Analytical Biochemistry 510 (2016) 79–87

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Label-enhanced surface plasmon resonance applied to label-free interaction analysis of small molecules and fragments

Lars Eng, Linnéa Nygren-Babol, Anders Hanning*

Episentec, 194 61, Upplands Väsby, Sweden

ARTICLE INFO

Article history:
Received 23 March 2016
Received in revised form
24 May 2016
Accepted 6 June 2016
Available online 17 June 2016

Keywords:
Surface plasmon resonance
SPR
Label-enhanced SPR
Sensitivity
Specificity
Kinetics

ABSTRACT

Surface plasmon resonance (SPR) is a well-established method for studying interactions between small molecules and biomolecules. In particular, SPR is being increasingly applied within fragment-based drug discovery; however, within this application area, the limited sensitivity of SPR may constitute a problem. This problem can be circumvented by the use of label-enhanced SPR that shows a 100-fold higher sensitivity as compared with conventional SPR. Truly label-free interaction data for small molecules can be obtained by applying label-enhanced SPR in a surface competition assay format. The enhanced sensitivity is accompanied by an increased specificity and inertness toward disturbances (e.g., bulk refractive index disturbances). Label-enhanced SPR can be used for fragment screening in a competitive assay format; the competitive format has the added advantage of confirming the specificity of the molecular interaction. In addition, label-enhanced SPR extends the accessible kinetic regime of SPR to the analysis of very fast fragment binding kinetics. In this article, we demonstrate the working principles and benchmark the performance of label-enhanced SPR in a model system—the interaction between carbonic anhydrase II and a number of small-molecule sulfonamide-based inhibitors.

The surface plasmon resonance (SPR) technology for biomolecular interaction analysis was introduced some 25 years ago. Originally, due to the inadequate sensitivity, the use of SPR was limited to studying the binding of larger molecules such as antibodies and other proteins. However, during these 25 years, the sensitivity of commercial SPR instruments has been continuously improved, and today SPR is a well-established research tool to study the binding also of low-molecular-weight (LMW) compounds. One particularly important, but also challenging, application of SPR is fragment screening in the early drug development process. In fragment screening or fragment-based drug discovery (FBDD), medium-sized libraries (a few hundred to a few thousand compounds) of very small molecules (molecular weight on the order of 100–300 Da; 8–23 non-hydrogen atoms) are screened for binding against the drug target of interest [1–3]. The advantage of fragment screening as opposed to high-throughput screening is that the sampling of the chemical space is more efficient and, hence, smaller libraries can be used. The fragment hits, considered as building blocks of more complex drug-like compounds, are then grown and developed into somewhat larger lead molecules and taken downstream in the drug development process. In FBDD, SPR is used in a screening cascade to initially screen for the binding of fragments to the target, to confirm binding and estimate binding kinetic and affinity constants in a secondary screen, and to assess the specificity of the binding event in competitive assays in a third step. Today, SPR is a well-established technology in FBDD—indeed, SPR has become one dominant method for fragment finding—and there are numerous articles published on the topic [4–15].

However, there are still LMW applications where the state-of-the-art performance of modern SPR instruments constitutes a limitation. One such application is fragment screening on difficult drug targets, that is, targets that are difficult to immobilize on the sensor chip surface at a high density with a retained high binding activity. The number of targets that can be fragment screened today is limited to the targets that can be immobilized in a sufficiently large amount with a retained high functional binding activity. There is a strong desire and a trend within the pharmaceutical

Abbreviations used: SPR, surface plasmon resonance; LMW, low-molecular-weight; FBDD, fragment-based drug discovery; RU, resonance unit; DMSO, dimethyl sulfoxide; eRU, enhanced resonance unit; CAII, carbonic anhydrase II; MeSA, methanesulfonamide; SAA, sulfanilamide; AEBSA, 4-(2-aminoethyl)benzenesulfonamide; CBS, carboxybenzenesulfonamide; Nap, naproxen; Phe, phenytoin; War, warfarin; Fur, furosemide; Azo, azosulfamide; Dig, digitoxin.

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

http://dx.doi.org/10.1016/j.ab.2016.06.008
0003-2697/

industry to be able to extend the range of screenable targets, with a particular interest relating to the highly druggable but not so easily screenable G-protein-coupled receptors and other membrane proteins [7,8,12,15–20]. Furthermore, a high immobilization level of the protein target is frequently required, in particular for large proteins, to obtain a measurable signal on fragment binding even though it is well known that low immobilization levels are recommendable to avoid protein–protein interaction effects and steric effects due to crowding, to minimize the effect of mass transfer limitations, and to minimize the nonspecific binding [10,15]. Sparsely immobilized surfaces may also give a more correct representation of the biological state of proteins in cell membranes. Moreover, because fragments bind with low affinities, with binding dissociation constants often being 0.1–10 μM or even weaker, the occupation rate is inherently low. This, in combination with the small size, may require the use of high sample concentrations that cause problems with solubility, aggregation, and nonspecific binding [8,10,13–15]. When it comes to kinetic measurements, the measurable time constant of today's instruments is on the order of 1 s due to limitations in the microfluidic system rather than to limitations of the optical measurement system. The binding kinetics of low-affinity fragments is often considerably faster than this and consequently cannot be monitored [5–7,10,12,14,15,19]. Finally, the impressively low refractive index short-term noise of modern SPR instruments, down to approximately 15×10^{-9} refractive index units or 15×10^{-3} resonance units (RU), is seldom realizable in practical experiments due to effects of, for example, nonspecific binding, changes in target conformation, variations in bulk solvent composition (e.g., the ubiquitous variations of dimethyl sulfoxide (DMSO) concentration in fragment sample solutions), and instrument baseline drift, which effects may be orders of magnitude larger than the refractive index detection limit per se. In practice, and depending on the specific system at hand, binding signals below 1 to 5 RU are difficult to quantify accurately [4,6,7,11,21,22].

