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IMMOBILISATION FREE IN-SOLUTION KINETICS USING FLOW INDUCED DISPERSION ANALYSIS (FIDA)

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In this tech note we describe a new technology for in solution kinetics quantification. The technique is based on flow induced dispersion analysis and it allows determination of k_{on} and k_{off} using very small sample volumes (nL-to-microliter).

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INTRODUCTION

Understanding the interaction between biomolecules is of great interest within biomolecular research. A key parameter is to understand the affinity between drug and target, but also quantifying the underlying kinetics that define the affinity. Kinetics describes at which rate the drug and target interact. For a 1:1 interaction the interaction is described as follows:

$$[A]_t + [B]_t \rightleftharpoons [AB]_t$$

Where *t* denotes time. Instead of looking at a single timepoint, one can also look at the change of complex concentration [AB] over time:

$$\frac{d[AB]_t}{dt} = k_{on}\,[A]_t\,[B]_t - k_{off}\,[AB]_t$$

Where k_{on} is the rate constant for the association of the complex and k_{off} is the rate constant for the dissociation of the complex. By looking at a specific time where the rates are equal, we observe:

$$k_{on}\,[A]_{eq}\,[B]_{eq} = k_{off}\,[AB]_{eq}$$

Where *eq* means equilibrium, hence there is no net change in the system. At equilibrium the dissociation constant, or the affinity, is described as the following:

$$K_D = \frac{k_{off}}{k_{on}} = \frac{[A]_{eq}\,[B]_{eq}}{[AB]_{eq}}$$

Where K_D is the dissociation constant and describes the affinity between A and B.

Technologies for assessing kinetics can be divided into two groups: surface-based and in-solution based techniques.

In this tech note we describe a new technology for in solution kinetics quantification. The technique is based on flow induced dispersion analysis and it allows determination of k_{on} and k_{off} using very small sample volumes (nL-to-microliter). The results are compared to Surface Plasmon Resonance (SPR), a surface-based technique often recognised as the golden standard to evaluate kinetics.

This tech note will give an overview of how to utilise FIDA for determining the kinetics of a system. FIDA measures the diffusivity of a species, which has been shown in multiple cases. The diffusivity can also be used to obtain the hydrodynamic radius by using the Stokes Einstein relation.

The protocol involves two distinct analysis steps:

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STEP 1:
By premixing the two interacting molecules we can ensure that the equilibrium has been established prior to measuring the diffusivity, thus eliminating any time components. Doing so we get the following equation:

$$D_{app} = D_I + (D_{AI} - D_I) \cdot \frac{A_T}{A_T + K_D}$$

Where, D_{app} is the apparent diffusivity observed at a given concentration of analyte (A) and constant concentration of indicator (I). A_T describes the total analyte concentration, whereas D_I and D_{AI} are the diffusivity of the indicator and the indicator-analyte complex, respectively. K_D is the equilibrium affinity constant. Using a premix protocol at different A_T, the equilibrium K_D is thus determined.

STEP 2:
In contrast, if the interacting molecules are not allowed to reach equilibrium prior to detection, we need to extend the model to describe the formation of complex with time, which depends on the k_{on} and the k_{off} as shown below.

$$D_{app}(t) = D_I + (D_{AI} - D_I) \cdot \frac{A_T}{A_T + K_D} + (D_I - D_{AI}) \cdot \frac{A_T}{A_T + K_D} \cdot e^{-(k_{off} + k_{on} \cdot A_T) \cdot t}$$

Where k_{on} and k_{off} are introduced along with t, describing the time, and the remaining components remain identical as in the equilibrium case.

One can express the k_{off} as a product of the K_D and k_{on}, hence leaving only 1 fitting parameter, namely k_{on}. Doing so results in the following expression:

$$D_{app}(t) = D_I + (D_{AI} - D_I) \cdot \frac{A_T}{A_T + K_D} + (D_I - D_{AI}) \cdot \frac{A_T}{A_T + K_D} \cdot e^{-(K_D\, k_{on} + k_{on} \cdot A_T) \cdot t}$$

The last thing that should be addressed is the time component in the equation above. The Fida Neo instrument is a pressure driven instrument; hence the user is in full control of how much time is allowed for the reagents to interact in the capillary as the pressure controls the flow rate. By increasing the pressure, less time is allowed for the reaction, hence shifting the reaction away from the equilibrium.

