Fluidic Analytics

Application note

Quantifying the stoichiometry of protein–protein interactions

Microfluidic Diffusional Sizing was used to determine the hydrodynamic radius (R_h) of protein A (SpA) in solution—both when free and when bound to IgG. This enabled the stoichiometry of the SpA–IgG complex to be determined. Furthermore, the size data was used to infer the overall conformational arrangement in solution based on models derived from the crystal structure. Thus, the Fluidity One-W is able to provide valuable insights on structural arrangements of individual proteins and protein complexes.

Authors
Sebastian Fiedler[1], Maren Butz[1], Haris Choudhery[1] & Sean Devenish[1]

www.fluidic.com

1. Fluidic Analytics Ltd. Cambridge, UK

Introduction

Characterizing the stoichiometry of protein complexes is essential to fully understand cellular pathways; from fundamental biological processes, to disease pathology and the action of drugs.

Protein-A (SpA) is a small protein of 42 kDa that binds Immunoglobulin G (IgG) with a stoichiometry of 1:3.[1] However, no protein structure exists that would explain how three IgG antibodies would bind to SpA without causing steric clashes. To understand how SpA binds to multiple IgGs and to observe whether its conformation changes upon binding, the Fluidity One-W was first used to determine the hydrodynamic radius (R_h) of free SpA in solution and then measure the R_h of the SpA–IgG complex.

Typically, determining the stoichiometry in an equilibrium binding assay relies on identifying the shift of the binding curve along the x-axis (ligand concentration). That is, a higher number of binding sites require more ligand to reach saturation, which shifts the transition of the binding curve to higher ligand concentrations. In practice, however, the analysis of such binding data demands additional information on complex composition since fits to different stoichiometries often describe the data equally well.

Here, the Fluidity One-W reports the size of a labeled protein or its complex in solution. Such measurements inform researchers directly of the stoichiometry of an interaction simply by measuring the size of the complex formed.

Methods

Sample preparation

SpA (*Sigma*) was diluted into labeling buffer (0.2 M $NaHCO_3$, pH 8.3), mixed with Alexa Fluor™ 488 NHS ester (*Thermo Fisher Scientific*) at a dye-to-protein ratio of 3:1. After overnight incubation at 4 °C, the solution was purified using a 1 mL Pierce® Desalting Column (*Thermo Fisher Scientific*) using phosphate buffer solution (pH 7.4) with 0.05% Tween 20. Anti-EGFR IgG (*Absolute Antibody*) was used directly from stock.

Size prediction

Models for several different arrangements of free and IgG-bound SpA were generated using PyMOL.[2] Starting from the two-domain substructure of SpA (PDB: 4NPF), models of five-domain SpA were manually built in both compact and extended conformations.

Binding of one, two and three IgG proteins to the five-domain SpA was guided using an IgG Fc–SpA structure (PDB: 5U4Y) and a structure of complete IgG (PDB: 1IGT). All structural models were output, subjected to energy minimisation[3] and analysed using HYDROPRO[4] to determine the predicted translational radii.

Experimental protocol

The Alexa Fluor™ 488-labeled SpA at a concentration of 100 nM was measured on the Fluidity One-W using the slow flow rate setting to obtain the size of the unbound protein.

A second sample containing 100 nM of labeled SpA and 3.0 µM IgG was incubated overnight to reach equilibrium. This mixture was measured on the Fluidity One-W using the slow flow rate setting to obtain the size of the SpA–IgG complex.

Results

SpA carries five different IgG binding domains formed by three-helix bundles that are connected by unstructured loops.[1] Since, to the best of our knowledge, no three-dimensional full length SpA model exists, two different structural models of SpA were generated using the PyMOL software package.[2] In the first model, a two-domain structure of SpA (4NPF) was repeated to generate a compact five-domain structural model (Figure 1A). The second model was generated by opening out the compact structure to give an extended arrangement (Figure 1A). After energy minimisation of both structures, the HYDROPRO[4] software package was used to calculate the hypothetical R_h value of each model.

MDS analysis on the Fluidity One-W provided the in-solution, experimental R_h for comparison to the models. As shown in Figure 1B, the experimental R_h clearly indicates that SpA adopts an extended conformation in solution, suggesting that its five IgG binding domains do not interact with each other.

