Nanomedical DIAGNOSTICS

Comparison Study

Field Effect Biosensing versus Isothermal Titration Calorimetry Binding Measurements for Rho GTPase

- In this comparison study, Agile R100 and MicroCal iTC200 gathered binding data for Rho GTPase interacting with GAP.
- The affinity values for the binding pair determined by Agile R100 and the MicroCal iTC200 were consistent.
- Agile R100 used substantially less sample material and less experiment time than the MicroCal iTC200, while providing additional kinetic on- and off-rate data that was unattainable through ITC.
- This white paper expands on data that was published in a scientific journal: Lerner, et. al., Large Scale Commercial Fabrication of High Quality Graphene-Based Assays for Biomolecule Detection, Sensors & Actuators B (2016), DOI: 10.1016/j.snb.2016.09.137

ABSTRACT

Agile R100 is an optics-free binding assay system based on Field Effect Biosensing (FEB) with unprecedented capabilities. In this study, we compare Agile R100 to a standard binding analysis tool, isothermal titration calorimetry (ITC), by measuring immobilized Rho guanosine triphosphate hydrolase (GTPase) interacting with a GTPase-activating protein (GAP) on both platforms. The affinity value measured by Agile R100 was within a factor of 2 of the MicroCal iTC200 result, and both were within a factor of 2 of previously-published findings. FEB technology has the advantage over ITC of requiring a drastically smaller amount of protein and less experiment time.

INTRODUCTION

Agile R100 uses Field Effect Biosensing (FEB), a breakthrough electrical technique that measures the change in current that occurs during a binding interaction.[1] This change in conductance across a graphene biosensor surface is monitored in real-time, enabling fast and clear binding conclusions.

Isothermal titration calorimetry (ITC) measures the heat generated during molecular binding. The target is held in the chamber of the ITC system, and analyte is injected into the chamber at high concentrations. The bulk heat change associated with a chamber of molecules binding is measured. The dissociation constant (K_D), stoichiometry (N), change in enthalpy (ΔH), and change in entropy (ΔS) are calculated from the bulk heat change.

In the experiments detailed below, the binding interaction of Rho guanosine triphosphate hydrolase (GTPase) and a GTPase Activating Protein (GAP) is characterized with Agile R100 and with MicroCal iTC200. Rho GTPase catalyzes the conversion of GTP to GDP only in the presence of GAP. GAP controls the rate of movement from the active to inactive conformation of Rho GTPase.[2,3]

MATERIALS AND METHODS

Protein Expression and Purification

Recombinant, His-tagged Rho GTPase and GST-tagged GAP were expressed in *E. coli* bacteria and purified using standard methods.[3]

From 1.5 L bacterial culture, protein purification yielded approximately 1.1 mg Rho GTPase and approximately 0.35 mg GAP. Rho GTPase and GAP were diluted to working concentrations in assay buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 25 µM GTP, and 1 mM βME) for the Agile R100 and MicroCal iTC200 experiments.

ITC requires both the target protein and the analyte to be in the same buffer, and that the buffers contain the same additives (surfactants, chelators, etc.). To keep the comparison simple here, the same buffer and additives were used for target and analyte on both the FEB and ITC systems. However, use of different buffers and additives for target and analyte is supported on Agile R100, and may be necessary to maintain protein stability or function.

FEB Biosensor Immobilization

COOH biosensor chips were used with an Agile R100 system for data acquisition. The graphene surface of the COOH biosensor chips was activated using a standard EDC/sNHS crosslinker protocol.[4]

Immediately after activating the surface, 50 µL 10.7 nM Rho GTPase (12.6 ng total protein) in cell culture grade 1X phosphate buffered saline (PBS) pH 7.4 was incubated on

the graphene surface for 15 minutes at room temperature to covalently link and immobilize the target. This is a typical concentration to use for a small protein (~21 kDa) like Rho GTPase.

Next, 50 µL polyethylene glycol (PEG)-based "Quench 1" was added to block exposed graphene to nonspecific binding, followed by 50 µL ethanolamine based "Quench 2" to inactivate any remaining surface binding sites. Both quenching steps were incubated at room temperature for 15 minutes.

FEB Biosensor Measurement

Assay buffer was used to create a baseline measurement for the Rho GTPase functionalized chips. A GAP concentration series was measured with each exposure consisting of 50 µL of 16.5 ng, 165 ng, 825 ng, 1650 ng, and 10,000 ng total protein of GAP in assay buffer. The association sensing response was recorded. GAP containing solutions were aspirated off, and assay buffer was added to initiate dissociation. A calibration step for the next concentration of GAP was performed, and the association and dissociation were repeated for all concentrations of GAP in a continuous measurement process.

Agile Plus software graphed sensing responses with respect to each concentration, generated a standard curve, and was used to export data for second-order fits and further processing.

