Nanomedical DIAGNOSTICS

Application Note

Reproducible Kinetic Characterization of Small Molecule Compounds in Serum with Agile R100

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- Understanding how a small molecule performs in the presence of serum is fundamental to creating a successful drug. Early insight into how a compound binds to a target in the presence of serum enables better decisions for candidate selection and eventual development, potentially decreasing attrition rate and reducing expense and time to market.
- Assays often need to be greatly modified when studying a compound in serum versus buffer. Frequently, different tools are used, necessitating the development of entirely new experimental conditions. Even when the same platform is utilized, limited ability to measure in complex matrices forces additional assay re-optimization, expending precious time and resources.
- In this application note, we show that Agile R100 provides reproducible kinetic binding data for small molecules with **no assay adjustment when transitioning from buffer to serum.** We examine 3 low molecular weight compounds interacting with their targets – a CR GPCR, a cytokine, and an aptamer in both buffer and serum. **The kinetic constants between buffer and serum are within a factor of 1.9**.
- Agile R100 leverages breakthrough FEB technology to easily sense in complex matrices, providing earlier information about the behavior of a drug in serum without extensive assay development and optimization.

ABSTRACT

Obtaining small molecule kinetic information in serum earlier in the drug discovery process gives researchers a significant edge in determining the likelihood of success of a drug.[1] Agile R100 leverages Field Effect Biosensing (FEB) technology to detect small molecule interactions in both buffer and serum, without the interference and noise common on other platforms. Minimal to no protocol changes are required to transition from buffer to serum which drastically reduces development time and cost, bringing early kinetic serum data within the reach of most labs. For this application note, 3 unique target and small molecule binding pairs were chosen to represent common interactions in the drug discovery space. The pairs are: a chemokine binding G-protein coupled receptor (CR GPCR) binding with small molecule Compound A, the cytokine tumor necrosis factor alpha (TNFα) protein binding with small molecule SPD304, and a DNA aptamer against colistin (i.e. colistin aptamer) binding with the antibiotic drug colistin. Agile R100 provides reproducible kinetic characterization of small molecules in serum with no assay adjustment from buffer, illuminating the ability to use the system throughout the drug discovery and development process with minimal modifications.

INTRODUCTION

Kinetic characterization of small molecule interactions in serum is fundamental to understanding parameters for clinical efficacy, and early access to this vital information enables improved drug candidate development and earlier attrition of non-performing compounds.[1] Serum interference can cause small molecule compounds to behave differently than they do in buffer. For example, some initially promising compounds may bind to common components in serum, such as albumin, leaving less available active drug and reducing efficacy.[2,3] The earlier this can be discovered, the more effective decisions are about leads that advance in drug discovery.

Many kinetic binding assay platforms suffer from serum interference.[4,5] Surface plasmon resonance (SPR) and Bio-Layer Interferometry (BLI) are useful for kinetic characterization in buffer, but these optical systems are greatly impacted by dense matrices such as serum.[6,7] High performance liquid chromatography-mass spectroscopy (HPLC-MS) performs precise analysis of small molecules in serum, but does not provide information-rich kinetic data including affinity and on- and off-rates, only the amount of drug present in a sample. In addition, HPLC-MS requires rigorous and time-consuming experimental design because the platform is highly sensitive to changes in buffer, separation technique, flow rate, and volume of fractions, among other factors.[8] Often, multiple detection platforms are used to attain kinetic binding data initially in buffer and later in serum, but assay development and optimization are required at every transition, which adds costs, time, and resources. A platform is needed that can characterize kinetic binding interactions across buffer and serum conditions with minimal to no assay adjustment.

Agile R100 is based on Field Effect Biosensing (FEB), which is an electrical technique, not an optical one.[9] The target is immobilized to the biosensor surface, and change in conductance is measured in real-time as analyte bind to and dissociate from the target.[10] This produces reliable kinetic binding data with high accuracy, sensitivity, and reproducibility.[11] Optically-dense serum causes large amounts of background noise on SPR and BLI tools, which necessitates time-consuming and error-prone reference subtraction measurements. Agile R100 easily circumvents this because the response is not impacted by the optical density of the sample; the system only registers changes in conductance, an electrical property. Because serum does not interfere with the electrical sensing mechanism of the platform, minimal to no assay adjustment is needed between buffer and serum conditions. Therefore, Agile R100 is an optimal kinetic binding platform for early stage drug discovery through preclinical development that minimizes the time and cost of assay optimization.

