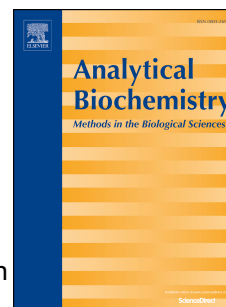


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Interpolation method for accurate affinity ranking of arrayed ligand – analyte interactions

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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 affected by the ligand density. This paper outlines an SPR- imaging method that yields high-throughput globally fitted affinity ranking values using a 96-plex array. A kinetic titration experiment without a regeneration step has been applied for various coupled antibodies binding to a single antigen. Globally fitted rate (k_d and k_a) and dissociation equilibrium (K_D) constants for various ligand densities and analyte concentrations are exponentially interpolated to the K_D at $R_{\max}=100$ RU response level (K_D^{R100}).

Key words: Biosensor, Label free, Kinetics, Affinity, SPR imaging

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. Since the K_D is used for the determination of the effective dosage levels of biomolecules that are applied as drugs, the determined K_D should reflect the K_D in solution. However, the constants that are derived from current, immobilized ligand based assays are affected by the immobilized state of the ligand [1]. 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 [2]. These interfering effects include rebinding effects, mass transport limitation, non-specific binding and deviation from the 1:1 model binding [3]. 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 [4]. The same holds for the analyte concentration - interfering effects will occur when multiple analyte molecules compete for interaction with a single immobilized ligand molecule.

The IBIS MX96 instrument (IBIS Technologies, Enschede, The Netherlands) applies a valve-less consecutive injection of samples [5]. "Back-and-forth" flow-based fluidics enables unlimited interaction times using only 100 microliter sample that can be recovered. Further the MX96 applies in the dissociation phase so-called co-pumping to keep the concentration of dissociating analyte zero to reduce rebinding effects. The continuous flow microspotter (CFM, Wasatch Microfluidics, Salt Lake City, Utah, USA) enables high reliability printing of ligand molecules under flow conditions [6]. SensEye® sensors (gel-type E2S, Ssens, Enschede, The Netherlands) using preactivated surface chemistry were applied for printing an array of monoclonal antibodies in a ligand density gradient (3x dilution series). The instrument enables multiplex, up to 96 spots, kinetic analysis of interactions using "kinetic titration" without a regeneration step. The latter can be used for capture surfaces (E.g. anti-IgG or anti-tag SensEye®) and can be applied to test supernatants directly from crude cultivation broths. The 1:1 Langmuir interaction model is applied in Scrubber2 software (BioLogic Software, Campbell, Australia) by global analysis, which has become a global standard for SPR-users e.g. Biacore. Scrubber2 has been

adapted for the IBIS MX96 to calculate the affinity constants from the sensorgrams generated on 96 spots simultaneously and to globally fit all these 96 interaction data at once.

A so-called K_D^{RO} method for the determination of affinity constants has been published in 2011 [1,2] in which the contribution of interfering effects is minimized or theoretically zeroed, so that the constants are a better estimate of the true constants of bio-molecular interactions in solution. This method is based on the extrapolation of the number of immobilized ligand and analyte molecules to zero, thus mimicking the interaction in which only one ligand and one analyte molecule are involved, enabling a true 1:1 binding model with theoretically not any interfering effect.

Recognized practical effects are additional ligand immobilization artefacts and heterogeneity of surface binding sites. 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 [7].

As already indicated, the calculation of the “true” affinity equilibrium constant will become more reliable at lower densities, preferably at a “density” of only a single immobilized ligand molecule acting as a free ligand [1,2]. Then the contribution of the interfering effects will be zero and will no longer influence the rate- and affinity equilibrium constants. Practically, this condition cannot be measured and by decreasing the ligand density the more noisier and less reliable the sensorgrams become. Additionally the quality of fits to noisy curves cannot be judged adequately. It should be noted that immobilization artefacts and heterogeneity of surface binding sites should be prevented, for instance by oriented capturing of the ligands by applying high affinity anti-ligand antibodies or using tag – anti-tag interactions.

As proof of principle for interpolating the affinity values to a fixed R_{max} value an experimental ranking test of eight monoclonal antibodies directed against the Epithelial Cell Adhesion Molecule (Active human EpCAM protein fragment (ab155712) Abcam Cambridge, UK) was performed. The monoclonal

