Biophysical Characterization of Protein-Aptamer Interactions

MicroScale Thermophoresis

Clemens Entzian and Thomas Schubert

2bind GmbH, Regensburg

2bind
molecular interactions

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Our mission is to give researchers, developers, and decision makers the power to accelerate and streamline key research processes. We offer tailor-made biophysics services and solutions that are perfectly adjusted to your precise needs in the key, high-value areas of Drug Discovery, Antibody Research, Aptamer Characterization, and Protein Biophysics.

A state-of-the-art portfolio of biophysical technologies enables us to provide you with a complete and comprehensive picture of your specific question: From affinity and stability to kinetics and thermodynamics. This portfolio includes MicroScale Thermophoresis (MST), nano-Differential Scanning Fluorimetry (nanoDSF), Biolayer Interferometry (BLI), and Isothermal Titration Calorimetry (ITC).

Work with the Experts in Biophysics

Technology

MicroScale Thermophoresis (MST) is an optical fluorescent method. It records the changes in fluorescence of a target molecule as a function of temperature and the concentration of the cognate ligand molecule. Thus, in MST, the target must be fluorescent and the ligand must not be fluorescent in the same wavelength range as the target. The fluorescence changes are the result of two effects:

First, molecules exhibit directed movement along temperature gradients in solution, an effect called thermophoresis (Duhr & Braun, 2006). Such thermophoretic movement of a molecule is characterized by its size (hydrodynamic radius), its surface charge, and its hydration shell. All three parameters can be affected by binding of a ligand molecule. Thus, a concentration-dependent titration of the target molecule's ligand induces changes in the thermophoretic movement. These changes translate in quantifiable, spatial fluorescence changes, which are easily tracked optically via the target fluorescence.

Second, fluorescence is a function of the temperature. Thus, the fluorescence of the target molecule varies with the temperature (Baaske *et al.*, 2010). This temperature-dependence is additionally affected by the local molecular surroundings of the fluorophore. Thus, binding of a ligand molecule to the fluorescent target can change the chemical environment of the target fluorophore and thus the overall detected temperature-dependence of the fluorescence. This effect is known as TRIC (temperature-related intensity change; Gupta, Duhr & Baaske, 2018).

The thermophoresis and TRIC signals are additive and thus both contribute to the high sensitivity and robustness of MST measurements towards molecular binding events of all kinds. Thus, MST can be used for determining the affinity and binding strength of almost any kind of molecular interaction with very low sample consumption and very high sensitivity.

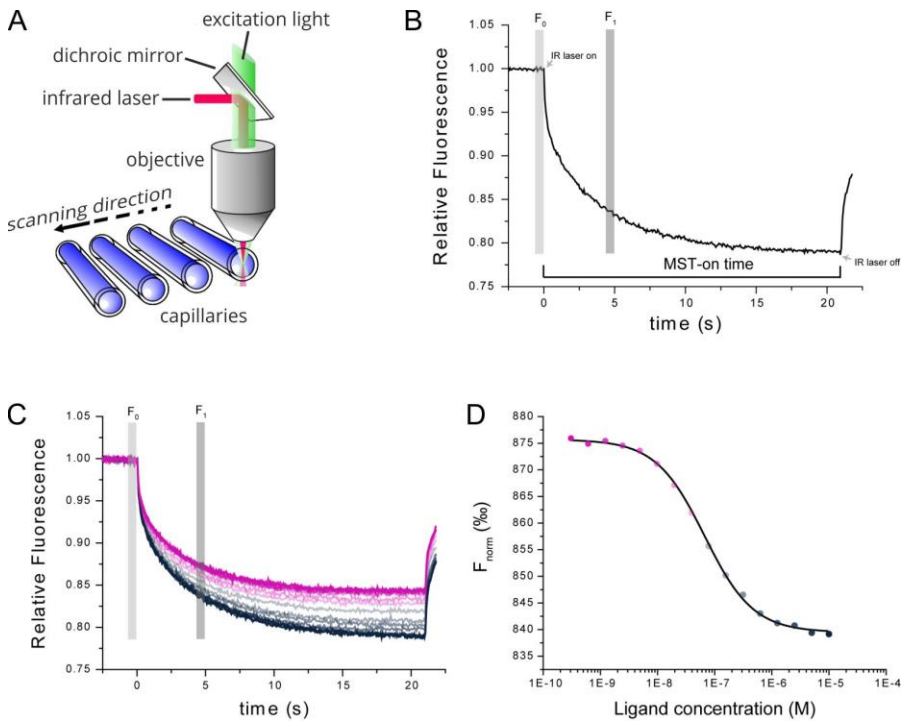

Figure 1. Technical MST setup. (A) MST measurements take place in small glass capillaries. Infrared and fluorescence lasers are used for generation of the MST effect and sample tracking. (B) TRIC and thermophoresis together account for a time-dependent change in fluorescence upon infrared-heating of the sample capillaries. (C) Multiple MST traces are recorded for different mixture ratios of target and ligand molecules. (D) Dose-response analysis of the MST traces allows for determination of the steady-state affinity of the target-ligand interaction.