ITC Measurement

Using MicroCal iTC200, 300 µL 1 mg/mL GAP (300 µg total protein) was injected into the ITC well. This concentration is typical for an expected K_D in the low micromolar range.

Next, highly concentrated analyte (300 µM Rho GTPase) was titrated into the ITC well until the concentration of Rho GTPase was ~2.5 times greater than the concentration of GAP. 19 injections were used, consuming about 500 µg total protein of Rho GTPase. The heat in the system is monitored and allowed to come to equilibrium between steps.

ITC measures the heat generated during binding to determine the dissociation constant K_D, stoichiometry N, enthalpy ΔH, and entropy ΔS.

RESULTS

Agile R100 Binding Data

Agile R100 performs real-time direct measurements of biochemical interactions on the sensor surface. Figure 1A depicts the association curves for all 5 analyte concentrations tested.

The data in Figure 1A show clear concentration-dependent binding. We then fit second-order fit lines to the raw

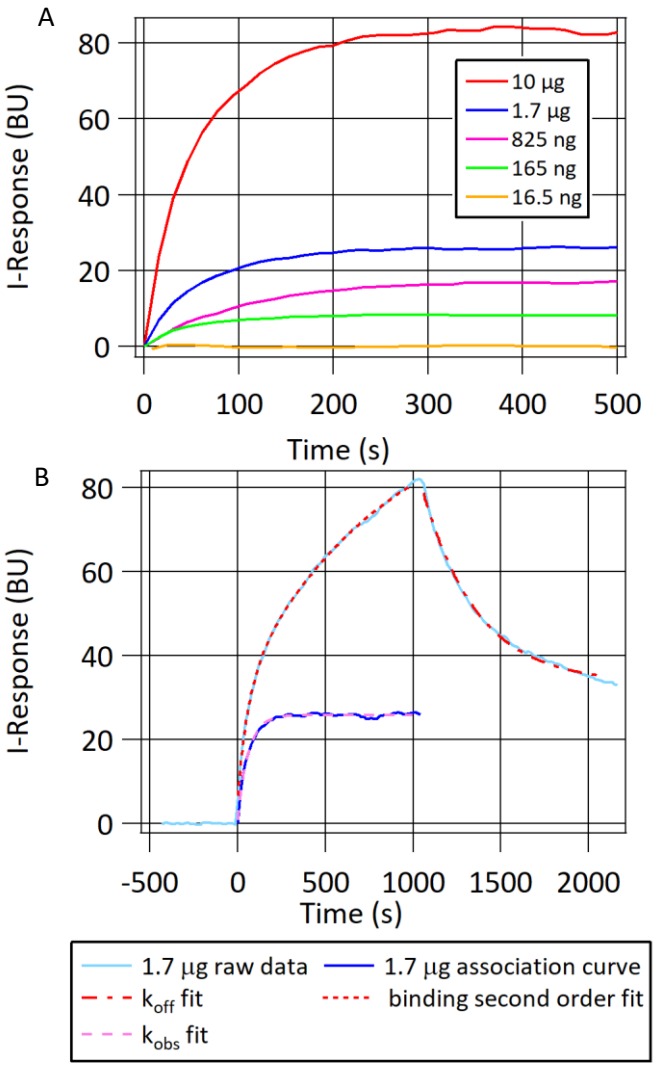

Figure 1: Kinetic binding data of the Rho GTPase and GAP interaction using Agile R100. A) The association curves are graphed for different amounts of GAP, each in 50 µL of buffer. C) The kinetic experiment method calculated k_{on} and k_{off} with second-order and first-order fit curves, respectively, to generate specific and accurate K_D values.

association curves, and subtracted a concentration-independent background signal, yielding binding association curves for each concentration. Figure 1B shows this process demonstrated using the 1.7 µg data. k_{on} was calculated as $k_{on} = (k_{obs} – k_{off}) / [\text{Concentration}]$, where k_{obs} is the observed binding rate at a given concentration, and k_{off} is obtained from a fit of the dissociation step.[5] K_D was calculated as $K_D = k_{off} / k_{on}$. This process was done for each concentration measurement performed with Agile R100, and the results are shown in Table 1. The average of those values is a K_D of 2.0 µM, within a factor of 2 of the ITC result.

Table 1: Summary of kinetic binding data of Rho GTPase and GAP using Agile R100 and MicroCal iTC200

Agile R100		MicroCal iTC200	
Conc.	**K_D**	**Conc.**	**K_D**
10 µg	1.5 µM	30 µM	2.7 µM
1.7 µg	1.4 µM		
825 ng	3.8 µM		
165 ng	1.1 µM		

MicroCal iTC200 Thermodynamic Data

As measured by MicroCal iTC200 (Fig. 2), K_D of 2.7 µM, within a factor of 2 of the Agile R100 result. Stoichiometry is measured, and N = 0.66 ± 0.01 sites. Energy parameters such as enthalpy and entropy are also characterized with ITC, and $\Delta H = 1.5 \pm 0.4$ calmol^{-1} and $\Delta S = 76.7$ calmol^{-1}deg^{-1}. ITC does not provide k_{on} and k_{off} values.

