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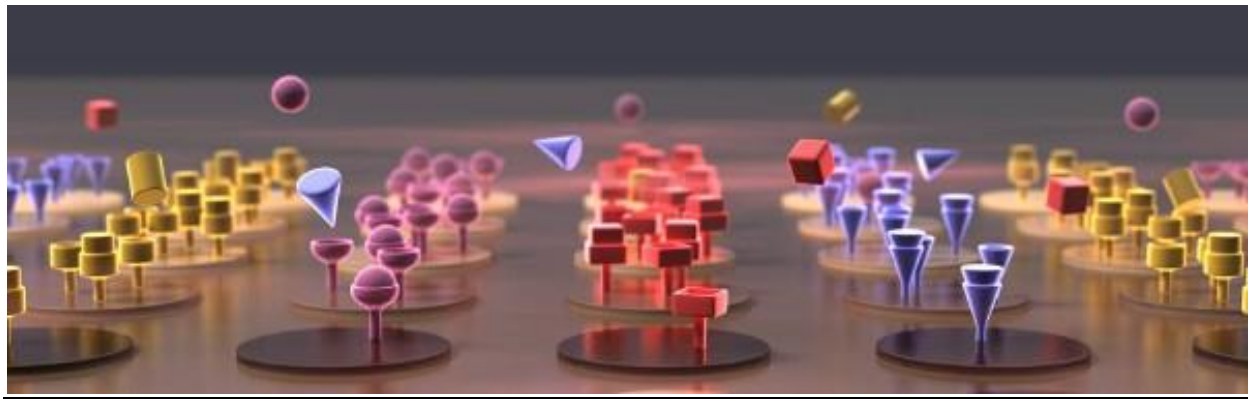

IBIS White paper

Affinity of Fc – VH interactions

 # 10.140325

1. Abstract

This white paper describes the determination of the kinetic rate and equilibrium affinity constants of Fc – VH interactions as performed in the labs of Crescendo Biologics, Cambridge, UK. For determining affinity constants it is well known to apply low ligand densities to reduce rebinding effects and factors that influence kinetics. Two approaches were applied for determining the affinity constants of VH antibodies for a Fc fusion protein: (a) A capture approach using Protein G and (b) direct immobilization of the Fc fusion protein (Mw ~ 60 kD) on the sensor surface; both followed by the VH (MW~ 12 kD) injection. Both experiments were performed in a single run and the data obtained was comparable with Biacore T200 data. The capture of the Fc fusion protein on the protein G spots lead to a decaying baseline for the VH binding. The decaying baseline correction was performed by a double referencing principle using blank subtraction in both Scrubber and SPRintX software. The equilibrium dissociation constants obtained with the IBIS MX96 were similar for VH binding to directly coupled Fc fusion protein, or to Fc fusion protein captured with Protein-G. Earlier blind control measurements using a Biacore T200 in a kinetic titration experiment showed similar values.

2. Introduction

Crescendo's proprietary VH technology combines two highly desirable features of antibody platforms, in vivo generation and human origin. The platform is based on the generation of human heavy chain-only antibodies in transgenic mice, which is a source of candidate-quality V_H fragments that have matured in vivo to have high affinity, stability and solubility, yet do not require humanisation.

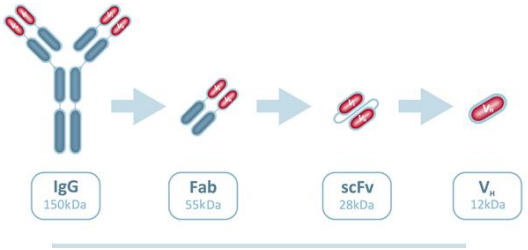

Figure 1. *A V_H is the smallest part of an antibody that retains antigen binding specificity and affinity*

VH fragments are the smallest antibody fragments that retain binding affinity and specificity. They have significant advantages in product robustness and ease of engineering. In this study the rate and affinity constants of a selected VH84A4, which binds to an Fc fusion protein (~60 kD) was determined using the IBIS MX96. Various spots with VHs, Fc fusion protein and protein G were created with the Continuous Flow Microspotter (CFM) in order to generate a variety of ligand densities and qualities of a spot. Protein G was immobilized in various concentrations in order to create captured ligand densities of Fc. The VH84A4 was tested on various label free interaction platforms in order to validate the rate and to determine binding constants (K_D).