Features	Advantages
✓ Wide molecule size range	Analyze molecules from 100 Da up to 1 MDa
✓ Real-time quality controls	Online aggregation, precipitation, and sticking controls
✓ No immobilization required	Measurement is done truly in solution
✓ Very short analysis time	High analysis speed enables high throughput
✓ Free choice of assay buffer	Biological liquids such as serum or cell lysate possible
✓ Low sample consumption	Minimum of only 6 µl per sample, nM concentrations
✓ Wide affinity range	Affinities can be analyzed in the pM-mM range
✓ Wide temperature range	Analysis possible from 20°C to 45°C

Introduction

Monoclonal antibodies (mAbs) are the gold-standard of macromolecule-based drug treatments of many diseases. However, mAb development is a time and cost-intense process. Moreover, the therapeutic use of mAbs is somewhat limited due to mAb stability, specifity, and deliverability. Nucleic acid aptamers are a promising alternative to antibodies because of smaller size, reduced development cost, up to equal sensitivity, and reusability. Aptamers are short DNA or RNA sequences binding their target via a unique tertiary structure. The assessment of binding characteristics of aptamers towards their targets is the pivotal initial step in the selection of aptamers (Entzian & Schubert, 2016).

Here, we demonstrate the potential of MST for examining the binding affinity of RNA and DNA aptamers. We show different assay format and types that allow for a fast and precise aptamer binding characterization. Aptamers can be easily used in MST as the fluorescent target, because they can be readily synthesized with appropriate fluorescent dyes attached (e.g. Cy5, Alexa-647).

RNA-Aptamers

Extracellular signal-regulated kinase 2 (ERK2) vs C5.71 Aptamer

ERK2 is a major determinant in the control of cell growth, cell differentiation, and cell survival. The ERK pathway is often upregulated in human tumors and, as such, ERK2 represents an attractive target for anticancer therapy (Kohno & Pouyssegur. 2006). Here, we analyzed the binding affinity of the C5.71 aptamer against ERK2 using an MST assay. Although aptamers can be easily labeled with a fluorescent dye during synthesis, it is also possible to use an aptamer in the completely native form.

In this case, the aptamer target (here the ERK2) has to be labeled with a fluorescent dye. The C5.71 aptamer showed a moderate binding affinity to ERK2 with a K_D-value in the low micromolar concentration range (K_D: 171.1 ± 36.0 µM) (Figure 2).

Assay type	MST
Target	ERK2 (MAPK1, Mitogen-activated protein kinase 1)
Labeling	NHS-chemistry
Ligand	C5.71 Aptamer (Mayer lab, Bonn)
Measured K_D [µM]	171.1 ± 36.0

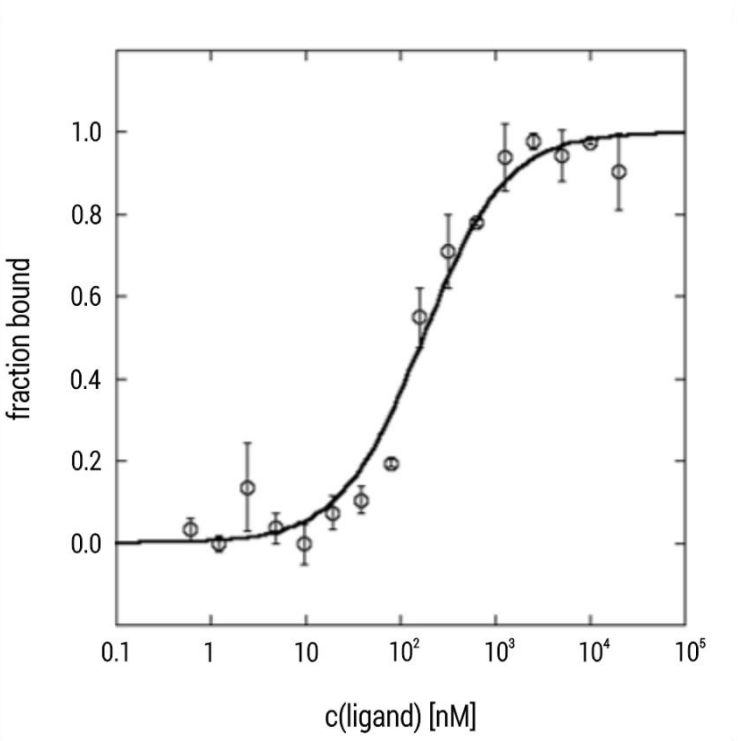

Figure 2: MST dose-response curve of ERK2 and C5.71 aptamer. Data represent the mean (+/- standard deviation) from three independent experiments and were fitted to a K_D-binding model assuming a 1:1 binding stoichiometry.

