Antibody-Antigen Interaction Analysis

Application Note NT0011

Using MST to analyse the binding of Nanobodies and Nanobody-Fc fusion proteins to human CD38

Mandy Unger[1], Friedrich Koch-Nolte[1], Hon Cheung Lee[2], Stephen Blanke[3], Moran Jerabek-Willemsen[3]

[1] Department of Immunology, Universitätsklinikum Hamburg-Eppendorf, Hamburg, Germany
[2] Department of Physiology, University of Hong Kong, Hong Kong, China
[3] NanoTemper Technologies GmbH, Munich, Germany

Abstract

Antibody affinities play an important role in diagnostic, basic research and medical applications. Conventional antibodies are composed of two heavy and two light chains. Both chains contribute to binding of the antigen by their variable domains. In addition to conventional antibodies, llamas produce functional heavy chain antibodies that lack light chains and interact with antigens solely via the variable domain of the heavy chain, designated VHH. Recombinant VHHs are also called single domain antibodies or Nanobodies because of their size in the nm range. In this study we compared the binding of Nanobodies and Nanobody-Fc fusion proteins to human CD38 using MicroScale Thermophoresis (MST). This study highlights the potential of MST Technology in affinity determination of antibody-antigen interactions.

Introduction

CD38 is a multifunctional cell surface ecto-enzyme involved in diverse functions (1). Clinically, CD38 is used as a prognostic marker of B-cell chronic lymphatic leukemia (2).
Conventional antibodies consist of two heavy and two light chains (**Fig. 1**). Both chains contribute to the antigen-binding site. In addition to these conventional antibodies, llamas and other camelids produce antibodies composed only of heavy chains (3). The antigen-binding domain of these unusual heavy chain antibodies (hcAbs) is formed only by a single domain, designated VHH. VHHs are easily produced as recombinant proteins, designated single domain antibodies (sdAbs) or Nanobodies (Nb) (4-6). The CDR3 of these sdAbs possesses the extraordinary capacity to form long protruding extensions that can extend into cavities on antigens, e.g., the active site of enzymes (7). Other advantageous features of Nb include their small size, high solubility, thermal stability, refolding capacity, and good tissue penetration *in vivo* (5). The aim of this study was to analyse the binding of a monovalent Nb and a bivalent Nb-Fc fusion protein to human CD38 using MicroScale Thermophoresis or MST (11-13).

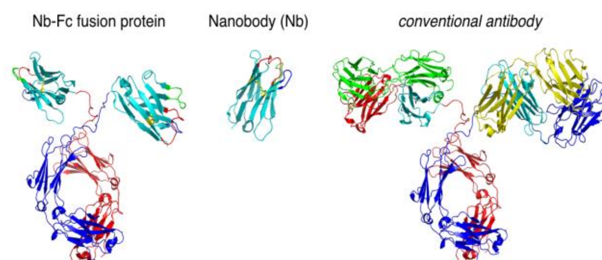

Fig. 1 Schematic diagram of the 3D-structures of a Nb-Fc fusion protein, Nanobody, and a conventional antibody. The three CDR regions of the Nanobody are depicted in red (CDR1), green (CDR2) and blue (CDR3), and the canonical disulfide bond in yellow. The figure was assembled with Pymol using the coordinates of pdb codes 3kik (lama VHH "GFP-enhancer") and 1igt (mouse IgG2a mAb "231").

Results and Discussion

In order to investigate the binding of a Nb and a bivalent Nb-Fc fusion protein to purified CD38 using MicroScale Thermophoresis, the two proteins were mixed in different molar concentrations. The concentration of Alexa^{647}-labeled CD38 was kept constant at 5 nM while the concentration of the unlabeled binding partner was varied. To this end, the Nb, Nb-Fc fusion protein, or a conventional control monoclonal antibody (rat-α-GFP mAb) were diluted in MST optimized buffer and incubated for 10 min at room temperature. Samples were loaded into Monolith

NT.115™ hydrophilic glass capillaries. In order to find the best thermophoretic setting, we analyzed the binding of the Nb to Alexa^{647}-labeled CD38 at low (20 %), middle (40 %), and high (80 %) MST power. The best signal to noise ratio was obtained by using 80 % MST power. Therefore all other binding experiments were performed using these MST-settings.