Figure 1 shows the principle behind this when applied to a full titration series. The left image in Figure 1 shows how the apparent size changes (can be calculated from the apparent diffusivity) upon titrating increasing amounts of binding partner until saturation is reached at full complex formation. From the titration curve, the K_D is extracted as shown. This binding curve is projected to the right image in Figure 1 shown as a dashed line. Furthermore, additional binding curves are drawn in here as well. These represent the same interaction, however, the reaction has been controlled to a certain amount of time, making sure the reaction is not at equilibrium. This results in a shift in the apparent K_D toward a weaker interaction. If the shift in observed K_D is sufficiently different from the premix, it is possible to estimate the kinetics of the system.

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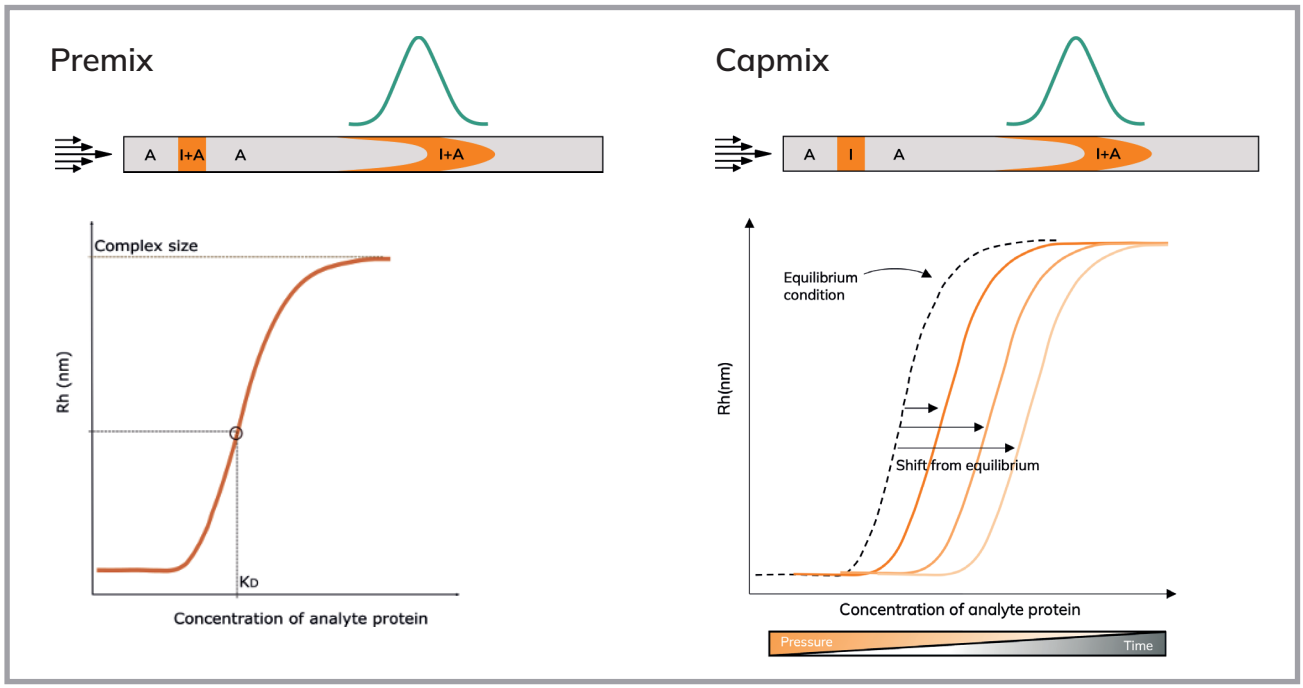

Figure 1: The left image shows a typical binding curve under premix conditions (equilibrium – step 1) used to determine the K_D. The right image shows the same typical binding curve as in the left image, but under non-equilibrium conditions using capmix (step 2). The less amount of time allowed for interaction between binding partners the weaker apparent K_D is observed (opaque orange) compared to the equilibrium K_D (dotted line).

MATERIAL & METHODS

Fida Neo equipped with a 480 nm LED fluorescence detector. A Fida dynamic coated capillary (L: 1 m, ID: 75 µm, Leff: 84 cm). The buffer used was PBS for all experiments. Indicator concentration was 20 nM affibody labelled with ALC480. The experiment was run as either a premix at 400 mBar pressure or capillary mix with pressures ranging from 50 to 600 mBar. The experiments were performed in triplicates and the data was analysed using Fida Software V3.0 with a standard fit to the raw data and the kinetics module for kinetics data.

The SPR data was obtained using a BiacoreX100. A CM5 chip was used and the immobilisation of the affibody was performed with pH screen ending at pH 4.5 for optimal immobilisation concentration of 1µg/mL. PBS-T was used for these experiments.