DNA-Aptamers

β-CBA II aptamer vs β-Conglutin

Conglutins represent the major storage protein family in Lupin seeds and are part of carbon, sulphur, and nitrogen supply for energy metabolism of the germinating seedling (Foley *et al.* 2015). Thus, β-conglutin has been reported to be one of the most frequent causes of severe food-associated anaphylaxis (Rubio *et al.* 2016). Here, we determined the binding affinity of the β-CBA II aptamer against β-conglutin using an MST assay. In this case, the aptamer was synthesized with a Cy5 fluorescent dye on its 5' end. The aptamer showed a strong binding affinity towards β-conglutin with a K_D-value in the low nanomolar concentration range (K_D: 11.8 ± 2.4 µM) (Figure 3).

Assay type	MST
Target	β-CBA II aptamer
Labeling	Cy5
Ligand	β -Conglutin
Measured K_D [nM]	11.8 ± 2.4

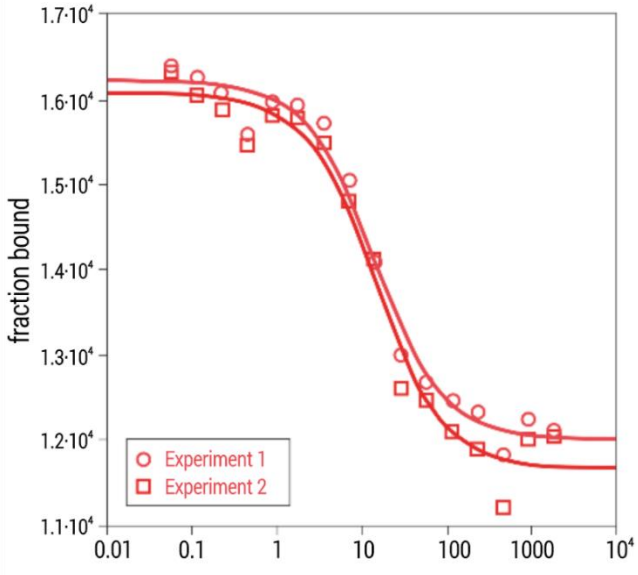

Figure 3: MST dose response curve of β-conglutin and β-CBA II aptamer. Data represent the mean (+/- standard deviation) from two independent experiments (shown individually in the graph) and were fitted to a K_D-binding model assuming a 1:1 binding stoichiometry.

HD1 & HD22 aptamers vs Thrombin

The serine protease thrombin plays a crucial role in coagulation, inflammation, and cellular proliferation events (Siller Matula *et al*., 2011). Due to its regulatory function in the conversion of fibrinogen to fibrin, and concomitant platelet aggregation, thrombin is an important therapeutic target (Breitspeicher *et al*., 2016). Here, we analyzed the binding affinity of two DNA aptamers (HD1 & HD22) for thrombin using an MST assay (Figures 4, 5, blue curves). The aptamers were synthesized with a Cy5 fluorescent dye attached to their 5' ends. Both aptamers showed strong binding affinities to thrombin under aequeous buffer conditions (HD1, K_D: 104.7 nM; HD22, K_D: 0.71 nM).

Importantly, the developed MST assay also allowed for assessing the specificity of the two aptamers for thrombin in a close-to-native setting. For this, instead of buffer, a 50% fetal calf serum solution was used (FCS, Figures 4, 5, red curves). Whereas the HD22 aptamer was not influenced by the presence of serum (almost unchanged K_D value of 132.7 nM), the HD1 aptamer showed a drastic decrease in its affinity of approximately 100-fold (K_D value in presence of FCS 90.5 nM). Thus, this aptamer is significantly affected by off-target effects. In a therapeutic scenario, this aptamer would strongly be impaired in its intended function by binding not only to thrombin but also to other serum proteins. This quick and easy MST assay demonstrated the importance of checking off-target effects of aptamers.