The results of the binding experiment with the monovalent Nb and Alexa^{647}-labeled CD38 show a concentration-dependent change in the thermophoresis of the Alexa^{647}-labeled CD38 of the in case of the Nb, whereas no change in thermophoresis of the Alexa^{647} was observed in case of the anti-GFP control monoclonal antibody (**Fig. 2**). The calculated K_d of the Nb-CD38 binding was 5.2 ± 0.3 nM. This correlates well with the binding affinity determined by semiquantative ELISA (not shown). The lack of change in thermophoresis in case of the anti-GFP antibody confirms that this antibody does not bind to Alexa^{647}-labeled CD38.

The results of the binding of the bivalent Nb-Fc fusion protein to Alexa^{647}-labeled CD38 show a biphasic change in thermophoresis (**Fig. 3**). The calculated K_d for the high affinity binding event was 4.9 +/- 0.3 nM, which corresponds well with the K_d determined for the monovalent Nb. The calculated K_d of the second weaker binding event was 810 +/- 82 nM. It is conceivable that binding of CD38 to one of the two binding sites on the Fc-fusion protein sterically interferes with binding to the second site.

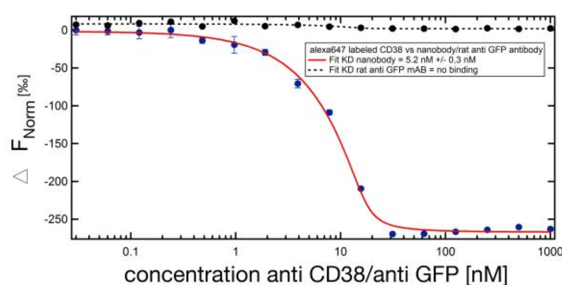

Fig. 2 Binding of monovalent Nanobody to Alexa^{647}-labeled CD38. The concentration of the fluorescently labelled CD38 was kept constant at 5 nM, while the concentration of the Nanobody or mAb Rat-α-GFP was varied from 0.3 nM – 1000 nM. After a 10 min equilibration, MST-analysis was performed (n = 2). The calculated K_d for the binding of the Nanobody to CD38 was 5.2 ± 0.3 nM. As expected, no binding of the Rat-α-GFP to CD38 was detected.

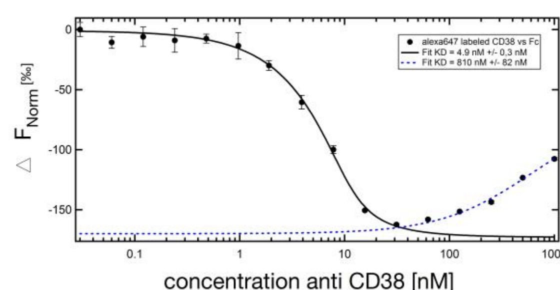

Fig. 3 Binding of bivalent Nb-Fc fusion protein to Alexa^{647}-labelled CD38. The concentration of the fluorescently labelled CD38 was kept constant, while the concentration of the Nb-Fc fusion protein was varied from 0.3 nM - 1000 nM. After a 10 min equilibration, MST-analysis was performed (n = 2). A K_d of 4.9 ± 0.3 nM was calculated for the high affine binding event (black) and a K_d of 810 nM ± 82 nM for the second, weaker binding event (blue) (n = 2).

Conclusion

This study demonstrates that MicroScale Thermophoresis is capable of measuring antibody-antigen interactions in solution. Other advantages of this approach include simple handling, low material consumption, and fast analysis.

Material and Methods

Assay conditions

The recombinant soluble ecto-domain of human CD38 (8) was labelled with Alexa^{647} according to the manufacturer's (Molecular Probes, Invitrogen, Germany) instructions. The concentration of the labelled CD38 was kept constant at a concentration of 5 nM. CD38-specific Nanobody 1067 and the 1067-mouse IgG1-Fc fusion protein were produced in *E. coli* and HEK cells, respectively as described previously (9, 10). A monoclonal antibody directed against GFP Rat-α-GFP (Chromotek, Germany) was used as control. The unlabeled binding partners were titrated in 1:1 dilutions starting at 1000 nM. Samples were diluted in MST optimized buffer (50 mM Tris-HCl buffer pH 7.6 containing 150 mM NaCl, 10 mM $MgCl_2$ and 0.05 % Tween-20). For the measurement the samples were filled into hydrophilic treated capillaries (K004, NanoTemper technologies, Germany) and measured after a 10 min equilibration at room temperature. The measurements were performed at 40 % LED, and 20 %, 40 %, and 80 % MST power. Laser-On time was 30 sec, Laser-Off time 5 sec.

