Regenerable biosensors for small molecule kinetic characterization using SPR

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Keywords: Surface immobilization, Surface regeneration

Abstract

A key activity in small molecule drug discovery is the characterization of compound—target interactions. Surface plasmon resonance (SPR) is a flexible technique for this purpose, with a wide affinity range (mM – pM), low protein requirements and the ability to characterise the kinetics of compound binding. However, a key requirement of SPR is the immobilization of the target protein to the surface of the sensor chip. The most commonly used immobilisation techniques (covalent immobilisation, Streptavidin—biotin) are irreversible in nature, which can afford excellent baseline stability but impose limitations on throughput for slowly dissociating compounds or unstable targets. Reversible immobilisation (e.g. His-tag—Ni-NTA) is possible but typically precludes accurate quantification of slow dissociation kinetics due to baseline drift.

Here we present our investigation of three immobilisation strategies (dual His-tagged target protein, His-tagged Streptavidin and Switchavidin) that combine the robustness of irreversible immobilisation with the flexibility of reversible immobilisation. Each has its own advantages and limitations, and whilst a universal immobilisation procedure remains to be found, these strategies add to the immobilisation toolbox that enable previously out-of-scope applications. Such applications are highlighted in two examples which greatly increased throughput for kinetic characterization of potent kinase inhibitors and kinetic profiling of covalent inhibitors.

Introduction

Surface plasmon resonance (SPR) based biosensors serve as a key component of the biophysics toolbox during the drug discovery process [1]. In a typical SPR experiment, the drug target is tethered to the chip surface using irreversible immobilisation strategies such as EDC/NHS-mediated covalent cross-linking to the chip matrix or pseudo-irreversible capture to covalently pre-immobilised streptavidin [2, 3]. Whilst these strategies can enable stable baselines with minimal drift—a pre-requisite for robust kinetic characterisation—their irreversible nature preclude the iterative use of the biosensor surface in the case of slowly dissociating compounds or unstable target proteins.

Reversible immobilization typically involves capture approaches, where affinity tags on the target protein (such as a poly-His tag or antigen peptide motifs) enable tethering via a Ni^{2+}-NTA matrix or via irreversibly immobilized antibodies respectively [4-6]. Most of these techniques have limited use for biosensing purposes, as they are either too weak (e.g. Ni^{2+}-NTA capture) or complicated (e.g. protein DNA-tagging [7, 8]). While these approaches can generate protein surfaces that are efficiently regenerated after use, this reversibility is typically accompanied by increased drift due to dissociation of the tethered target protein. This drift is also larger at the surface densities required for the detection of small molecule binding. Not only does this drift complicate or even prevent the kinetic characterization of small molecules with small absolute response and slow dissociation rates, it also results in time-dependent variation of surface coverage and further complicating data analysis.

Different approaches for various degrees of regenerable immobilisation have been proposed over the years [9-11 7, 12, 13] but none of them have been widely applied in industry. This is largely due to shortcomings in these approaches and the challenge of ensuring a stable, drift-free baseline, whilst achieving complete and reproducible regeneration to enable iterative (re-)use of the biosensor surface.

Regenerable protein immobilisation would have several benefits. It would provide flexibility to rapidly switch between assays without the need to discard, or even remove, sensor chips from the instrument. Assay development would be accelerated, as various buffer conditions could be tested on freshly immobilized protein in an iterative fashion. Throughput for slowly dissociating compounds would be improved (or testing of irreversible compound enabled), as the surface could be regenerated for the next analyte without waiting for compound dissociation. Finally, inherently unstable targets could be enabled.

For regenerable protein surface strategies to be truly viable and broadly applicable in small molecule drug discovery, we defined the following set of requirements:

- **i)** **Stable baseline**: Baseline stability (also at high densities of target protein) should enable kinetic characterization of slow dissociating compounds. Drift should be indistinguishable from the drift specifications of the SPR platform.
- **ii)** **Fully regenerable**: The surface should be regenerable multiple times with the sensor chip remaining in the SPR instrument (>99 % regeneration efficiency) with

no significant loss in capture capacity (>99 % capture levels retained on regenerated surfaces).

iii) **Mild conditions during immobilization**: The target should not be subjected to any harsh chemical environment during immobilization, such as low or high pH or organic solvents, so it can be used for less stable targets.

iv) **Site-specific attachment**: It should be possible to immobilize the target site-specifically to, as far as possible, retain target conformational flexibility and maximise ligand-binding competence.

v) **Use existing consumables:** All considered approaches must be compatible with existing SPR consumables (e.g. biosensor chips) and should utilize cost-efficient reagents.

vi) **Minimal protein engineering:** The approach should be compatible with current best-practice protein expression and purification strategies.