RESULTS

As a first step, a premix experiment with a fixed indicator concentration of 20 nM of ALC488-affibody was used against 0 – 1000 nM rituximab to obtain an equillibrium K_D of 0.31 nM (data not shown). Secondly, a series of capillary mixing (capmix) experiments were carried out at two different mobilisation conditions of 400 mBar and 600 mBar. Upon applying a mobilisation pressure of 400 mBar, the affibody and rituximab were allowed to react for 110 seconds in the Fida capillary. The alternative condition, at 600 mBar mobilisation pressure, enabled the reaction time to be 74 seconds.

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Figure 2 shows the results of the capmix experiments, where the shift from equilibrium state can be clearly seen at two different mobilisation conditions, where 600 mBar shows an apparent K_D of approximately 6.18 nM compared to 4.12 nM for 400 mBar condition. Both capmix cases illustrated in Figure 2 reported a weaker K_D compared to the premix state K_D of 0.31 nM. Table 1 shows the extracted K_D for each of the mobilisation pressures shown in Figure 2.

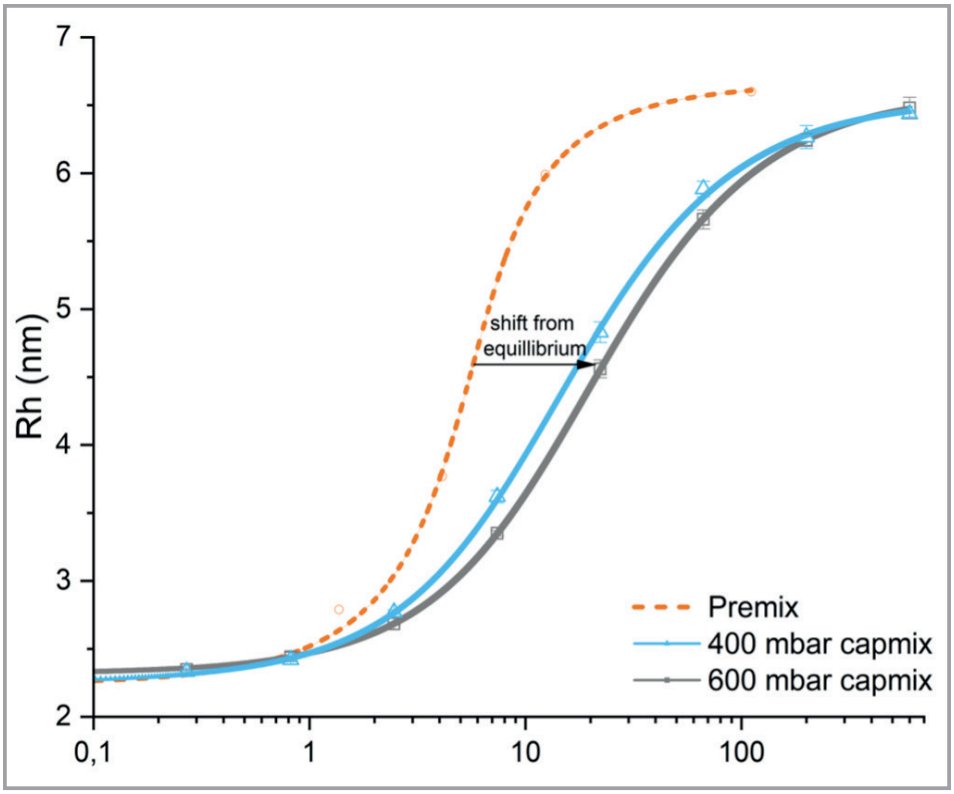

Figure 2: Shift from equilibrium state seen for affibody-rituximab system at 400 and 600 mbar mobilisation conditions.

Mobilisation	$K_{D, app}$ in nM*	Reaction time (in sec)
Premix	0.31	N/A
400 mBar	4,12	110,4
600 mBar	6,18	73,8

Table 1: Different mobilisation pressure conditions applied, apparent $K_{D, app}$ values and the reaction times used to generate the capmix binding curves in Figure 2.

At 600 mBar, we observe a 20-fold difference in $K_{D, app}$, which is significantly lower degree of binding than at equilibrium, indicating a shift from equilibrium at this condition. Thus, the 600 mBar capmix data was analysed using the Fidabio kinetics module shown in Figure 3.

