Strategic approaches to unlock binding kinetics in complex protein systems using grating-coupled interferometry (GCI)

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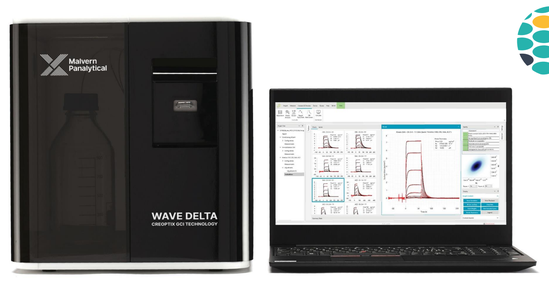
Introduction

- Accurate measurement of interactions between small molecules/biologics and targets of interest (TOI) is crucial for the development of effective therapeutic treatments
- Surface-based biophysical methods like surface plasmon resonance (SPR) and grating-coupled interferometry (GCI) are routinely used to determine the binding kinetics (**Fig. 1**)
- Though powerful techniques, SPR & GCI can face challenges when quantifying binding interactions such as when analytes exhibit very slow dissociation rates (long residency times) or when assessing the kinetics of larger, multivalent, complex biologics
- Methods have been developed to investigate challenging systems by SPR, but it remains to be seen if these can extend to GCI as a more recently-developed surface-based technique. We show the development of a fully regenerable GCI surface and streamlined workflows for antibodies/biologics & analytes with long residence times.

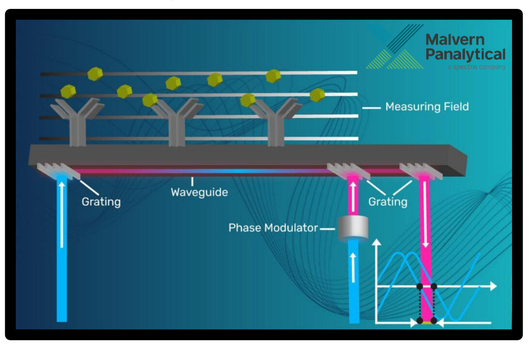


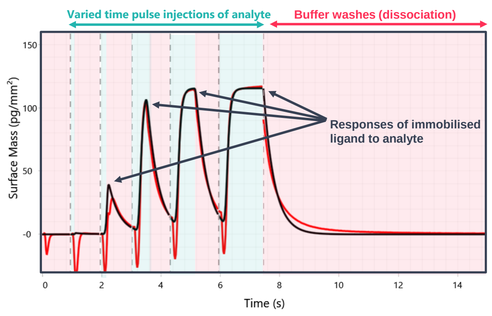
Assay Biology
Quality Data Throughout

Image source: Malvern Panalytical

Controlled release of biotinylated substrate from a regenerable GCI surface

Fig. 1. Principles of GCI.





Challenges of current immobilisation strategies

- Amine coupling is readily available but heterogeneous and can reduce protein activity
- NTA affinity capture possible for his-tagged proteins and regenerable surface, but baseline drift can complicate kinetic analysis
- Streptavidin capture of biotinylated substrate reduces baseline drift but not regenerable due to high biotin-streptavidin affinity
  - Non-specific binding of analytes to streptavidin
  - Enzymatic site-specific biotinylation of an Avi-tagged protein homogenises capture and maintains activity
- Regenerable surfaces can increase screening throughput and reduce consumables costs

Switchavidin

- Engineered for pH dependent affinity of avidin, with much faster dissociation under slightly acidic conditions
- Further mutated to reduce non-specific binding of analyte
- More flexibility by allowing multiple rounds of immobilisation on the same chip
- Improved efficiency of surface-based kinetic studies (**Fig. 2**)
- At time of experiments, no established method existed for GCI

Fig. 3a. Method validation of SwA capture using tool compounds.

