of the approach has previously been limited.

1. Introduction

Surface plasmon resonance (SPR) is today a well-established method for the study of molecular interactions involving biological macromolecules as well as small molecules. The present state-of-the-art of SPR technology and its applications have been the subject of a number of reviews and monographs [1–3]. SPR provides a highly sensitive method for measuring refractive index changes in the vicinity (e.g. a few hundred nanometers) of a solid sensor surface. SPR is a universal method in the sense that any molecule binding to the surface causes a refractive index change and can be detected. The binding reaction can be monitored in real-time, and kinetic and affinity constants can be conveniently extracted. As a consequence, SPR is often referred to as an information-rich technology.

Unfortunately, the universal nature of SPR detection also constitutes a fundamental limitation: since every binding event causes a similar refractive index signal, it is not possible to monitor the complex case of two different molecules binding simultaneously to different sites on a protein [4]. This inherent limitation has greatly reduced the application of SPR-based approaches to the study of allosteric binding mechanisms. Today, the study of allosteric effects using SPR requires extensive mathematical modelling based on one-dimensional refractive index data only [5]. Another area of rapidly growing interest where the information content of conventional SPR may be limited is in the study of bispecific binders (e.g. bispecific antibodies). Here, specialized assay formats are required, and the binding of the two species must be studied in sequence, rather than in parallel [6]. A related case is that of competition-based assays where analyte molecules bind the surface species at the same or similar binding sites. In principle, competition assays can be performed using SPR by calculating residual binding, however, this is non-trivial for small molecule competitors as minor concentration differences in organic solvent vehicle (i.e. DMSO) across samples can have a large negative impact on the achievable signal to noise ratio and interfere with the ability to accurately determine the residual binding signal. A final problematic area is when binding of low molecular weight analytes causes conformational change in sensor chip surface-immobilized ligands. Given the size disparity between small molecules (i.e. 1–10 Å) and proteins (i.e. 10–100 Å), the small refractive index contribution caused by small-molecule binding may be overshadowed by larger positive or negative refractive index changes caused by the mass redistribution of protein upon conformation

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* Corresponding author.
E-mail address: anders.hanning@episentum.com (A. Hanning).

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Abbreviations

DP	Dip Position (of the SPR dip) (eRU)
DW	Dip Width (of the SPR dip) (eRU)
eRU	Enhanced resonance unit
K_D	Binding dissociation constant (M)
LE-SPR	Label-Enhanced SPR
RU	Resonance unit
SPR	Surface Plasmon Resonance

change. In many cases this leads to false positives, false negatives, or even negative sensorgrams upon small-molecule binding [7,8].

In this article, we show how the Label-Enhanced SPR (LE-SPR) technique increases the information content of SPR by enabling qualitative discrimination and quantitative monitoring of two different, simultaneously binding species using standard, commercial single-wavelength SPR instruments. LE-SPR is based on labeling of a binding molecule with a specialized dye label in combination with readout and software-based shape analysis of the entire SPR dip during the binding reaction. Here we show three examples of dual-species detection: i) the accurate quantitative determination of two different species in mixed bulk solution samples; ii) the qualitative deconstruction of a conventional sensorgram of two different, simultaneously binding species into separate binding curves of the two species, followed by the faithful reconstruction of the original sensorgram; and iii) the simultaneous determination of binding dissociation constants of two different species in a mixture binding to a protein on the surface.

2. Material and methods

SPR measurements were performed on Biacore™ 2000 or Biacore T200 (GE Healthcare). BiaEvaluation and T200 Evaluation software (GE Healthcare), EpiGrammer™ software (Episentum), GraphPad Prism™ (GraphPad Software), JMP™ (SAS Institute), and Excel™ (Microsoft) were used for data evaluation. Dye reagent Episentec™ B23 (Episentum) was used as the colored species in all experiments according to the manufacturer's suggestions.