The interface of kinetics module is divided into 3 sections. The left section in Figure 3 concerns the input parameters, where the indicator size, complex size and equilibrium (premix) K_D are all known parameters, hence these parameters are fixed. This leaves k_{on} as the only fitting parameter. The remaining input parameters are constants specific to the experiment, such as temperature, injection pressure, mobilisation pressure, capillary dimensions, viscosity, indicator concentration, injection time of the indicator and the sample residence time. All of these parameters are either controlled by the user or are known prior to the experiment.

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By entering the various parameters for 600 mBar mobilisation condition we were able to extract a k_{on} of 2.6x10^{6} M^{-1}s^{-1} with less than 5% error bars and a k_{off} of 0.0008 s^{-1} (figure 3). To test if the other mobilisations/reaction conditions would give similar results, we also analysed the system at 400 mBar (with reaction time of 110 sec) condition from Figure 2, and the results are compiled in Table S1. In addition to the two reaction conditions, we also performed the same assay at a further longer reaction time of 222 seconds (at 200 mBar mobilisation) and obtained a k_{on} value of 1.04 x10^{8} M^{-1}s^{-1} with >20% error. The fitted curve at this reaction condition is illustrated in figure S1 and shows the mass transport limitation when the capmix times are long enough to facilitate equilibrium condition. At this stage, the kinetics measurements are not possible.

NOTE: The mobilisation pressure must be optimised for different systems. 600 mBar was sufficient to assess the kinetics of current system described in this tech note but the conditions may vary between systems. The main criteria for running a kinetics measurement on Fida Neo is that the pressure condition must meet the limits of Taylor's dispersion. If the complex formed is too big for the chosen pressure condition, there will be incomplete sample diffusion during measurement. An inbuilt software simulation enables quick assessment of dispersion limits, assisting in setting proper experimental (pressure) conditions.

For cases where complex sizes are too large for fast in-capillary mixing, alternative thinner and shorter capillaries will enable high measurement pressure (hence faster mixing), still meeting the ideal Taylor's criteria.

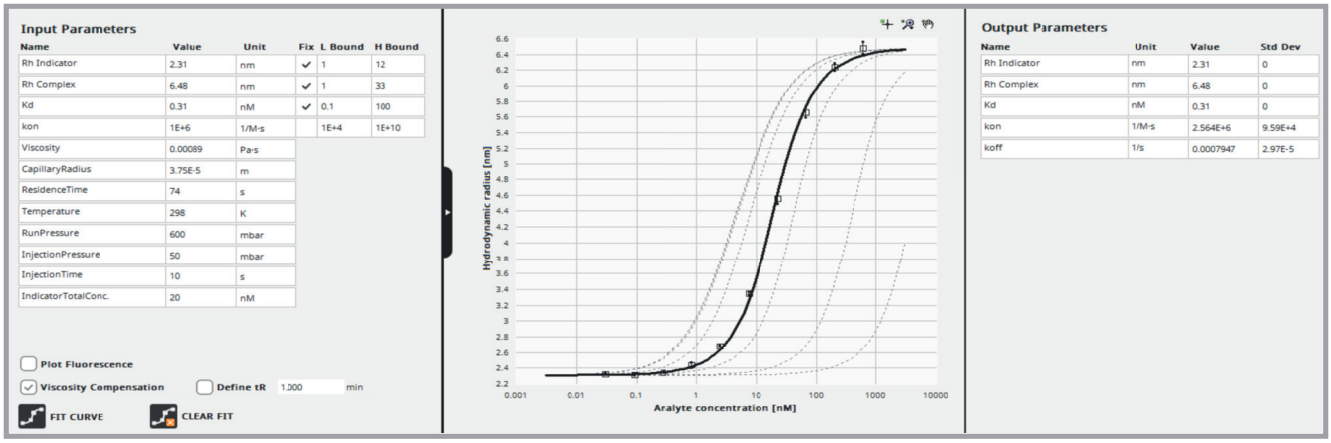

Figure 3. Using the kinetics module in the Fida data analysis software the k_{on} and k_{off} of the affibody-rituximab interaction was estimated. The black line represents the fit to the data (squares) and the dotted lines are simulated curves with different k_{on} rates based on the lower bound (L bound) and high bound (H bound) given in the input parameters.

To validate the measured parameters on Fida Neo, we performed the kinetics analysis of the same system using Surface Plasmon Resonance (SPR). Table 2 shows the comparison values from the two methods, where with FIDA we observe an affinity of 0.3 nM and with SPR we observe an affinity of 0.24 nM, agreeing strongly. The k_{on} extracted using FIDA was 2.6x10^{6} M^{-1}s^{-1} whereas the k_{on} using the SPR system was 4.7x10^{5} M^{-1}s^{-1}, which is approximately a factor 3 different. Lastly, using the FIDA to calculate the k_{off} based on the affinity and k_{on} we get a calculated k_{off} of 0.0008 s^{-1} compared to the 0.0001 s^{-1} from SPR, which is a factor 8 in difference.