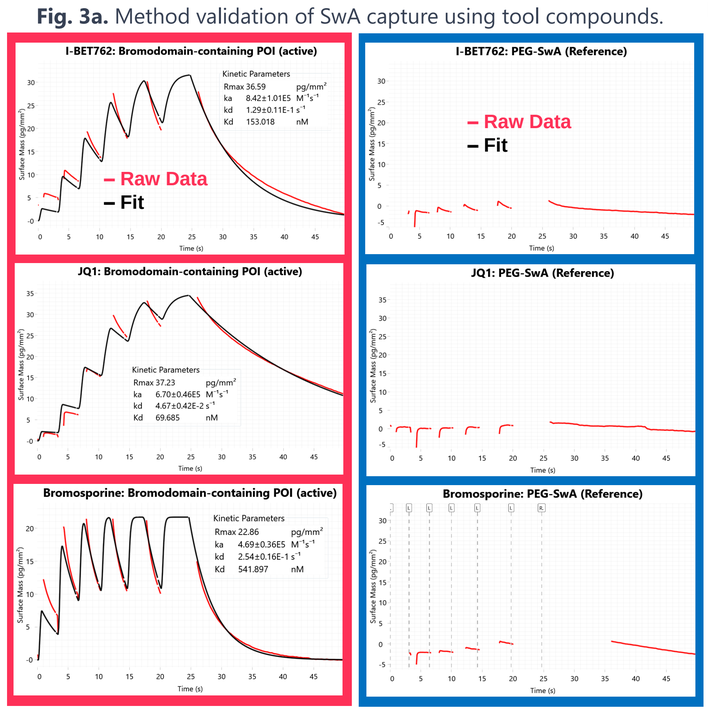
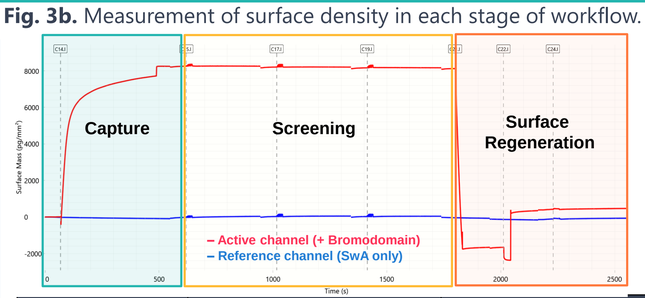


Fig. 3b. Measurement of surface density in each stage of workflow.



Analyte	Calculated K_D	Literature K_D
I-BET762	153 nM	low to mid nM
JQ1	70 nM	low nM
Bromosporine	542 nM	nM to low µM

Method Validation

- Biotinylated PEG2-amine was covalently attached to a polycarboxylate surface using NHS succinimide cross-coupling
- Biotinylated bromodomains were incubated with Switchavidin
- Three test compounds were screened against bromodomain proteins (**Fig. 3a**)
- Affinities closely match reported values in literature (**Fig. 3b**)
- Near complete removal of SwA-bromodomain protein complex using dilute citric acid + SDS
- No surface activity of compounds following chip stripping

Fig. 2. General workflow of switchavidin (SwA) capture.

Kinetic profiling of antibodies

Challenges

- Multivalent species can bind more than one binding partner, particularly in high-density environments
- Engaging multiple epitopes simultaneously will enhance the stability of antibody-antigen complexes and increase residence time. This phenomenon is known as avidity (**Fig. 4a**)
- Avidity can be avoided by capture of the antibody instead of the antigen
- Poor covalent immobilisation of antibodies can occlude the Complementarity-determining regions (CDRs) from binding partners (**Fig. 4b**)

Fig. 4a. Avidity effects with antigen immobilisation.

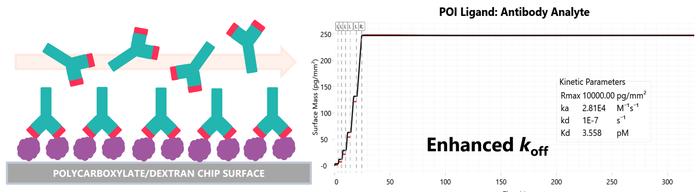
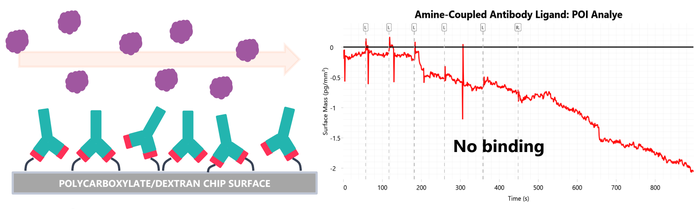


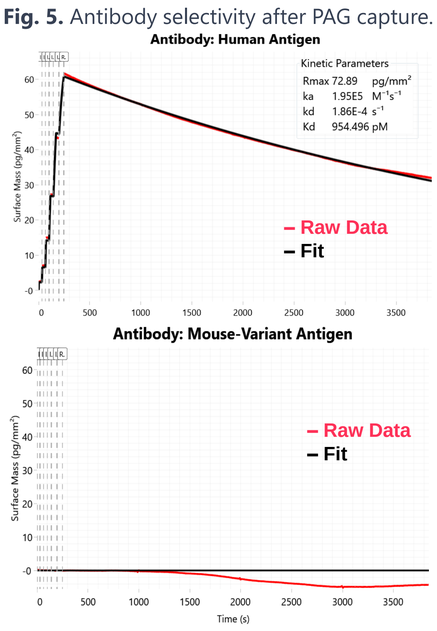
Fig. 4b. Poor antibody immobilisation strategy.