One way of increasing the performance of SPR instruments and extending the application area of the SPR technology is through the use of label-enhanced SPR. The basic theory of label-enhanced SPR and a conceptual demonstration of the technique have been published elsewhere [23]. Label-enhanced SPR is based on labeling of one interactant with a dye label possessing a strong absorbance and a high refractive index, followed by evaluation of the full shape of the SPR dip curve on binding of the dye-labeled species. In brief, the refractive index mainly influences the angular position of the SPR dip minimum, whereas the absorbance mainly influences the angular width of the SPR dip. By measuring both the position and width and then solving a simple linear equation system, both the refractive index and the absorbance on binding to the chip surface are monitored. This is in contrast to conventional SPR analysis, where only the angular position of the SPR dip is measured, resulting in monitoring of the refractive index only. Extracting absorbance information from the SPR dip yields an absorbance sensorgram—here termed “epigram”—that reflects the binding event with a very high sensitivity. Furthermore, the absorbance sensorgram displays a very high specificity with respect to the binding of the labeled species because most disturbing factors (e.g., temperature variations, variations in the composition of the running buffer, nonspecific binding of unlabeled sample components to the chip surface) generally affect only the refractive index but not the absorbance.

Dye labeling of an interactant may, to a larger or smaller extent, influence the binding behavior (i.e., the kinetics and affinity of binding) of the labeled species. However, by using an indirect assay format (i.e., a competition or inhibition assay), truly label-free data can be obtained. For example, by using a surface competition assay where the unlabeled analyte competes with binding to the surface with a dye-labeled competitor, and by monitoring the binding of the dye-labeled species only (the absorbance sensorgram or epigram), the true kinetic and affinity constants for binding of the unlabeled analyte can be obtained through the well-established Motulsky–Mahan equations for competitive binding originally developed for radiochemical competition assays [24–27]. Qualitative competition assays are quite well established in conventional refractive index SPR analysis [4,9,28,29]. Some examples of quantitative competition assays in conventional SPR analysis have also been published but are more complicated because more accurate data are needed and because the exact molecular masses and molecular refractive index increments of both competing species need to be exactly known [10,20,22,30].

In the current work, we report the application of label-enhanced SPR to monitoring of the binding of LMW compounds and fragments using a commercially available SPR instrument. We demonstrate the enhanced sensitivity and the ability to measure true kinetic and affinity constants of even very small *unlabeled* molecules with a high sensitivity in the competitive format. We perform a small, simplified LMW/fragment competition assay screen where the binding signal is independent of the size of the binding molecules, and we demonstrate the high specificity embodied in the absence of bulk refractive index disturbances caused by variations in the DMSO concentration of different samples. Finally, we show how the competitive format enables an extension of the measurable range of binding kinetics beyond what is possible for direct binding measurements. We have selected to work with the carbonic anhydrase II system because it is a frequently used benchmarking system for SPR-based assays; however, the focus is on the analytical technique as such, not on the specific target molecule.

Materials and methods

All experiments were performed on a standard Biacore 2000 instrument (GE Healthcare Life Sciences, Sweden) using CM5 carboxymethyl–dextran sensor chips at 25 °C. Kinetic curve fitting was performed using BiaEvaluation 4.1 software (GE Healthcare Life Sciences). Used in the conventional mode, the Biacore instrument provides a measure of the refractive index close to the sensor surface expressed in resonance units. The RU is not a unit that can be derived from first principles, but it is based on an arbitrary definition implemented in the Biacore software. 1 RU corresponds to approximately 1×10^{-6} refractive index units or approximately 1 pg/mm^2 of adsorbed protein.

Enhancement of sensorgrams—or, in other words, calculation of absorption sensorgrams—was performed using EpiGrammer 2.1 software (Episentec, Sweden). Enhanced sensorgrams, also termed epigrams, provide a measure of the absorbance close to the sensor surface and consequently are specific for dye-labeled species. The unit of the epigram is enhanced resonance units (eRU). In analogy with the RU, the eRU is based on a definition implemented in the EpiGrammer software. To comply with the Biacore convention, 1 eRU corresponds to approximately 1 RU for the labeling dye B23 (Episentec). However, for most substances, because the eRU is a measure of absorbance, it cannot be numerically compared with the ordinary RU.

For all experiments, the enzyme human carbonic anhydrase II (CAII, 30,000 Da) was immobilized on the chip surface using amine coupling chemistry. The immobilized amount of protein was approximately 1000 RU, which is considerably less than what is ordinarily used for this enzyme (i.e., 5000–10,000 RU); the reason for this was to demonstrate the achievable sensitivity using only a low surface density of protein.