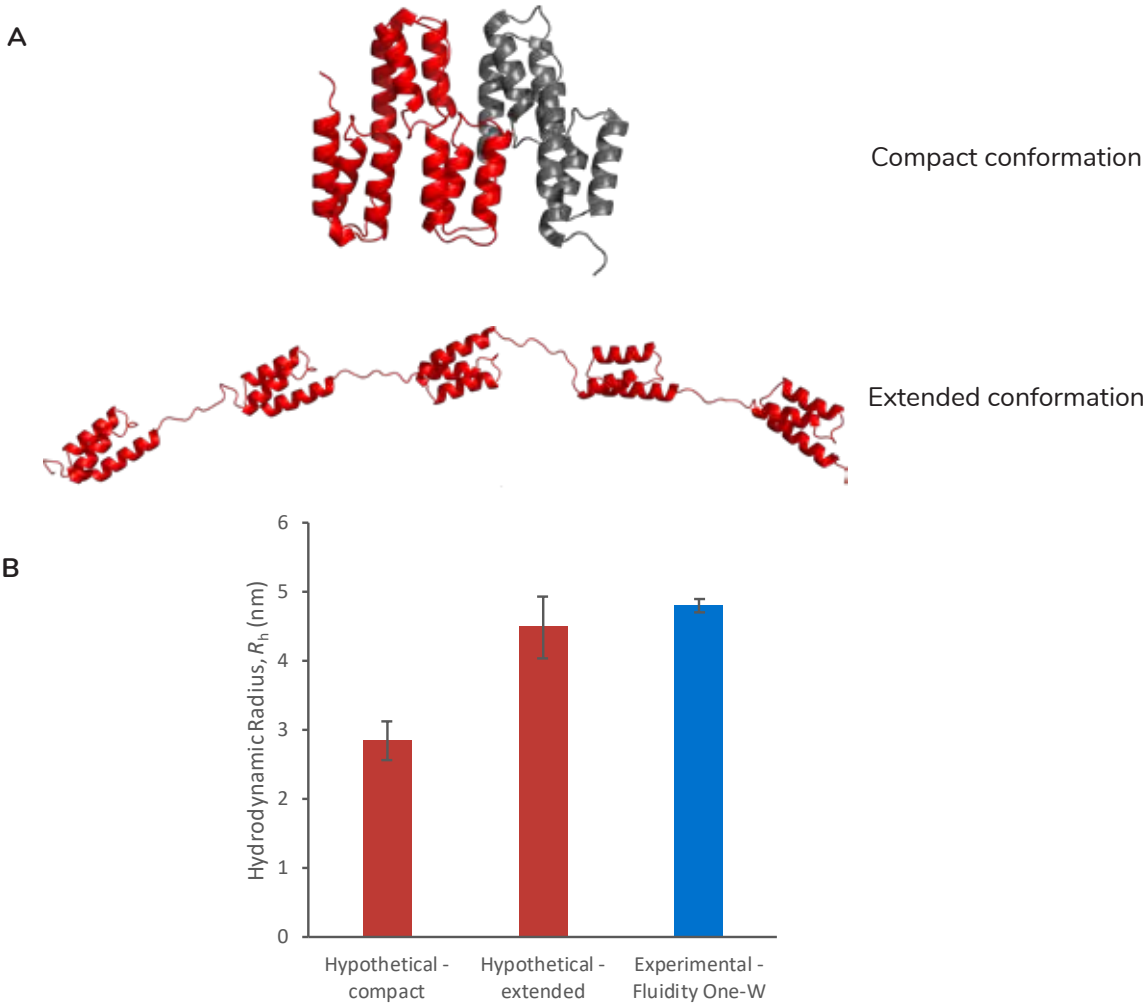

Figure 1: (A) The PyMOL rendered models of SpA in compact and extended conformations. The structure of 4NPF that was used as the starting point for model generation is shown in **grey** in the compact conformation. **(B)** A comparison of hypothetical R_h values for an extended or compact form of SpA derived from HYDROPRO (**red** bars) with the experimentally measured R_h (**blue** bar). Error bars of 10% of the predicted value have been added to indicate the minimal uncertainty in the models. The experimentally determined R_h was measured using the Fluidity One-W (triplicate measurement at 100 nM).

Although SpA carries five potential IgG binding sites, chromatographic analysis[5] indicates that on average it only binds between two and three IgG molecules due to steric conflicts caused by the considerable difference in molecular weight between SpA (~42 kDa) and IgG (~150 kDa). To examine whether this stoichiometry applied in solution phase, the hydrodynamic radius of the saturated complex was compared to predicted radii. Three different structural models of SpA were generated using PyMOL[2], with either one, two or three molecules of IgG bound to the extended conformation of SpA (Figure 2A). The respective hypothetical R_h values were then calculated using HYDROPRO.[4]

As displayed in Figure 2B, the experimental R_h obtained by MDS analysis on the Fluidity One-W agrees with the hypothetical R_h of a 1:3 SpA:IgG complex.