With these requirements in mind, we explored three immobilization strategies: the use of a dual His-tagged target [14-16], a biotinylated target in combination with His-tagged Streptavidin, and a biotinylated target in combination with Switchavidin [17] (**Figure 1**).

In choosing these three immobilization strategies for scrutinization, it is recognised that more could have been selected, such as DNA- or antibody-based approaches. While it is possible to obtain very tight interactions between antibodies and antigens, a prerequisite for stable baseline, suitable high affinity antibodies are not readily available for many target proteins. Similarly, antibodies derived against peptide tags (like anti-His-tag antibodies) are in general not potent enough to ensure minimal drift required for small molecule kinetic characterization. While both the His-tagged Streptavidin and Switchavidin approaches are similar to Biotin CAPture which utilizes DNA-tagged Streptavidin to stably capture Streptavidin to covalently immobilized DNA [9], the capture levels with the Biotin CAPture approach are typically not compatible with small molecule detection.

The dual His-tag approach consists of two consecutive His-tags fused to the target protein. Different variants have been described previously such as a long His-tag, two His-tags with a spacer or one His-tag in each terminus of the target protein [14, 15, 18]. While a traditional single His-tag is insufficient to achieve a stable baseline, two tags will improve the affinity of the target protein to a Ni^{2+}-NTA surface through bivalent avidity, leading to reduced baseline drift. The surface is typically regenerated using standard procedures for Ni^{2+}-NTA (e.g EDTA).

The second approach, His-tagged Streptavidin, operates by a similar principle. Each Streptavidin tetramer will carry four His tags resulting in large avidity effects when binding to a Ni^{2+}-NTA surface, resulting in a very stable baseline [18]. Biotinylated target proteins can subsequently be captured but in contrast to covalently immobilized Streptavidin, regeneration of the Ni^{2+}-NTA surface is possible.

The final approach requires Switchavidin [17], a mutant variant of Avidin, and utilizes a slightly different strategy. When subjected to acidic conditions, Switchavidin is monomeric , which greatly reduces its affinity to biotin, enabling efficient regeneration. Similar to His-tagged Streptavidin, Switchavidin mediates immobilization of biotinylated target protein, in this

case on biotin sensor surfaces, which enables regeneration of the entire protein complex. The Switchavidin procedure has been shown to work well with 2D surfaces and large molecule interactions (such as antibodies) but the procedure has, to our knowledge, not been described in the context of small molecule binding characterization.

In this report, we describe a comparison of the three approaches in the context of small molecule binding and kinetic characterization. We investigate how they compare to traditional immobilization procedures and to what degree they present a truly viable and broadly applicable approach in small molecule drug discovery (**Table 1**).

Materials and methods

His-tagged Streptavidin expression and purification

The His-tagged Streptavidin was designed to contain N-terminal His6 tag followed with Streptavidin residues 25-183. The construct was codon optimized and cloned into pET24a vector (GenScript) and transformed to *E. coli* BL21 Star (DE3) strain (Invitrogen). The culture was grown at 37 ^{o}C in Terrific Broth medium until the $OD_{600\ nm}$ was 1.0 then induced with 0.2 mM isopropyl β-D-1-thiogalactopyranoside for overnight incubation at 18 ^{o}C. The cells were harvested and purified with two step purification method of Ni^{2+}-affinity chromatography and size exclusion chromatography with a Superdex-200 column (GE healthcare) in the buffer of 50 mM Tris pH 8.0, 100 mM NaCl, 5 % glycerol, 1 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP).

Switchavidin was purchased from BioMediTech, (Finland) and produced as described by Taskinen et al [17].