To demonstrate Agile R100's ability to reproducibly measure kinetics in both buffer and serum, 3 binding interactions that represent unique and high-valued targets in the world of drug discovery are characterized in this application note. GPCRs are extensively involved in cellular signal transduction,[12] and Compound A is a small molecule drug antagonist for GPCR chemokine receptor (CR), involved in autoimmune diseases. TNFα is a cytokine well-known for its involvement in activation of inflammation pathways, and SPD304 is a small molecule inhibitor of TNFα.[13–15] Colistin is an antibiotic used to treat multi-drug resistant organisms (MDROs) common in sepsis and other serious infections,[16] and the colistin aptamer is a DNA aptamer against colistin being researched with the goal of improving therapeutic drug monitoring (TDM) of critically ill patients. Agile R100 measures the kinetics of all 3 of these compounds in both buffer and serum with no adjustments made from simple buffer to serum. This streamlined approach enables valuable kinetic characterization in serum without additional time and resources, accelerating drug discovery and development.

MATERIALS AND METHODS

Biosensor Chip Immobilization

CR GPCR

Using the "Immobilization – NTA" protocol in Agile Plus software, 14.3 µg/mL His-tagged CR GPCR (47.7 kDa) was immobilized onto NTA biosensor chips. The experiment was performed at room temperature, and the volume for each step was 50 µL. After calibration in diH_2O for 5 minutes, 11.3 mM $NiCl_2$ was incubated for 15 minutes. The biosensor chips were then rinsed with diH_2O followed by immobilization buffer (1X phosphate buffered saline [PBS] pH 7.4, 1% *n*-Dodecyl β-D-maltoside [DDM], 1% cholestryl hemisuccinate [CHS]) for 2 minutes each. The system was recalibrated in immobilization buffer for 5 minutes, followed by CR GPCR incubated for 60 minutes in immobilization buffer. Finally, the biosensor chips were rinsed 5 times with assay buffer to allow the system to equilibrate before measurement. Assay buffer for the buffer experiments consisted of 1X PBS pH 7.4, 1% DDM, 1% CHS, 0.2% DMSO. Assay buffer for the serum experiments consisted of the same: 1X PBS pH 7.4, 1% DDM, 1% CHS, 0.2% DMSO, with the addition of normal rat serum to a final concentration of 1%.

TNFα

Using the "Immobilization – NHS" protocol in Agile Plus software, 179 ng/mL tumor necrosis factor alpha (TNFα) (17.5 kDa) was immobilized onto NHS biosensor chips. The experiment was performed at room temperature and the volume for each step was 50 µL. After calibration in immobilization buffer (1X PBS pH 7.4) for 0.5 minutes, the target TNFα was incubated for 15 minutes in immobilization buffer. Then, Quench 1 (3 mM polyethylene glycol [PEG-amine] in 1X PBS pH 7.4) was incubated for 15 minutes to block exposed graphene from nonspecific binding, followed by Quench 2 (1 M ethanolamine in 1X PBS pH 8.5) for 7.5 minutes to deactivate any remaining binding sites. Finally, the biosensor chips were rinsed 5 times with assay buffer to allow the system to equilibrate before measurement. Assay buffer for the buffer experiments consisted of 1X PBS pH 7.4. Assay buffer for the serum experiments consisted of the same: 1X PBS pH 7.4, with the addition of normal rat serum to a final concentration of 1%.

Colistin Aptamer

Colistin aptamer (21.7 kDa) was immobilized onto bare biosensor chips, attached via a pyrene group on the 5' terminus of the aptamer. A custom DNA aptamer against colistin sulfate designed by BasePair Biotechnologies is used in this study. The π-π stacking between the graphene surface and the pyrene group ensures strong attachment of the aptamer to the biosensor chip. The experiment was performed at room temperature, and the volume for each step was 50 µL. A custom protocol was created in Agile Plus software as follows: After calibration in immobilization buffer (1X PBS pH 7.4, 1 mM $MgCl_2$) for 5 minutes, 2.17 µg/mL colistin aptamer was incubated for 15 minutes in immobilization buffer. Then, the biosensor chips were rinsed for 1 minute in immobilization buffer. Modified Quench 1 (3 mM PEG-amine in 1X PBS pH 7.4, 1 mM $MgCl_2$) was incubated for 15 minutes, serving as a block against nonspecific interactions. Finally, the biosensor chips were rinsed 5 times with assay buffer to allow the system to equilibrate before measurement. Assay buffer for the buffer experiments consisted of 1X PBS pH 7.4, 1 mM $MgCl_2$. Assay buffer for the serum experiments consisted of the same: 1X PBS pH 7.4, 1 mM $MgCl_2$, with the addition of normal rat serum to a final concentration of 1%.

