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Application note

Determination of K_D of aptamer protein interactions by microfluidic diffusional sizing

The interactions of proteins with secondary molecules are of key importance in biology. Here we show how microfluidic diffusional sizing (MDS) can be used to measure K_D experimentally, with no calibration or specialist preparation required. The K_D of thrombin interacting with two different aptamers is successfully assessed by this method, with the values obtained in good agreement with those previously reported.

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Introduction

The interactions of proteins with secondary molecules is of great importance across the life sciences.

These interactions may reflect a protein-protein interaction, for example in signal transduction, an interaction between a protein and a non-protein molecule such as a drug binding its target, or a small molecule binding to change a protein's function or stability. Whatever the reason, proper characterization of the dissociation constant, K_D, is integral to understanding the interaction.

While methods exist to determine K_D experimentally, reviews remark that these often require a degree of expertise to collect reliable data (1) and can have technique specific limitations – e.g. immobilizing a protein for surface plasmon resonance can render it inactive, Co-IP can see significant background noise unless appropriately specific antibodies are used, and spectroscopic techniques require prior knowledge of the expected change upon binding (be it fluorescence, NMR shift, circular dichroism etc) (2).

Here we present a new technique to characterize protein interactions in vitro; microfluidic diffusional sizing (MDS). Crucially this technique is extremely simple and rapid – with no calibration, specialist preparation or setup required. After titrating the two species (one pre-labelled) the average size of the labelled species is measured and is quickly related back to K_D by standard fitting.

In this work the binding of serine protease thrombin to two aptamers (HD22 and TBA) is assessed. The K_D values determined for each interaction are found to be in good agreement with previously reported values.

Methods

Thrombin preparation

250 NIH units of lyophilized Thrombin (Sigma Aldrich, product code T1063) was reconstituted in 100 µL purified H_2O. The concentration was verified to be 600 µg/mL (= 16.3 µM) by testing on a Fluidity One.

Aptamer preparation

Two aptamers were separately assessed for their binding to thrombin; HD22 and TBA. Each were purchased pre-labelled with Alexa Fluor 647 – full details in appendix.
For each species, 100 µM aptamer in H_2O was diluted to a final concentration of 1 nM aptamer in 50 mM Tris, pH 7.4, 100 mM NaCl, 1 mM $MgCl_2$, 0.1% BSA for the experiment.

Sample preparation for titration

A set of samples were prepared to assess the K_D of each aptamer binding thrombin. For these the concentration of aptamer was held constant at 1 nM in each sample, while the concentration of thrombin was varied from 0 nM to 1630 nM, to reflect values above and below the expected K_D. Full details are given in the appendix.
All samples were prepared at the same time and then tested in order from lowest to highest thrombin content. Samples were stored at 4 °C between preparation and testing.

Test method

To test each sample a 5 µL aliquot was pipetted onto a microfluidic chip and tested using a Fluidity One-W instrument. This reports the average hydrodynamic radius (R_h) of the labelled species following microfluidic diffusional sizing (MDS) (3).

In this instance it means that the average size of unbound and bound aptamers in any sample was reported. Hence we observe an overall change from the R_h of the aptamer alone (with 0 μM thrombin added), to the R_h of the bound aptamer-thrombin complex (when an excess of thrombin is added to ensure the K_D is exceeded).

Results

Each sample was tested in triplicate, and the average values were fitted to a standard binding equation – see Figure 1. Fit parameters are shown in Table 1. The K_D results obtained are in good agreement with literature values.