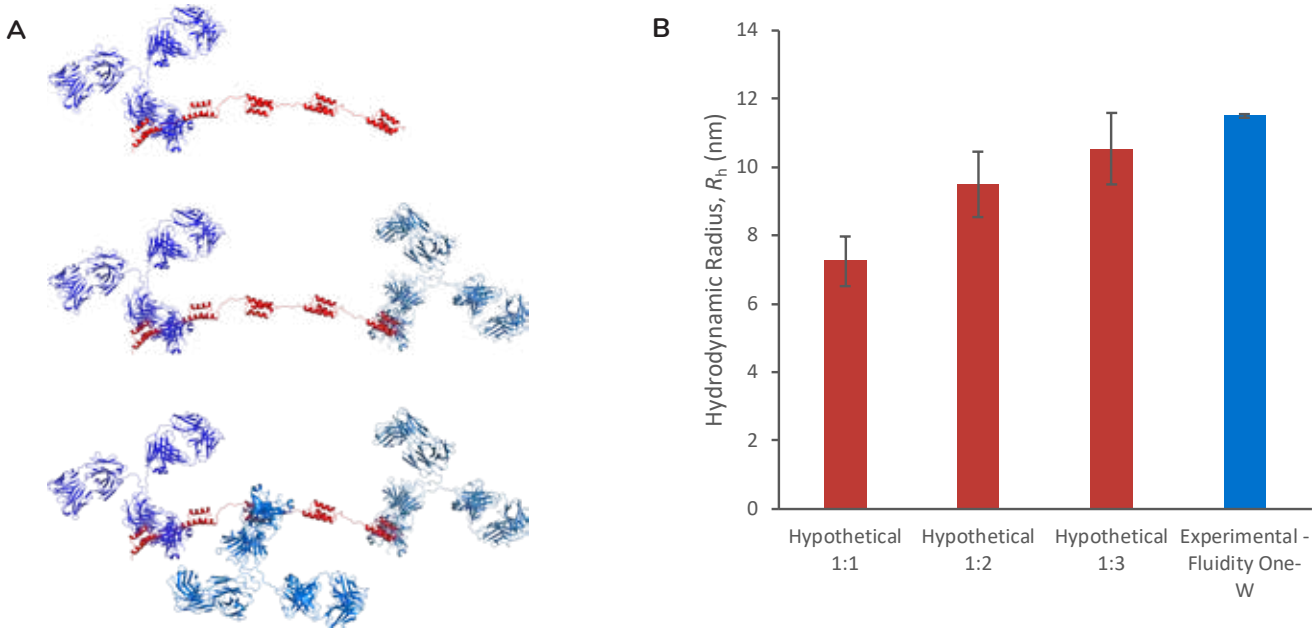

Figure 2: (A) PyMOL rendered models of SpA–IgG complexes at 1:1, 1:2 and 1:3 ratios where the **red** ribbon represents SpA and the **blue** ribbons represent IgG. **(B)** A comparison of the three hypothetical R_h values (**red** bars) calculated assuming a SpA:IgG binding stoichiometry of 1:1, 1:2 and 1:3, with the experimentally measured R_h (**blue** bar). Error bars of 10% of the predicted R_h values have been added to indicate the minimal uncertainty in the models. The experimental value was measured using the Fluidity One-W (triplicate measurement at 100 nM).

Conclusion

It has been demonstrated that R_h measurements on the Fluidity One-W can provide insights into both the conformation of individual molecules and the stoichiometry of protein complexes. The experimental R_h data are in good agreement with the hypothetical R_h values obtained from molecular models.

Both of these factors may be vital in fully understanding protein behaviour; for example in proteins that undergo large conformational changes, like intrinsically disordered proteins, or where the stoichiometry of a protein interaction is functionally or mechanistically important.

Furthermore, a titration of IgG to labeled SpA can simultaneously provide the K_D of the interaction, even in undiluted complex backgrounds—see separate application note (6). As such MDS analysis as employed on the Fluidity One-W presents a new tool for researchers to assess protein conformation and stoichiometry quickly and easily.

Fluidic Analytics

References

1. Deis, L. N., Pemble IV, C. W., Qi, Y., Hagarman, A., Richardson, D. C., Richardson, J. S., & Oas, T. G. Multiscale Conformational Heterogeneity in Staphylococcal Protein A: Possible Determinant of Functional Plasticity. *Structure*, **2014**, 22. 1467-1447.

2. The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.

3. Krieger E, Joo K, Lee J, Lee J, Raman S, Thompson J, Tyka M, Baker D, Karplus K. Improving physical realism, stereochemistry, and side-chain accuracy in homology modeling: Four approaches that performed well in CASP8. *Proteins*. **2009**, 77, 114-122.

4. Ortega, A., Amorós, D. and De La Torre, J.G. Prediction of hydrodynamic and other solution properties of rigid proteins from atomic- and residue-level models. *Biophysical journal*. **2011**, 101, 892-898.

5. Ghose, S., Hubbard, B., & Cramer, S. M. Binding capacity differences for antibodies and Fc-fusion proteins on protein A chromatographic materials. *Biotechnology and bioengineering*, **2007**, 96. 768-779.

6. Fluidic Analytics Ltd. Measuring the binding affinity of protein–protein interactions in simple and complex backgrounds. Application note reference AP0011.

+44 (0)1223 560 432
welcome@fluidic.com
www.fluidic.com
@fluidicanalytic