2.1. Bulk solution experiments

Phosphate-buffered saline (PBS) (Sigma) was used as the running buffer, and also to dissolve all samples. 25 different samples were prepared: sodium chloride (p.a., Merck) at 0, 0.1, 0.2, 0.5, and 1.0 mg/ml and Episentec dye B23 carboxylate (Episentum) at 0, 0.01, 0.02, 0.05, and 0.1 mg/ml in all possible combinations. Care was taken to minimize evaporation during sample preparation. SPR was run on a Biacore 2000 instrument, using a sensor chip Pioneer C1 (GE Healthcare), and flow rate 30 μl/min. Dip position (DP) and dip width (DW) were calculated using EpiGrammer software. No reference channel subtraction or blank subtraction was performed. The sensor chip surface is negatively charged as is the B23 dye, so no surface binding occurs; this was confirmed in separate control experiments. 15 blank injections of buffer only were interspersed between the sample injections; the standard deviation of these were 1.0 eRU in the DP domain and 0.4 eRU in the DW domain.

2.2. Deconstruction/reconstruction experiments

An ELISA-like immunoassay that included three steps was used: 1) immobilization of a small molecule, an analogue of 3,4-methylenedioxy-methamphetamine (MDMA), on the sensor chip surface; 2) injection of a primary anti-MDMA IgG antibody; and 3) either a) injection of a dye-labeled secondary anti-IgG antibody, or b) injection of avidin, serving as a colorless protein, or c) a mixture of dye-labeled secondary antibody and avidin. In separate control experiments, avidin had been shown to bind non-specifically but reversibly to the sensor surface and not to compete with the secondary antibody. A goat anti-mouse-IgG antibody (Agrisera) was labeled with Episentec dye B23 NHS ester (Episentum) to a labeling degree of 3–4 dye molecules per antibody. SPR was run on a Biacore 2000 instrument using a running buffer of PBS-P+ (GE Healthcare) and flow rate 10 μl/min. An MDMA analogue (a kind gift from Biosensor Applications) was immobilized via amine coupling on a CM5 sensor chip (GE Healthcare). Primary mouse anti-MDMA IgG antibody (a kind gift from Biosensor Applications) was injected, followed by injection of either a) 2 μg/ml labeled secondary goat anti-mouse-IgG antibody, or b) 50 μg/ml avidin (Sigma), or c) a mixture of the two. Dip position (DP) and dip width (DW) were calculated using EpiGrammer software. No reference surface subtraction or blank subtraction was performed.

2.3. Dissociation constant experiments

Purified complement protein C3b was site-specifically biotinylated using a CVF-based C3 convertase and EZ-link Maleimide-PEG$_2$-biotin (Thermo Scientific) using a protocol identical to that described in Ref. [9]. All purified complement reagents were obtained from Complement Technologies. On a Biacore T200, 700 RU of C3b-biotin was captured on a CMD-200 sensor chip (XanTec Bioanalytics) which had previously been amine coupled with NeutrAvidin Protein (Thermo Scientific). For the binding studies, the thioredoxin-compstatin fusion protein (TRX-4W9A) was produced and purified as described earlier [10]. For labeling of the protein, TRX-4W9A was dialyzed into phosphate buffered saline (pH 7.4) for 2 h at room temperature, and then incubated for 1 h in the dark with 5 molar excess of dye reagent Episentec B23 NHS ester (Episentum) followed by dialysis into HBS at 4 °C overnight. In separate control experiments, a two-fold variable concentration dilution series of pure unlabeled (HBST buffer, 30 μl/min flow rate, 2 min association time, 3 min dissociation time) and pure dye-labeled (HBS buffer, 10 μl/min flow rate, 1 min association and dissociation times) TRX-4W9A, respectively, were injected over C3b. Dissociation constants (K_D) were calculated using conventional SPR and a 1:1 Langmuir equilibrium binding model. Then, 16 mixtures of the two species (unlabeled TRX-4W9A at 0, 500, and a two-fold dilution series from 2-5000 nM; dye-labeled TRX-4W9A at 0 and 500 nM) were run in LE-SPR mode. No reference surface subtraction was performed, but blank subtraction was performed. The data was evaluated using EpiGrammer, and split into its epigram and ektogram components as described below. To exclude any bulk RI disturbances appearing during the injection, binding data was read during the dissociation phase only: binding level at 110 s minus binding level at 150 s (dissociation early - dissociation late) [11]. Finally, the binding data of the mixtures was evaluated using global fitting of a 1:1 competitive equilibrium binding model as described below.

3. Results and discussion

Label-Enhanced SPR (LE-SPR) is based on labeling of a binding molecule with a specialized dye in combination with read-out and software-based shape analysis of the entire SPR dip during the binding reaction. The basic theory of LE-SPR and applications involving detection of one labeled species only have been described elsewhere [12,13]. Here is presented a short theoretical account of

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