



Gradient method for accurate affinity determinations

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ABSTRACT

The value of the affinity constants (k_d , k_a , and K_D) that are determined by label free interaction analysis methods are strongly affected by the ligand density at the sensor surface [1]. This paper outlines a new SPR-imaging method that applies a ligand density gradient enabling the analyte response to be extrapolated to $R_{\max} = 0$ μ RIU. The mass transport limited region is used to determine the analyte concentration. Cumbersome optimization procedures for tuning the ligand density is prevented and surface dependent effects as rebinding, strong biphasic behavior etcetera are minimized. The method can be fully automated for e.g. accurate determination of the quality of antibodies from commercial sources.

For multiple applications, the quality of binding, expressed as the dissociation equilibrium constant (K_D), is of great importance. The K_D value (M, mol/L) in a 1:1 Langmuir model is the analyte concentration at which in equilibrium 50% of the ligand molecules are specifically bound with analyte molecules [1]. The constants that are derived from current, immobilized ligand based assays are affected by the immobilized state of the ligand [2]. This causes the thus determined, apparent constants to deviate from the true, “solution” constants due to interfering effects that result from the immobilization of the ligand [3]. These interfering effects include rebinding effects, mass transport limitation, non-specific binding and deviation from the 1:1 model binding summarized below. The higher the ligand density, the more pronounced these interfering effects become and it is generally accepted that the ligand density should be applied just above the limit of detection of the biosensor instrument [5]. The same holds for the analyte concentration - interfering effects will occur when multiple analyte molecules compete for interaction with a single immobilized ligand molecule.

This technical note describes a general method to determine simultaneously the concentration and the rate and affinity constant of an analyte to an immobilized ligand in a ligand density gradient. The so-called Vysion SPR imager of Vysens B.V. Hengelo, The Netherlands, applies a valve-less consecutive injection of samples in a so-called cuvette injection flow cell (CIF) (Fig. 1). Further it enables to generate two ligand density gradients on the sensor surface. “Back-and-forth” flow-based fluidics enables unlimited interaction times using only 100 μ L of sample. An automated kinetic titration experiment [8] of three analyte dilutions using the CIF, either with or without a regeneration step, can be applied.

Xantec sensors (gel-type HC30 M, Xantec Bioanalytics GmbH Dueseldorf, Germany) using EDC-NHS activated surface chemistry can be applied for immobilizing the ligand density gradient on the sensor surface or any other immobilization method. The 1:1 Langmuir interaction model is embedded in Scrubber2 (BioLogic Software, Campbell, Australia) [6] or Tracedrawer software (RidgeView Diagnostics, Uppsala, Sweden) by global analysis. Antibody binding follows a biphasic behavior at higher ligand densities, however at very low ligand densities the antibody will follow mostly monophasic behavior, because the antibody is not yet able to bridge the immobilized antigens. So in principle a user should analyze the biomolecular interaction with different models depending on the ligand density. At $R_{\max}$ at the limit to zero, one can consider the analyte ligand interaction as a single molecular event which can be analyzed by the most simple and favorable monophasic binding model.

Generally in complex media (patient samples e.g. serum, urine, synovial liquid etc.) non-specific binding often will result in false positives because abundant proteins other than the analyte interact (at lower affinity) with the sensor surface and ligand molecules. A general strategy applied in diagnostic tests is that the ratio of specific versus non-specific binding can be tuned by both the degree of sample (analyte) dilution and the ligand density. In label free POC devices the concentration should preferably be measured in the mass transport limited regime at lower analyte concentration because in this way fastest results (e.g. in five minutes) are obtained instead of incubating to depletion or equilibrium (as in ELISA).

The causes of non-specific binding and the influence to the binding kinetics can be grouped as follows [1]:

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- (1) Rebinding of dissociating molecules. (This results in an apparent slower off-rate)
- (2) Parallel binding processes and/or cross-reactivity and formation of multi-assembly complexes.
- (3) Steric hindrance and/or “avidity” effects. (apparent slower on-rate)
- (4) Ligand immobilization artifacts and heterogeneity of surface binding sites. (distribution of constants)
- (5) Mass transport limitation (e.g. analyte depletion of the layer close to the immobilized ligand surface).
- (6) Additional interfering effects that do not fit into one of these groups such as complex reactions (e.g., induced fit, allosteric conformational change, heterogeneity).

Selective measuring the binding interaction of an analyte to the ligand is by immobilizing the ligand to the surface. For protein ligands, this bears the possibility that its conformational ensemble may be skewed or even significantly altered, even when using chemically and/or structurally uniform attachment strategies. Further, while in free solution all molecules experience the same environment, the microenvironment at the surface may be strongly variable dependent on the location on the surface (e.g., from surface roughness), or dependent on the location within an inhomogeneous matrix. A so-called K_D^{RO} method for the determination of affinity constants has been published in 2011 [3,4] and an interpolation method paper in 2016 [5], in which the contribution of interfering effects is minimized so that the constants are a better estimate of the true constants of biomolecular interactions in solution.