antibodies HO-3 (Trion Research GmbH, Planegg, Germany), EpAb3-5, EpAb1-3, EpAb5-4, OC9-1 (BioMab Inc, Taipei, Taiwan), VU1D9 (kind gift from Immunicon Corp., Huntingdon Valley, PA, USA), 323/A3 (Pierce #MA5-12436, Waltham, MA, USA), SPM134 (Abnova #MAB12106, Taipei, Taiwan) [8,9,10,11,12]. Since the antibodies were not characterized for regeneration conditions, it is preferable to run a kinetic titration experiment where the 54 kDa EpCAM protein fragment was injected starting with a low concentration of 0.5 nM and ending with the highest concentration of 32 nM. Recently the analysis of such a kinetic titration experiment with a “floating align point fitting” was embedded in the analysis software for 96-plex interaction analysis. Floating align point fitting means that the subsequent injections of the antigen will be fitted without having the align point equal to the injection time point. During the measurement the antigen interaction will generate an increasing signal and each new baseline level in the kinetic titration is the result of the accumulated analyte bound to the ligand molecules. So the align point that is used for the fit calculations is not equal to the injection time zero, but always earlier in the concatenated series. The amount of immobilized protein will cause a shift of the SPR-dip as measured by the R_{LL} value (response of local ligand density) that can be considered as the total protein density per spot, whereas the amount of **functional ligand** is related to the fitted R_{max} value. Streamlining and selecting relevant data is carried out using the tile plots in SPRintX and Scrubber 2 (Top left of figure 1 for 8 spots only).

Injection of a very low antigen concentration followed by subsequent injections of higher concentrations of the antigen was performed (concatenated steps by factor 2).

*** Figure 1 here ***

*** Figure 2 here ***

This notes and tips article presents the results obtained from an affinity ranking experiment of a single antigen: EpCAM protein fragment (MW 54 kD) towards a panel of eight antibodies. Affinity ranking information was obtained by injections of the various antigen concentrations over the high affinity coupled antibodies without a regeneration step. This 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. Although theoretically correct, exponential *extrapolation* to $R_{\max}$ = zero may lead practically to deviated ranking values when different ranges of $R_{\max}$ are measured. Extrapolation to $R_{\max}$ =0 of these different ranges will result in less comparable ranking values and the lower values contribute highly how the exponential extrapolation intersects the Y-axis at $R_{\max}$ =0. Generally practical ranking of many antibodies simultaneously can be performed when the $R_{\max}$ ranges of all these antibodies overlap which is the case with $R_{\max}$ = 100 RU. Then an $R_{\max}$ =fixed value can be chosen so that these values can be better ranked with respect to each other and $R_{\max}$ values lower than 20 RU can be discarded.

Table 1	k_a ($s^{-1}M^{-1}$)	k_d (s^{-1})	K_D (M)	K_D (M)
			$R_{\max}=100$	$R_{\max}=0$
EpAB3-5	3.1E+05	1.1E-05	2.6E-11	1.6E-11
HO-3	1.5E+05	5.9E-06	4.0E-11	2.1E-11
VU1D9	3.1E+05	8.2E-05	2.7E-10	2.3E-10
SPM134	2.6E+05	9.3E-05	3.5E-10	3.9E-10
323/A3	2.9E+05	1.7E-04	5.8E-10	5.4E-10
OC-9-1	2.6E+05	1.9E-04	7.5E-10	6.8E-10
EpAB5-4	2.5E+05	2.5E-04	1.0E-09	9.3E-10
EpAB1-3	2.4E+05	2.5E-04	1.0E-09	9.0E-10
MJ37^{*)}	1.6E+05	4.3E-04	2.8E-09	3.9E-10

^{*)} Similar determined anti-EpCAM antibody but fit results are not shown in this notes and tips paper.

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 to 100, these values are considered to be reliable. In SPR imaging instruments the not-tunable range of ligand densities after off-line spotting can be used to calculate the accurate ranking affinity value of multiple antibodies by interpolating to $R_{\max}$ =fixed. A rule of thumb regarding low ligand densities but acceptable noise levels using SPR imaging instruments is to interpolate various ligand densities to a fixed value of $R_{\max}$ ~ 100 RU. Further it is important that the functional concentration, as applied in the fitting routine, is determined accurately for calculation of the absolute affinity values of K_D and k_a . The calibration free concentration analysis (CFCA) method recently introduced by Robert Karlsson [13] further improves

the accuracy of the kinetic values. However ranking values (with respect to each other) can be determined without knowing the exact functional analyte concentrations. Thus, the best practical ranking affinity values for comparing many antibodies to its target can be obtained by interpolating the globally fitted affinity values to $R_{\max}$ is 100 RU.

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Figure captions

Figure 1. Serial view of the rEpCAM ranking experiment over 48 spots consist of concatenated injections of rEpCAM (concentrations from 0.5 up to 32 nM as indicated) without regeneration. In total 6 spots per antibody x 8 Abs resulted in 48 x 7 = 336 sensorgrams. **Top left:** Results in tile view are shown for the 8 different mAbs of the injections in overlay per spotted antibody (auto-scaled). The R_{max} values are shown per spot in the inserted tiled overlay. The sensorgrams in overlay are globally fitted (red lines in the tiled overlay) using the floating align point fitting procedure in Scrubber2 software.

Figure 2. Equilibrium dissociation constant K_D (y-axis) as a function of R_{max} (x-axis) of rEpCAM to antibody EpAb3-5, HO-3, VU1D9, SPM134, Clone 323/A3, OC-9-1, EpAB5-4 and EpAB1-3. Each point is the result of global fitting of 6 injections of the kinetic titration experiment. An exponential fit to $R_{max} = 100$ RU is applied for the different ligand density spots.