Measurement

The "Kinetic Binding with Zero" protocol in the Agile Plus software was used for all 3 experiments. The protocol consists of 5 steps, beginning with a 5-minute calibration in the corresponding assay buffer. Then a zero-concentration measurement was taken in assay buffer. The biosensor chips were then recalibrated in assay buffer for 5 minutes, followed by the addition of the small molecule compounds to measure the association kinetics. Compound A (434.89 g/mol) and SPD304 (620.53 g/mol) were measured for 5 minutes, and colistin sulfate (2801.3 g/mol) was measured for 7 minutes. Finally, assay buffer was added to the biosensor chips to promote dissociation of the relevant compound, and incubated for 5 minutes for each binding pair. Compound A binding to CR was measured at a concentration of 100 µM, SPD304 binding to TNFα was measured at 5 µM, and colistin sulfate binding to the aptamer was measured at 10 µM.

Assay buffers for the buffer experiments were developed using information from previous experiments and literature. The assay buffer for the serum experiments used the same respective buffers with the single addition of 1% normal rat serum. No additional modifications were necessary to successfully transition from buffer to serum experiments.

Analysis

Three replicates were measured for each interaction in buffer, and 4 replicates were measured for each interaction in serum. The average sensor response for both buffer and serum is plotted with standard deviation shown as grey lines. The kinetic binding values for the association rate (k_{on}), dissociation rate (k_{off}), and dissociation constant (K_D) for each measurement are calculated by Agile Plus software. For the CR GPCR and Compound A interaction, the C-Response[10] was analyzed, and for the TNFα cytokine and SPD304 interaction and the colistin aptamer and colistin interaction, the I-Response[10] was analyzed.

RESULTS AND DISCUSSION

For each interaction, reproducible kinetic binding results were calculated with n = 3 for each interaction in buffer and n = 4 for each interaction in serum. The average k_{on}, k_{off}, and K_D for each measurement in buffer and serum are summarized in Tables 1, 2, and 3, and the average sensorgrams and standard deviation for each interaction in buffer and serum are shown in Figures 1, 2, and 3. Importantly, no modifications were needed to transition from buffer to serum experiments.

Table 1: Summary of the average kinetic binding data (k_{on}, k_{off}, and K_D) generated from the CR and Compound A interaction in the relevant assay buffer (either in assay buffer without serum or with serum).

	k_{on} ($M^{-1}s^{-1}$)	k_{off} (ks^{-1})	K_D (µM)
Buffer	155 ± 54.9	5.04 ± 1.79	38.4 ± 18.8
Serum	134 ± 56.0	7.81 ± 2.95	59.1 ± 6.25