Recognized practical effects are additional ligand immobilization artefacts and heterogeneity of surface binding sites [7]. The method will not compensate for this and the alternative route is by capturing ligands followed by the target interaction. When a harsh regeneration step is included the R_{max} value will decrease after the subsequent injections of the analyte concentrations and can again affect the kinetic affinity constants. Preferably any regeneration step of the surface should be avoided and this is achieved using kinetic titration [8].

Injection of a low analyte concentration followed by two subsequent injections of higher concentrations of the analyte can be performed (e.g. concatenated steps by factor 2). The kinetic titration method with global fits to the on- and off-rates for various levels of R_{max} showed a significant improvement in reliability when *interpolation to R_{max} = fixed value* is applied [5] It turned out that it is rather difficult to immobilize low ligand densities at discrete spots for reliably applying the extrapolation

method to $R_{max} = 0$ according to Ref. [4] while interpolating to a fixed R_{max} value is more convenient using the method of [5].

As the ligand density of an antibody captured to the sensor surface is tuned in Biacore instruments to obtain affinity values at $R_{max} = 50\text{--}100$ RU [9], these values are considered to be reliable.

In SPR imaging instruments the gradient enables that all ligand densities are available from very high to low up to zero (Fig. 2). The zero ligand density is used as the reference signal to compensate for common mode signals as bulk refractive index shifts, temperature effects, non specific binding to the hydrogel, etc. Further it is important that the functional concentration, as applied in the fitting routine, is determined accurately for calculating the absolute affinity values of K_D and k_a . The functional concentration is determined instantly under mass transport controlled conditions and an additional analysis is not necessary [7]. So, the steepness of the gradient should be as high that the high ligand density in the CIF meets mass transport limitation conditions for analyte binding. It is not necessary to tune the level of high ligand density but tuning the concentration of the analyte is necessary to get “curvature” at low ligand density. When the flow cell is calibrated for antibody concentrations in the mass transport controlled regime interactions in the CIF then dual analysis can be carried out at the location of high ligand density for concentration measurements. Additionally at several locations of low ligand density kinetic analysis can be performed where the analyte interaction meets $R_{max} = 0\text{--}100$ μ RIU to enable to extrapolate to $R_{max} = 0$ μ RIU. The more levels close to 0 are applied in the calculation the more accurate the fitting to zero response will be performed automatically by the software. So from hundreds of biomolecular interaction events on the gradient the single molecular affinity parameters can be determined. It is also theoretically possible to compare experiments measured e.g. with Biacore instrument with a “wrong” ligand density.

If the method was available during the COVID19 pandemic then we could measure the quality of antibodies of COVID-19 patients faster and more consistent. It took us more than a year to measure the COVID-19 patients of paper Hendriks et al. [10]. Applying the plug and play method it is foreseen that an unexperienced user will generate highly accurate effective concentration and reliable affinity or avidity parameters of the ligand-analyte interaction of interest from a single experiment in about 15 min.

Author contributions - CRediT

The author confirms sole responsibility for the following: study conception and design, data collection, analysis and interpretation of

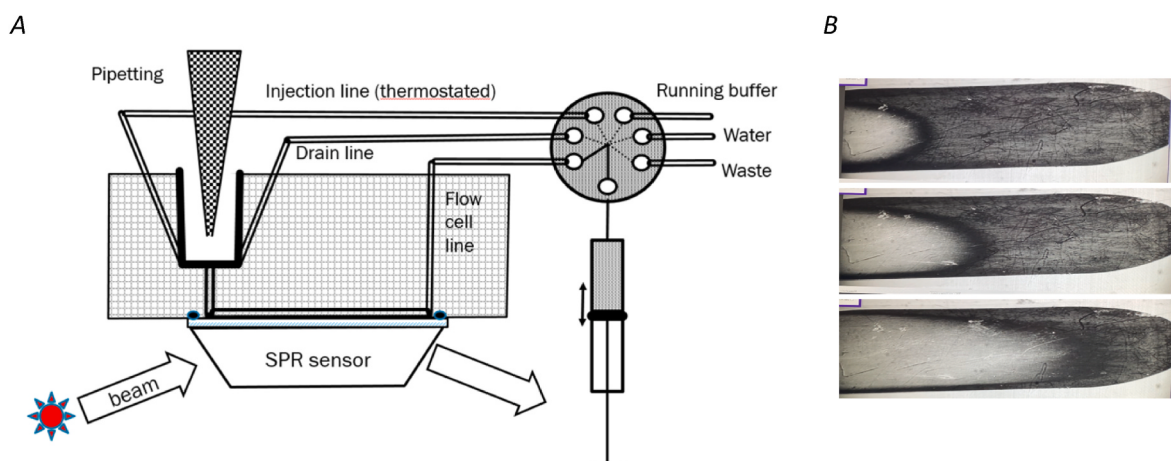


Fig. 1. Panel A: Schematic presentation of the fluidics of the Vysion SPR imager of Vysens B.V (patent pending). The cuvette can be considered as a micro-bioreactor and is connected to an injection line and a drain line. It enables to generate automated dilutions of the sample. The cuvette is closely connected to the entrance of the flow cell by a low volume channel. The sample can be manually injected into the cuvette or by means of an autosampler. Panel B: Fully controlled injection to generate a ligand density gradient on an activated sensor surface for timely exposure of the ligands. Images were taken at 1 min (top), 2 min (middle) and 3 min (bottom) after injection.