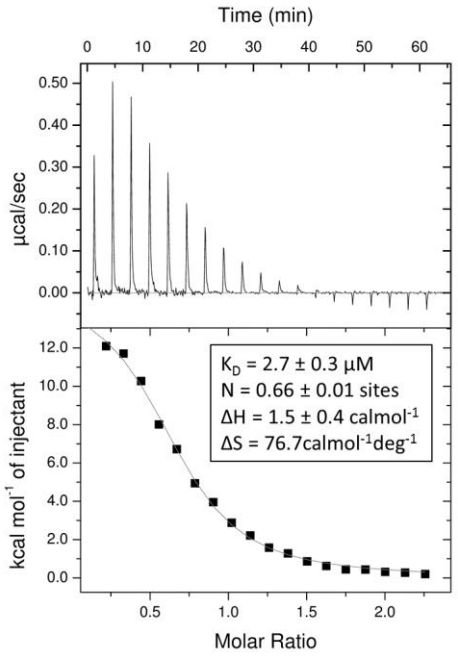

Figure 2: Thermal binding data of the 300 µM Rho GTPase and 30 µM GAP interaction using Microcal iTC200.

Discussion

Agile R100 delivers fast binding data comparable to alternative techniques, as indicated by the similarity of K_D values. Schonegg et al. reports K_D = 4.0 µM for the Rho GTPase and GAP interaction.[3] When a quick validation or kinetics check is needed, or a minimal amount of sample is available, the demonstrated approach of measuring responses for a few concentrations using Agile R100 is preferable to running an entire ITC measurement.

As shown in Table 2, the total experiment time and total amount of material used was significantly lower when using FEB compared to ITC.

Table 2: Rho GTPase and GAP Experimental Specifications

	Agile R100	MicroCal iTC200
Amount of Rho GTPase used	13 ng	500,000 ng
Amount of GAP used	13,000 ng	300,000 ng
Experiment Time	4 hours	16 hours

CONCLUSIONS

Table 3 displays the advertised system specifications of the two platforms. Agile R100 has an 11-log dynamic range versus MicroCal iTC200's 7 logs. Agile R100 uses much less target material, conserving precious samples. The platform is compact and lightweight so that benchtop space is preserved, and portability is possible.

Table 3: Agile R100 and MicroCal iTC200 System Specifications

	Agile R100	MicroCal iTC200
Dynamic Range of Detection	100 fM – 10 mM	1 nM – 10 mM
Amount of Target Material	0.5 – 500 ng	10,000 – 1,500,000 ng
Sample Volume	10 – 50 µL	200 – 300 µL
Total Time Per Measurement	15 – 45 min	40 – 60 min
Samples Per Day	9 – 28 per 8 h day	8 – 12 per 8 h day
Operating Temperature Range	-20°C – 100°C	10°C – 28°C
Weight of System	0.9 kg	9.4 kg
Dimensions (W x D x H)	7.6 cm x 21.2 cm x 3.6 cm	21 cm x 35 cm x 34 cm

With a minimal amount of sample needed for detection, fast assay troubleshooting is now possible with Agile R100. In general, Agile R100 compared to MicroCal iTC200:

- Detects interactions with greater sensitivity
- Uses significantly less sample material
- Requires less experiment time
- Detects in a wider temperature range, enabling more flexible experimental functionality
- Has easy bench-to-bench portability
- Saves benchtop space

REFERENCES

1. Lerner MB, Pan D, Gao Y, et al. Large scale commercial fabrication of high quality graphene-based assays for biomolecule detection. *Sensors Actuators, B Chem*. 2016. doi:10.1016/j.snb.2016.09.137.
2. Jaffe AB, Hall A. RHO GTPASES: Biochemistry and Biology. *Annu Rev Cell Dev Biol*. 2005;21(1):247-269. doi:10.1146/annurev.cellbio.21.020604.150721.
3. Schonegg S, Constantinescu AT, Hoege C, Hyman AA. The Rho GTPase-activating proteins RGA-3 and RGA-4 are required to set the initial size of PAR domains in Caenorhabditis elegans one-cell embryos. *Proc Natl Acad Sci U S A*. 2007;104(38):14976-14981. doi:10.1073/pnas.0706941104.
4. Goldsmith B, Lcascio L, Walker A, et al. *AGILE R100*. 1st ed. (Nokes J, Shue A, Barron F, Lerner M, eds.). San Diego: Nanomedical Diagnostics; 2016.
5. Pollard TD. A guide to simple and informative binding assays. *Mol Biol Cell*. 2010;21(23):4061-4067. doi:10.1091/mbc.E10-08-0683.