3. Materials and methods

Standard protocols were employed to create a 6x8 feature array containing a checkerboard configuration of VH, Fc fusion protein, Protein G, anti-His and control HSA spots using the continuous flow micro-spotter (CFM). The CFM applies "flow cells" to homogeneously deposit biomolecules onto the biosensor surface. Unlike other systems that deposit droplets to create non-homogenous features on a surface, the CFM 'circulates' the sample back-and-forth over the surface, until complete binding saturation occurs wherever the sample is in contact with the sensor surface.

To immobilize the proteins a G-COOH SensEye® (Ssens bv) was used. After creating the array, the sensor was inserted in the IBIS MX96 and in an overnight run a capture of Fc fusion protein was first applied followed by the injection of each VH in three concentrations.

Figure 2. *Disposable SensEye® biosensor inserted in the slider*

4. Results and discussion

After loading the sensor in the IBIS MX96, the sensor surface was evaluated using the MX96 imaging feature. Then, the ligand density was determined by evaluating the difference between empty spots and spots immobilized with the protein. This so-called local ligand density response abbreviated as R_{LL} value can be determined for each signal spot.

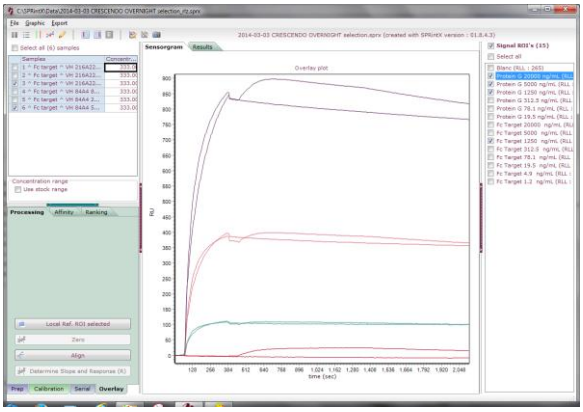

Figure 3. *Capture of Fc fusion protein on ~~a~~ protein G- spot in three different ligand densities. The lowest spot is an Fc fusion protein spot and binding of the VH was observed for the VH84A4 and not for the VH21E7*

A decaying surface of Fc fusion protein captured by Protein G showed specific binding of VH84A4 and not VH21E7. In order to calculate the affinity constant of the binding event on both spots the decaying surface should be corrected using double referencing. In this way a subtraction of a blank injection was performed and analysed in Scrubber software.

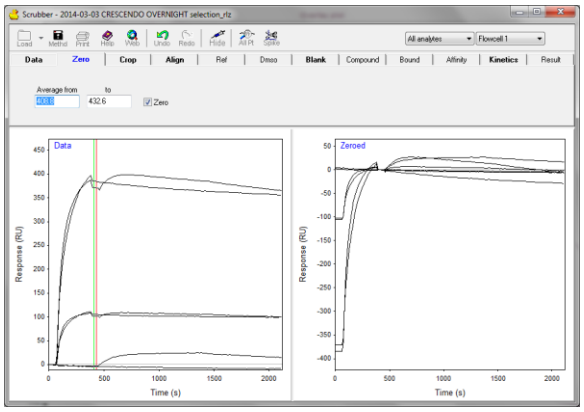

Figure 4. *Imported data in Scrubber followed by zeroing on the baseline of the second injection.*