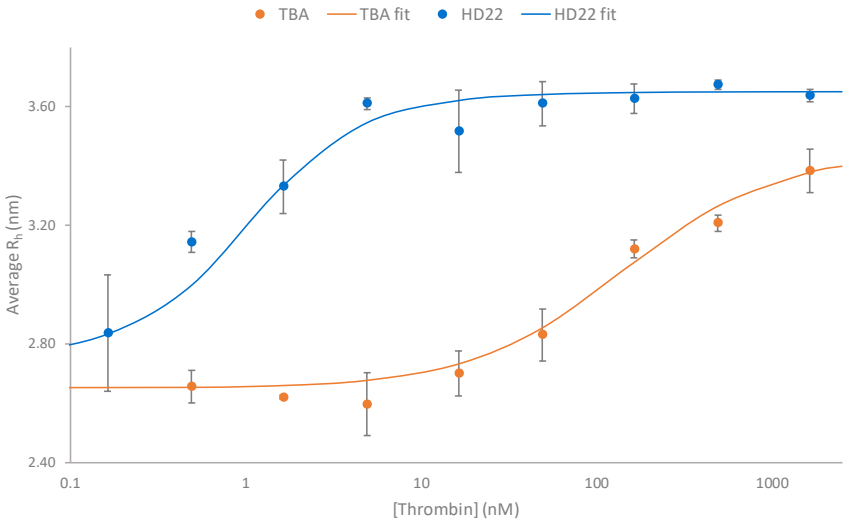

Figure 1: Results from the titration of HD22 and TBA with Thrombin.

Full breakdown of results and the fitting equation used are given in Appendix 1.

	Parameter	Present Work	Literature
HD22	$R_{h\text{-free}}$ (nm)	2.76	-
	$R_{h\text{-complex}}$ (nm)	3.64	-
	Binding affinity K_D (nM)	0.52 ± 0.08	0.5 (4)
TBA	$R_{h\text{-free}}$ (nm)	2.68	-
	$R_{h\text{-complex}}$ (nm)	3.43	-
	Binding affinity K_D (nM)	140 ± 20	25 - 200 (5)

Table 1: Comparison of the measured K_D found in this work and the literature reported K_D values.

The fit parameters are also shown; $R_{h\text{-free}}$, the hydrodynamic radius of the free aptamer, and $R_{h\text{-complex}}$, the hydrodynamic radius of the aptamer-thrombin complex.

Conclusion

Two pre-labelled aptamers were mixed with an unlabelled protein and using microfluidic diffusional sizing (MDS) on a Fluidity One-W the K_D of each interaction was successfully calculated. The calculated K_D values are in good agreement with previously reported values, and the difference in binding affinity is clearly visible.

The technique presents a simple means to experimentally determine K_D, and crucially does so without the need to alter the molecules beyond addition of a standard fluorescent label to one binding partner. Measuring size gives confirmation of on-target binding, by quick comparison of the expected and observed size. The method has no surface fixing, and no complex preparation steps – allowing binding to be observed rapidly and in near-native conditions.

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References

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Appendix

The HD22 and TBA used in this work were purchased pre-labelled with Alexa Fluor 647 from IBA Lifesciences GmbH.

In all instances the amount of aptamer was held constant at 1 nM while the amount of Thrombin varied as shown.

HD22 samples prepared

Thrombin added (nM)	Average R_h from triplicate testing (nm)	Standard deviation of R_h
0	2.76	0.11
0.0163	2.71	0.08
0.049	2.63	0.20
0.163	2.84	0.04
0.49	3.14	0.09
1.63	3.33	0.02
4.9	3.61	0.14
16.3	3.52	0.08
49	3.61	0.05
163	3.63	0.02
490	3.67	0.02
1630	3.64	0.08

TBA samples prepared

Thrombin added (nM)	Average R_h from triplicate testing (nm)	Standard deviation of R_h
0	2.68	0.06
0.49	2.66	0.01
1.63	2.62	0.11
4.9	2.60	0.08
16.3	2.70	0.09
49	2.83	0.03
163	3.12	0.03
490	3.21	0.07
1630	3.38	0.04

Binding equation used to calculate K_D:

$$y = R_{h\text{-}free} + (R_{h\text{-}complex} - R_{h\text{-}free}) \, x \, \frac{(K_D + A + x) - \sqrt{((K_D + A + x)^2 - 4 \, x \, A \, x \, x)}}{2A}$$

Where;

y = hydrodynamic radius of mixture measured on Fluidity One-W
$R_{h\text{-}free}$ = hydrodynamic radius of free aptamer
$R_{h\text{-}complex}$ = hydrodynamic radius of aptamer bound to thrombin
A = concentration of aptamer, which was kept constant at 1 nM
x = concentration of thrombin

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