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Overall, k_{on} and k_{off} parameters obtained with multiple different mobilisation conditions using Fida Neo are in good agreement with those obtained from SPR. Thus, the Fida Neo platform offers reliable orthogonal way of measuring kinetics parameters without the need of surface immobilisation, in any kind of matrix or buffer systems.

	K_D [nM]		k_{on} [M-1^{s-1}]		k_{off} [s^{-1}]	
	FIDA	SPR	FIDA	SPR	FIDA	SPR
Affibody-Rituximab	0.3	0.24	$2.6x10^6$	$4.7x10^5$	0.0008	0.0001

Table 2: Comparison of in-solutions kinetics using FIDA and a surface-based technique using SPR.

CONCLUSION

In this tech note we have presented the application of Fida Neo instrument to obtain kinetics parameters using an immobilisation free assay setup. The Fida Neo kinetics module was tested using a fluorescently labelled anti-IgG affibody binding to rituximab with high affinity (0.3 nM). By controlling the mobilisation pressure, and thus the reaction times in capillary mixing mode, the setup enables measurement of off-equilibrium affinity, which reflects on the kinetics of the system. We extracted a k_{on} of $2.6x10^6$ $M^{-1}s^{-1}$ and a k_{off} of 0.0008 s^{-1}, which was consistent over different mobilisation conditions, showcasing the reproducibility of the system. Furthermore, the interaction parameters of the affibody and rituximab system were validated using SPR, where the kinetics and affinity are in good agreement with the results obtained from Fida Neo.

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SUPPLEMENTARY INFORMATION

Mobilisation	K_D in nM	k_{on} [$M^{-1}s^{-1}$]	k_{off} [s^{-1}]
400 mbar	4,12	2.924 E+6	0.0009064
600 mbar	6.18	2.6 E+6	0.0008

Table S1: Showing the extracted kinetics using two different mobilisation conditions from Figure 2.

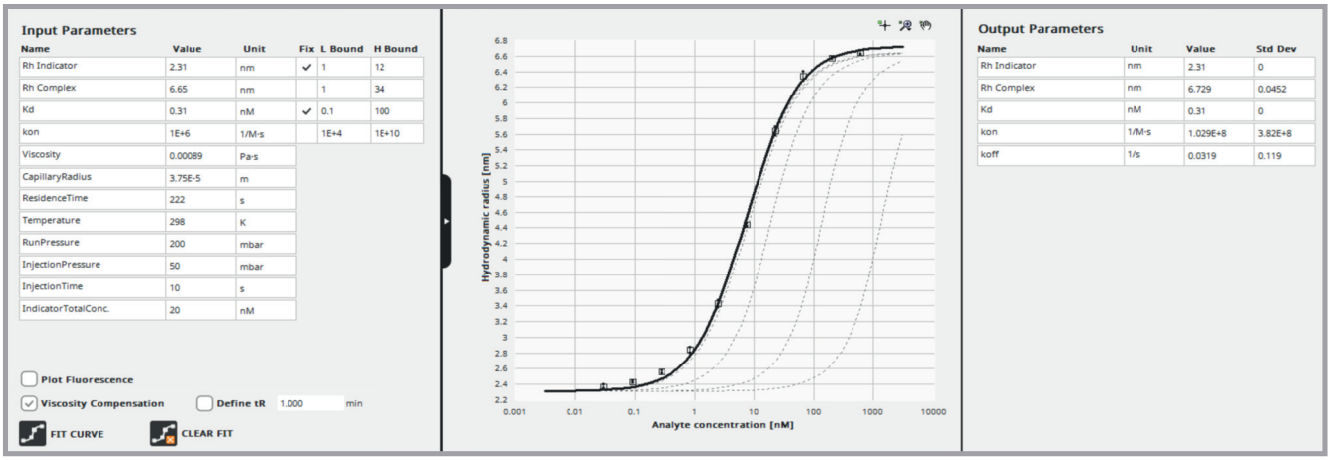

Figure S1: Fida Neo kinetics fit for mobilisation condition of 200 mBar with interaction time of 222 seconds. The simulated (dotted) curves illustrate the 'shift from equilibrium' states. The obtained experimental curve (solid curve) is skewed to the left, close to the predicted equilibrium state of the system. Therefore, 200 mbar condition does not provide accurate measure of kinetics for the affibody-rituximab system.

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