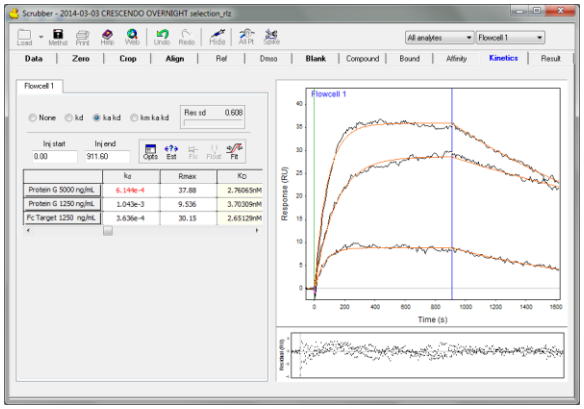

Figure 5. *Sensorgrams after blank correction obtained in Scrubber of binding and fitting the VH interaction on two protein G – and 1 Fc fusion protein spot after single analyte injection of 50 nM. The affinity constant was in the range of ~ 3 nM.*

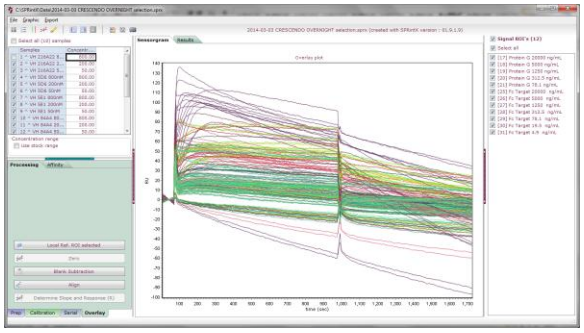

Figure 6. *In SPRintX all analytes can be analysed simultaneously. The decaying surface can be corrected using a blank subtraction. Analyte VH 216A11 (no binding to Fc) was used to correct for the Fc-decaying surface.*

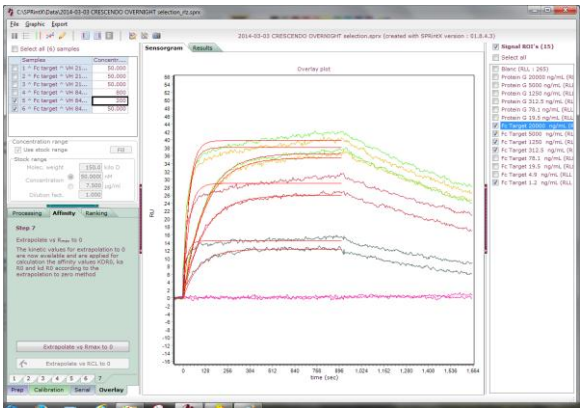

Figure 7. *Sensorgrams in SPRintX of direct interaction on Fc fusion protein spots to VH84A4. Fit lines (red) are drawn for calculating the affinity constants. Extrapolation to R_{max} is zero gives K_D^{R0} values.*

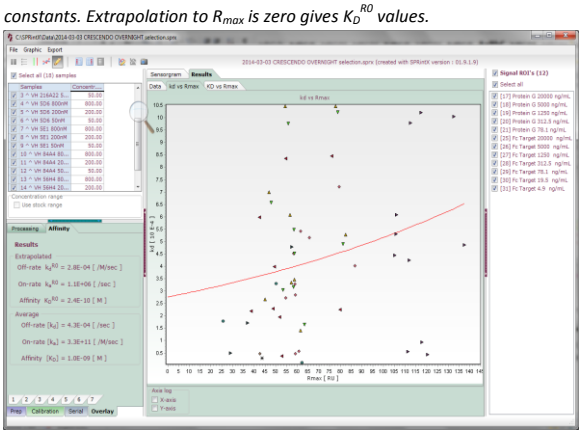

Figure 8. *Extrapolation to R_{max}=0 is based on the local affinity values of each sensorgram.*