Dual His-tagged ERK2 expression and purification

The dual-8His-tagged ERK2, with a construct name 2XHis8-ZZ-TEV-ERK2 was designed to contain two N-terminal His8 tags separated by a multi-amino acid spacer; a ZZ tag (domain of Protein A), a TEV protease cleavage sequence, and human ERK2. The construct was cloned into a pET vector (GeneArt) and transformed to *E. coli* BL21 GOLD (DE3)(Agilent Technologies). The culture was grown at 37 ^{o}C in auto-induction medium until the $OD_{600\ nm}$ was 0.6 and then the temperature was reduced to 18 °C and incubated overnight to allow protein expression. The culture was harvested, and the protein was purified by IMAC followed by size exclusion chromatography with a Superdex-200 column (GE healthcare) in the buffer of 20 mM HEPES pH 7.9, 150 mM NaCl.

SPR experiments with His-tagged Streptavidin and Dual His-tagged ERK2

All SPR experiments were conducted on BIAcore instruments (GE Healthcare) using NTA sensor chips (GE Healthcare). All NTA surfaces were prepared by washing with 0.5 M EDTA prior to loading with 200 µM $NiCl_2$. The normal running buffer was HBS-P+ (10 mM HEPES pH

7.4, 150 mM NaCl, 0.05 % Tween-20). For buffer tolerance experiments, the capture of dual-8His-ERK2 was typically kept below 4000 RU to prevent baseline drift (see result and discussion) and for His-tagged Streptavidin the levels where kept below 5000 RU.

Surfaces were regenerated by injecting 0.5 M EDTA, 5 M GuSCN (Sigma-Aldrich, CAS 593-84-0) with 5 mM TCEP (Sigma-Aldrich, CAS 51805-45-9), 1 mg/ml Pepsin (Sigma-Aldrich, CAS 9001-75-6) in 1 M Glycine pH 2.5, and 50 mM NaOH with 1 M NaCl using a contact time of 300 seconds for each solution.

For ERK2 compound interaction studies, 10 mM HEPES, 150 mM NaCl, 0.05% Tween-20, 1 mM TCEP and 1% DMSO pH 7.4 was used as a running buffer. Compound (SCH772984) was diluted 2-fold from 100nM and injected over the surfaces at 30µl/min, 120 seconds contact time followed by 1200 second dissociation. Resulting sensorgrams were reference and blank subtracted prior to fitting to a 1:1 binding model.

PAD4 (Peptidylarginine deaminase 4) expression and purification

PAD4 with the construct name N-6xHis-TEV-Avi-hPADI4(A2-P663) was designed to contain a N-terminal Avi-Tag to allow for a controlled enzymatic biotinylation after the purification of the protein. The construct was inserted into a pET24 expression vector and transformed into an E.coli BL21 Star expression strain. The culture was grown at 37 ^{o}C in auto-induction medium buffer until an $OD_{600\,nm}$ of 0.6 was reached, upon which the temperature was lowered to 18 °C and the culture was left for incubation for an additional 80 h. After the cells were harvested, the protein was purified with a two-step purification method using Ni^{2+}-affinity chromatography and size exclusion chromatography by employing a HiLoad Superdex-200 column (GE Healthcare) in a running buffer of 50 mM Tris pH 8.0, 300 mM NaCl and 1 mM TCEP. Site-specific biotinylation was achieved through a 12 h incubation with Biotin ligase (BirA) in the presence of biotin and ATP, which enabled to covalently link the free biotin to the lysine of the Avi-Tag with a yield of >99 %.