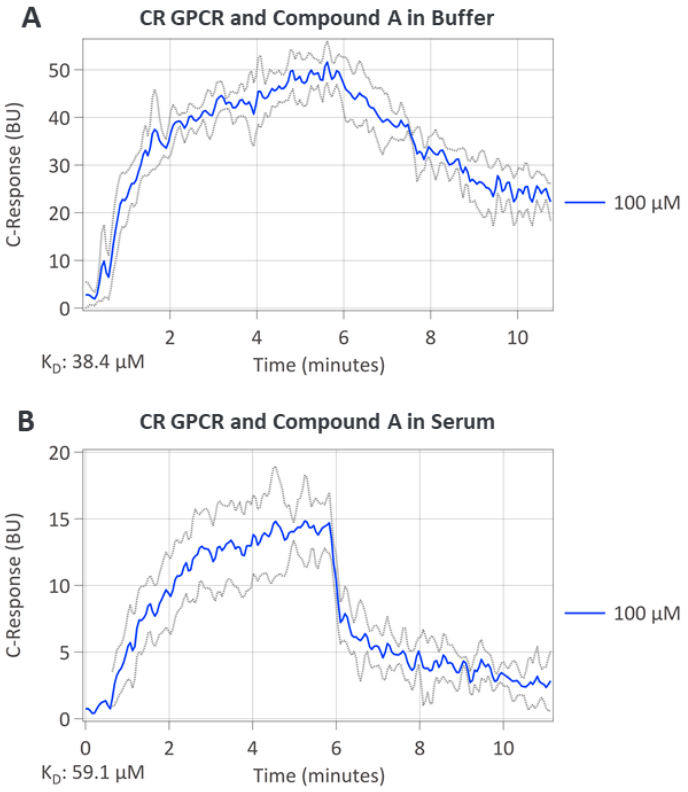

Figure 1: Average sensorgram and standard deviation of 100 µM Compound A added to immobilized CR. A) Interaction in 1X PBS pH 7.4, 1% DDM, 1% CHS, 0.2% DMSO, and the K_D is 38.4 ± 18.8 µM. B) Interaction in 1X PBS pH 7.4, 1% DDM, 1% CHS, 0.2% DMSO with the addition of normal rat serum to a final concentration of 1%, and the K_D is 59.1 ± 6.25 µM.

The average kinetic binding data in buffer compared to serum for the CR GPCR and Compound A interaction are comparable and reproducible, as shown in Table 1. All kinetic binding data were **within a factor of 1.5 for buffer and serum**, showing that Agile R100 produces precise kinetic binding data for small molecule compounds in both buffer and serum with no modification when transitioning from buffer to serum.

Table 2: Summary of the average kinetic binding data (k_{on}, k_{off}, and K_D) generated from the TNFα and SPD304 interaction in the relevant assay buffer (either in assay buffer without serum or with serum).

	k_{on} ($M^{-1}s^{-1}$)	k_{off} (ks^{-1})	K_D (µM)
Buffer	2500 ± 198	8.02 ± 1.27	3.19 ± 0.278
Serum	2520 ± 491	14.3 ± 1.23	5.94 ± 1.48

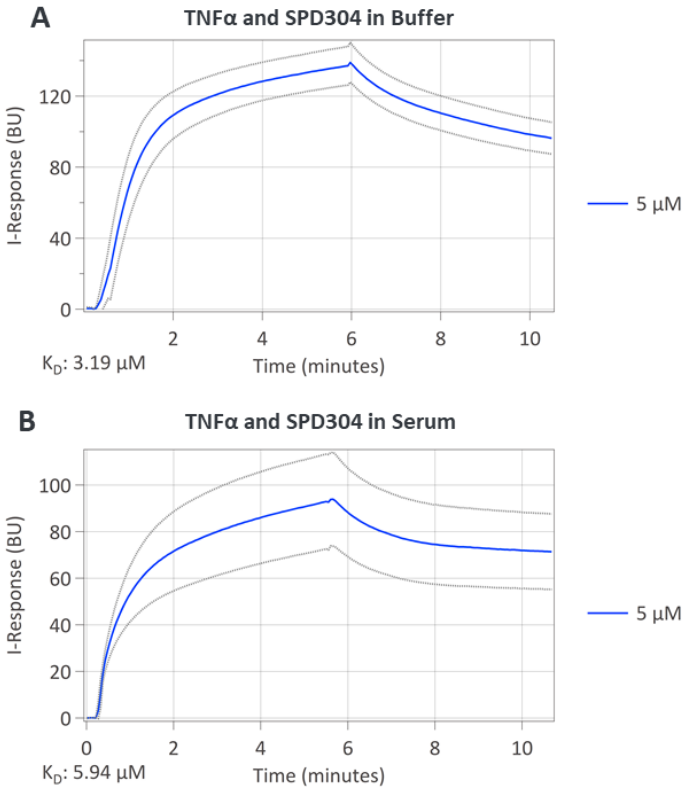

Figure 2: Average sensorgram and standard deviation of 5 µM SPD304 added to immobilized TNFα. A) Interaction in 1X PBS pH 7.4, and the K_D is 3.19 ± 0.28 µM. B) Interaction in 1X PBS pH 7.4 with the addition of normal rat serum to a final concentration of 1%, and the K_D is 5.94 ± 1.48 µM.

The average kinetic binding data in buffer compared to serum for the TNFα cytokine and SPD304 interaction are comparable and reproducible, as shown in Table 2. All kinetic binding data were **within a factor of 1.9 for buffer and serum.** Additionally, the kinetic binding data for buffer are in line with previously-published K_D values, which range from 5.4 ± 0.2 µM to 24.9 ± 2.0 µM when the small molecule SPD304 is solubilized in buffers with different solvents.[17] Agile R100 displays high reproducibility and accuracy in both buffer and serum.