VH affinity	*[k_d]*	*[K_D]*	*k_d^{R0}*	*K_D^{R0}*
VH 84A4	*5.1E-4*	*2.5E-9*	*5.2E-4*	*2.3E-9*
VH 56H4	*1.2E-3*	*7.7E-9*	*1.3E-3*	*8.2E-9*
VH 21E7	*1.3E-3*	*1.1E-8*	*1.1E-3*	*1.0E-8*

Tabel. *Data tabel of the determined affinity values of VH binding to Fc fusion protein spots. Redundancy of many spots guarantees reliability.*

From every curve a local affinity constant could be calculated for each VH –Fc interaction both on direct Fc spots and captured Fc (Protein G) spots. In Scrubber software global affinity constants can be determined. These values should be in the order of the extrapolated and averaged values as measured in SPRintX. The 1:1 interaction model is applied for both software packages. The residual plot should result in no curvature but only noise. The extrapolation method should correct for the ligand density dependence.

R_{MAX} values are in the low RU range (10-40 RU) and the V_H binding showed good 1:1 interaction behaviour as shown by the residual plot. The decaying behaviour of the Protein G surface was corrected both in Scrubber and SPRintX and similar values were found on the directly immobilized Fc fusion protein spots and a solid value for the equilibrium affinity constant was determined with many cross-checks. The IBIS MX96 showed similar values (2 - 3 nM) for the direct immobilization and the capture using the protein G spot (blank subtraction).

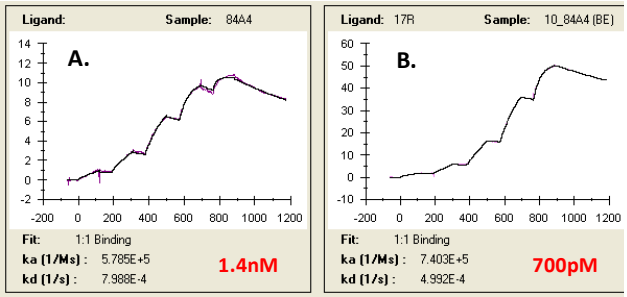

Figure 9. *BIAcore sensorgram traces to determine binding kinetics of VH 84A4 for Fc-fusion protein. (A) directly coupled Fc-fusion protein, (B) protein G captured Fc-fusion protein.*

However in Biacore a factor 2 difference (1.4 nM and 700 pM respectively) was measured for the two approaches. Because the R_{max} values in Biacore differ a factor of 5, it may cause the difference in the apparent affinity constant as measured with Biacore T200. In the IBIS MX96 the combination of analyte and ligand concentrations are available and the extrapolation to zero ligand density shows an independent behaviour for the direct vs capture approach.

5. Conclusion

Using the multiplex feature of the IBIS MX96, a reliable and reproducible equilibrium affinity constant for the interaction of a relative small VH protein (~12 kD) to an Fc fusion protein ligand has been determined.

This experiment demonstrates the ability of the IBIS MX96 platform to perform biomolecular interactions of small proteins to generate accurate affinity parameters. The multiplex feature of the IBIS MX96 enables coupling of ligand at a variety of different densities and presentation methods, e.g. a capture approach in combination with a directly coupled ligand. Here the IBIS MX96 has been used to determine the affinities of Fc fusion protein–VH interactions after spotting a single chip. Using a checkerboard of ligand densities and combination of various binding partners, a single spotted SensEye sensor can be installed for multiple interactions creating unrivalled robustness and reliability of the data.

6. Summary

The IBIS MX96 has been demonstrated in the labs of Crescendo Biologics ltd and a 48-spot SPR array was created to determine affinities of various VH test compounds to an Fc fusion protein target. A capture approach of Fc fusion protein to protein-G spots was applied and compared to a directly coupled Fc fusion protein spot. The global affinity approach as well as the extrapolation to R_{max} =0 shows similar values for each VH-Fc interaction. It has been shown that the equilibrium affinity constants and off- and on-rates determined by the IBIS MX96 are consistent for direct versus capture immobilization and were in agreement with the values obtained using the Biacore T200.

7. References

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