Assay type	MST
Target	HD1 aptamer
Labeling	Cy5
Ligand	Thrombin *(human)*
Measured K_D [nM]	104.7 ((in buffer) 132.1 (in FCS)

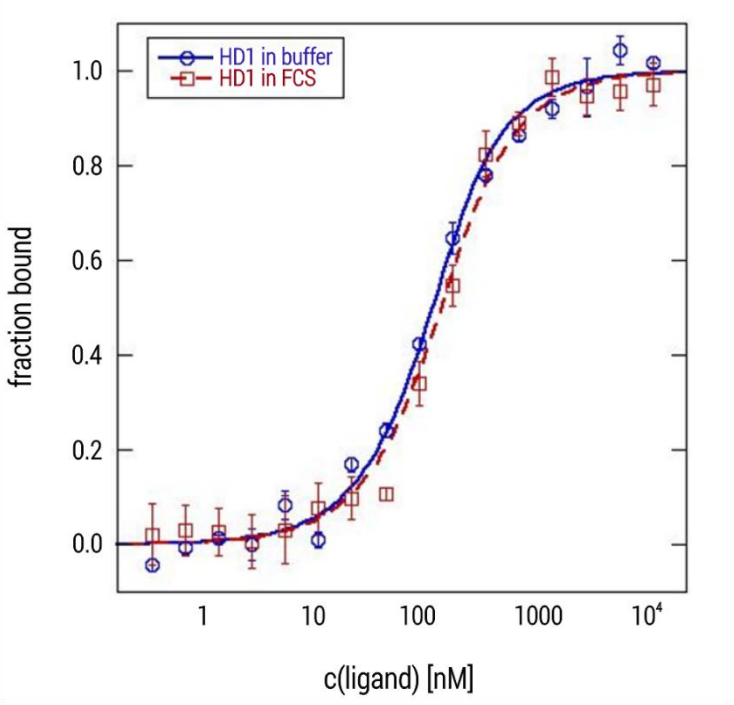

Figure 4: MST dose response curve of thrombin and HD1 aptamer. Data represent the mean (+/- standard deviation) from three independent experiments and were fitted to a K_D-binding model assuming a 1:1 binding stoichiometry.

Assay type	MST
Target	HD22 aptamer
Labeling	Cy5
Ligand	Thrombin *(human)*
Measured K_D [nM]	0.71 ((in buffer) 90.5 (in FCS)

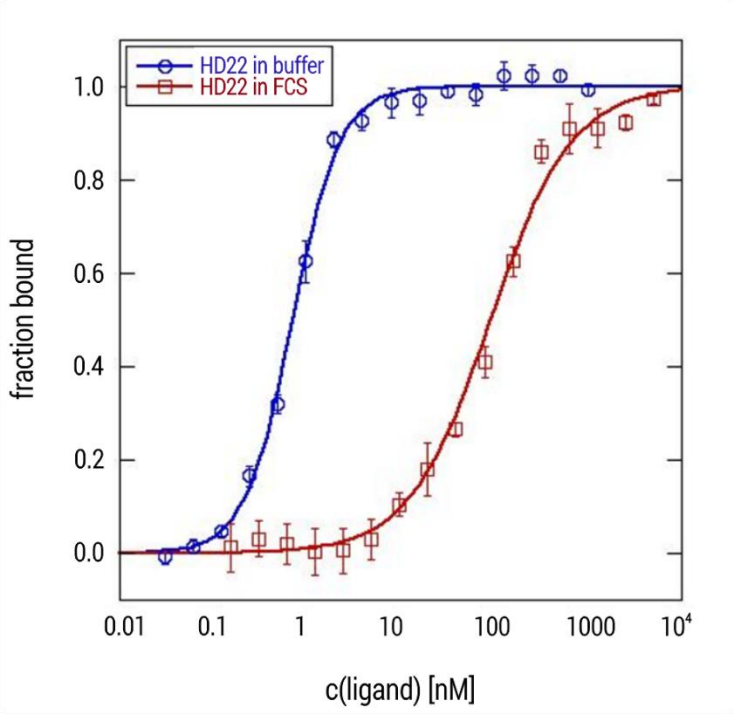

Figure 5: MST dose response curve of thrombin and HD22 aptamer. Data represent the mean (+/- standard deviation) from three independent experiments and were fitted to a K_D-binding model assuming a 1:1 binding stoichiometry.

Conclusion

In this application note we show MST assays that allow for the efficient, rapid, and precise characterization of the binding affinities of RNA or DNA aptamer-protein interactions. With only minor tweaks such assays can be adapted for almost all protein-aptamer interactions.

Additionally, this application note highlights the potential of MST assays for discovering previously unseen off-target effects of aptamers in native-like biological solutions.

Literature

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2bind.com
info@2bind.com
+49 941 2000 0890

Am Biopark 11
93053 Regensburg
Germany