Proteins A & G (PAG)

- Strong affinity for non-binding (F_c region) of antibodies – typically nM
- Covalent immobilisation of PAG can properly orient antibody to measure 1:1 binding events between paratope & antigen

Fig. 5. Antibody selectivity after PAG capture.



Key Findings

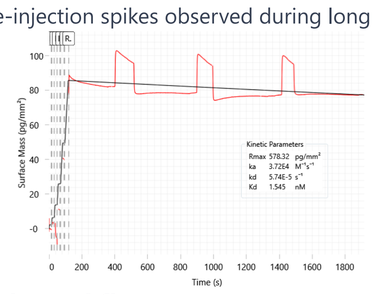
- PAG capture of antibody minimises avidity & rebinding effects; correct orientation of paratope
- pM to nM affinity of antibodies for human variant antigen (**Fig. 5**)
- Antibody selective for human variant antigen; no binding observed for mouse variant antigen
- Low immunoresponse to alt. mammalian variant antigens may predict performance in pre-clinical studies

Chaser assays for a slow k_{off}

Challenges

- Rebinding of a compound can artificially enhance the k_{off}
- Extended dissociation times requires syringe re-filling & re-injection
- Re-injections of buffer introduces noise (**Fig. 6**) during the wash (dissociation) phase, further decreasing confidence in k_{off} calculations
- k_{off} may be more accurately determined by measuring the return of surface activity using a control/tool compound

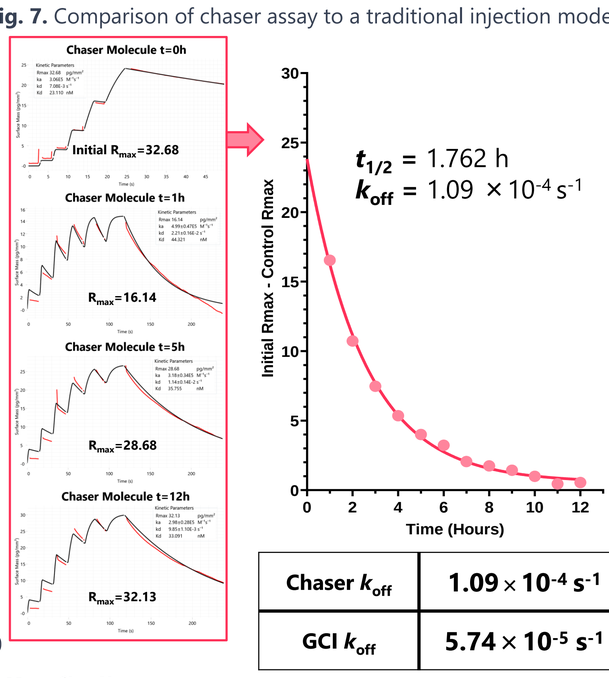
Fig. 6. Re-injection spikes observed during long wash phases.



Principle workflow

1. Protein captured to chip surface
2. The R_{max} of a tool compound with a faster k_{off} is recorded at t_0
3. Protein saturated with slow dissociation (k_{off}) analyte (compound A)
4. The tool compound is injected at regular intervals (t_n) over a pre-calculated time window
5. As compound A dissociates, more protein available for tool compound to bind to
6. R_{max} will increase over time window
7. An R_{max} correction is applied: R_{max} (t_0) – R_{max} (t_n)
8. Half-life calculated using an exponential decay model (**Fig. 7**)

Fig. 7. Comparison of chaser assay to a traditional injection mode.



Chaser k_{off}	1.09×10^{-4} s^{-1}
GCI k_{off}	5.74×10^{-5} s^{-1}

Key findings

- Careful selection of tool (chaser) compound required for successful execution of assay
- Use of chaser assay more accurately calculates dissociation rate constants by reducing effects of re-binding for compounds with long half-lives
- Injection spike interference due to syringe refills eliminated

Summary

- We have developed a fully regenerable, GCI chip for biotinylated molecule, whereby their controlled release is regulated via the pH-dependent affinity of an engineered avidin, switchavidin. This increases screening throughput and cost-effectiveness for our clients.
- Proteins A & G (PAG) capture of the F_c region correctly orients the antibody and avoids acidic conditions required for efficient amine cross-coupling. This maintains antibody activity and reduces avidity & rebinding effects to more accurately binding kinetics via a 1:1 model.
- A chaser can be used to more accurately determine the dissociation rate constant of a lead candidate small molecule by reducing re-binding and eliminating re-injection spikes from syringe refills

Domainex welcomes interest from any potential collaborators, industrial or academic. If you would like to learn more about our drug-discovery platform, please contact: **enquiries@domainex.co.uk**

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