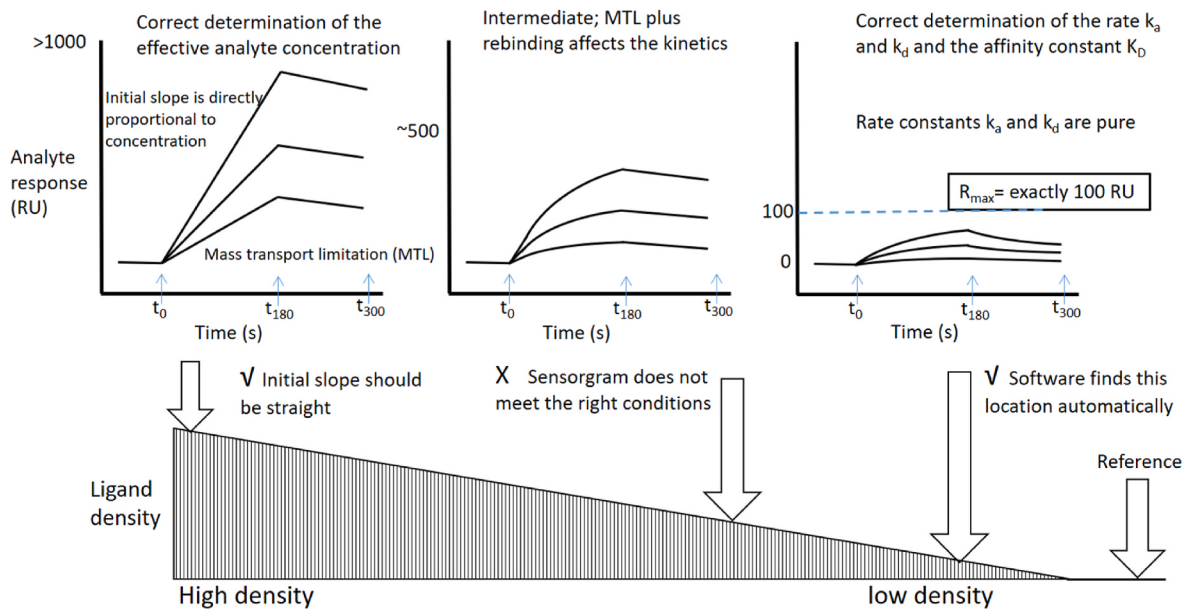


Fig. 2. Explanation of the method with a regeneration step. Sensorgrams from three analyte injections at various locations on the ligand density gradient; The reference location is at zero ligand density. Plug & play determination of the concentration and affinity constant in ~15 min.

results, and manuscript preparation.

Data availability

Data will be made available on request.

References

- [1] R.B.M. Schasfoort (Ed.), *Handbook of Surface Plasmon Resonance*, Royal Society of Chemistry, London, 2017.
- [2] H. Zhao, L.F. Boyd, P. Schuck, Measuring protein interactions by optical biosensors, *Curr. Protoc. Protein Sci.* 88 (1) (2017) 20–22.
- [3] W. de Lau, et al., Lgr5 homologs associate with Wnt receptors and mediate Responding signaling, *Nature* 476 (2011) 293–297.
- [4] R.B.M. Schasfoort, et al., "Method for estimating the single molecular affinity.", *Anal. Biochem.* 421 (2012) 794–796, 2.
- [5] R.B.M. Schasfoort, et al., Interpolation method for accurate affinity ranking of arrayed ligand–analyte interactions, *Anal. Biochem.* 500 (2016) 21–23.
- [6] Rich R.L. Myszkowski D. Survey of the year (1999 upto 2011) optical biosensor literature. *J.Mol.Recognit.* See <https://www.sprpages.nl/reviews/surveys>.
- [7] P. Schuck, H. Zhao, The role of mass transport limitation and surface heterogeneity in the biophysical characterization of macromolecular binding processes by SPR biosensing, in: *Methods in Molecular Biology* vol. 627, Springer protocols, 2010, pp. 15–54.
- [8] R. Karlsson, P.S. Katsamba, H. Nordin, E. Pol, D.G. Myszkowski, Analyzing a kinetic titration series using affinity biosensors, *Anal. Biochem.* 349 (2006) 136–147.
- [9] B. Watts, K. Cronin, Guide to Running an SPR Experiment Version 1.3, Duke Human Vaccine Institute Biomolecular Interaction Analysis Core Facility. 4. Lower Surface Density ($R_{max} = 100\text{--}150$ RU) for Kinetic Titration, 2022.
- [10] J. Hendriks, et al., High titers of low affinity antibodies in Covid-19 patients are associated with disease severity, *Front. Immunol.* (2022) 1620.