Instrumentation

The measurements were performed using a NanoTemper Monolith NT.115 instrument.

References

1. Partida-Sanchez, S., L. Rivero-Nava, G. Shi, and F.E. Lund. 2007. CD38: an ecto-enzyme at the crossroads of innate and adaptive immune responses. *Adv Exp Med Biol* 590:171-183.

2. Malavasi, F., S. Deaglio, A. Funaro, E. Ferrero, A.L. Horenstein, E. Ortolan, T. Vaisitti, and S. Aydin. 2008. Evolution and function of the ADP ribosyl cyclase/CD38 gene family in physiology and pathology. *Physiol Rev* 88:841-886.

3. Hamers-Casterman, C., T. Atarhouch, S. Muyldermans, G. Robinson, C. Hamers, E.B. Songa, N. Bendahman, and R. Hamers. 1993. Naturally occurring antibodies devoid of light chains. *Nature* 363:446-448.

4. Saerens, D., G.H. Ghassabeh, and S. Muyldermans. 2008. Single-domain antibodies as building blocks for novel therapeutics. *Curr Opin Pharmacol* 8:600-608.

5. Wesolowski, J., V. Alzogaray, J. Reyelt, M. Unger, K. Juarez, M. Urrutia, A. Cauerhff, W. Danquah, B. Rissiek, F. Scheuplein, N. Schwarz, S. Adriouch, O. Boyer, M. Seman, A. Licea, D.V. Serreze, F.A. Goldbaum, F. Haag, and F. Koch-Nolte. 2009. Single domain antibodies: promising experimental and therapeutic tools in infection and immunity. *Med Microbiol Immunol* 198:157-174.

6. Huang, L., S. Muyldermans, and D. Saerens. 2010. Nanobodies(R): proficient tools in diagnostics. *Expert Rev Mol Diagn* 10:777-785.

7. De Genst, E., K. Silence, K. Decanniere, K. Conrath, R. Loris, J. Kinne, S. Muyldermans, and L. Wyns. 2006. Molecular basis for the preferential cleft recognition by dromedary heavy-chain antibodies. *Proc Natl Acad Sci U S A* 103:4586-4591.

8. Liu, Q., I.A. Kriksunov, R. Graeff, C. Munshi, H.C. Lee, and Q. Hao. 2005. Crystal structure of human CD38 extracellular domain. *Structure* 13:1331-1339.

9. Koch-Nolte, F., J. Reyelt, B. Schossow, N. Schwarz, F. Scheuplein, S. Rothenburg, F. Haag, V. Alzogaray, A. Cauerhff, and F.A. Goldbaum. 2007. Single domain antibodies from llama effectively and specifically block T cell ecto-ADP-ribosyltransferase ART2.2 in vivo. *FASEB J* 21:3490-3498.

10. Scheuplein, F., B. Rissiek, J.P. Driver, Y.G. Chen, F. Koch-Nolte, and D.V. Serreze. 2010. A recombinant heavy chain antibody approach blocks ART2 mediated deletion of an iNKT cell population that upon activation inhibits autoimmune diabetes. *J Autoimmun* 34:145-154.

11. Moran Jerabek-Willemsen, Christoph J. Wienken, Dieter Braun, Philipp Baaske and Stefan Duhr. Molecular Interaction Studies Using Microscale Thermophoresis. *ASSAY and Drug Development Technologies, DOI:10.1089/adt.2011.0380 (2011)*

12 Christoph J. Wienken, Philipp Baaske, Ulrich Rothbauer, Dieter Braun and Stefan Duhr .Protein Binding Assays in Biological Liquids using Microscale Thermophoresis. *Nature Communications, DOI: 10.1038/ncomms1093 (2010)*

13. Stefan Duhr and Dieter Braun. Why molecules move along a temperature gradient. *PNAS 103, 19678–19682 (2006)*

© 2012 NanoTemper Technologies GmbH