Table 3: Summary of the average kinetic binding data (k_{on}, k_{off}, and K_D) generated from the colistin aptamer and colistin sulfate interaction in the relevant assay buffer (either in assay buffer without serum or with serum).

	k_{on} ($M^{-1}s^{-1}$)	k_{off} (ks^{-1})	K_D (µM)
Buffer	797 ± 313	5.00 ± 1.04	6.68 ± 1.11
Serum	471 ± 144	3.53 ± 0.934	7.58 ± 0.853

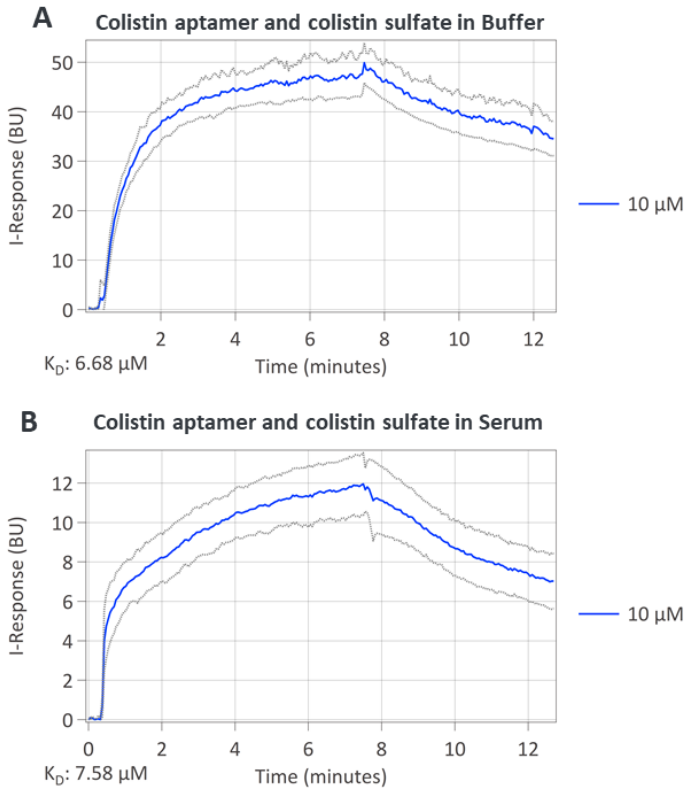

Figure 3: Average sensorgram and standard deviation of 10 µM colistin sulfate added to immobilized colistin aptamer. A) Interaction in 1X PBS pH 7.4, 1 mM $MgCl_2$, and the K_D is 6.68 ± 1.11 µM. B) Interaction in 1X PBS pH 7.4, 1 mM $MgCl_2$ with the addition of normal rat serum to a final concentration of 1%, and the K_D is 7.58 ± 0.853 µM.

The average binding kinetics in buffer compared to serum for the colistin aptamer and colistin interaction are comparable and reproducible, as shown in Table 3. All kinetics were **within a factor of 1.7 for buffer and serum**, illustrating Agile R100's ability to measure interactions in both buffer and serum across multiple biosensor chips with high precision.

The difference in sensor response magnitude between buffer and serum could possibly be due to the attenuation of signal caused by the presence of albumin, which can cause a decrease in sensor response magnitude. Serum could be bioloading onto the sensor surface, which would affect the charge profile and alter the sensor response magnitude when small molecules bind to the target. Though the sensor response magnitudes differ between buffer and serum, the kinetic binding data are

within a factor of 1.9 for all interactions, showing that serum does not interfere with the kinetic characterizations. Agile R100 provides highly reproducible kinetic characterization of small molecules in serum, letting researchers easily gain information-rich data without extensive time and expense.

CONCLUSIONS

The ability to study the interactions of small molecule compounds with their target proteins is crucial for successful drug discovery. By eliminating compounds that do not perform well in serum, researchers can make more confident decisions while reducing time and cost.[1] Agile R100 reproducibly characterizes small molecules in serum using FEB, an electrical label-free technique that is not subject to the serum interference that affects other assay tools. In this application note, Agile R100 precisely measures the binding kinetics of 3 low molecular weight compounds interacting with their targets in both buffer and serum with no assay modification, and the kinetic binding data compared between buffer and serum are within a factor of 1.9 for all 3 binding pairs. This demonstrates the **ability to measure in serum with little to no assay adjustment from buffer,** highlighting the possibility for increased candidate success rate with FEB technology.

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