SPR experiments with biotinylated PAD4

All SPR experiments were conducted on a BIAcore 8K instrument (GE Healthcare). Biotin sensor chips (BD200M, Xantec Bioanalytics GmbH) were washed with 2.5% citric acid and 0.25% SDS prior to use. 250 nM Biotinylated PAD4 was pre-incubated with 125 nM Switchavidin before immobilization on the Biotin sensor chip at 25 °C using all 8 flow channels, resulting in similar coupling densities of 10200 RU ± 300 RU in all flow channels. After a stabilization period of 1 h, compounds were injected in 2-fold dilutions in running buffer (50 mM Tris/HCl, 250 mM NaCl, 1 mM TCEP, 1 % DMSO, pH 8.0) using parallel kinetics, i.e. all eight concentrations of the dose-response experiment were injected simultaneously over the sensor surface resulting in one specific compound concentration per flow channel. The highest concentration tested was 50µM and the compound was diluted two-fold. The flowrate was 30 ml/min with an injection time of 240 s. After a 720 s dissociation period, the surface was challenged with regeneration solution (2.5 % citric acid (pH=2), 0.5 % SDS) for 60 s to enable a new immobilization cycle. The resulting sensorgrams

were reference- and blank subtracted prior to fitting to a 1:1 binding model (Biacore evaluation software).

SIK3 (salt inducible kinase 3) expression and purification

SIK3 construct 10His-SIK3-(M1-R327)-Avi was expressed in insect cells. The construct was inserted into a pFastBac expression vector and transfected into Sf21 cells, with a 48 h time of harvest for the expression. The protein was purified and biotinylated with a multiple step purification scheme containing affinity on Ni2+ resin, removal of the His-tag, size exclusion chromatography (Superdex-75), ion exchange chromatography (Resource Q) and site-specific biotinylation. The purification was finalized with a second size exclusion step (Superdex-75, GE Healthcare) in buffer 40 mM Bis-Tris Propane, pH 7.8, 200 mM NaCl, 10 % glycerol, 1 mM TCEP.

SPR and Mass Photometry experiments with biotinylated SIK3

All SPR experiments were conducted on BIAcore instruments BC3000 and S/T200 (GE Healthcare) using HBS-P+ (10 mM HEPES, 150 mM NaCl, 0.05% tween-20) as running buffer at 20 °C. SA sensor chip (SAD200M Xantec Bioanalytics GmbH) was washed with mixture of 10mM NaOH and 1M NaCl before immobilization of biotinylated SIK3 at 100 nM. NTA sensor chip (NID500M, Xantec Bioanalytics GmbH) was washed with mixture of 10 mM NaOH and 1 M NaCl and 0.5M EDTA before addition of 3 mM $NiCl_2$ and subsequent immobilization of His-tagged SA at 100 nM follow by biotinylated SIK3 at 100 nM. Biotin sensor chips (BD200M, Xantec Bioanalytics GmbH) were washed with a mixture of 2.5% citric acid and 0.25% SDS prior to use. Biotinylated SIK3 was pre-incubated with SwitchAvidin in 2:1 ratio for 5 min before diluted to 100 nM and immobilized. Compounds were injected in 3-fold dilutions in running buffer before 60 sec regeneration (2.5% citric acid, 0.25% SDS). Resulting sensorgrams were reference- and blank subtracted prior to fitting of a 1:1 binding model (Biacore evaluation software).

Biotinylated SIK3 was pre-incubated with SwitchAvidin in 2:1 ratio for 2 min before diluted to 100 nM and analyzed using a Refeyn One mass photometry (MP) instrument (Refeyn Ltd, UK) at 60 sec acquisition time. Resulting histogram was calibrated using NativeMark protein ladder (ThermoFisher) and fitted to multiple Gaussians to extract peak mass.

Results and Discussion

All investigated approaches result in high and stable capture levels

Dual 8His tag and His-tagged Streptavidin

Dual-8His ERK2 and His-tagged Streptavidin could both be captured to high levels before significant drift was observed (**Figure 2A** and **2B**).

Dual His-tagged proteins and His-tagged Streptavidin behaved similarly, in that drift is significantly reduced compared to a single His-tag due to avidity effects [14, 15]. However, as

the capture level increases the level of drift also increases, as the opportunities for avidity decrease due to neighboring Ni-NTA sites being already occupied[18]. Nonetheless, the capture levels achievable with the dual His-tagged protein and His-tagged Streptavidin are sufficient for typical small molecule applications.

Capture procedures that rely on avidity may be more sensitive to buffer conditions, as a small weakening in the interaction may result in a significantly weaker overall interaction. Dual-8His tagged ERK2 and His-tagged Streptavidin showed similar baseline stability with a variety of buffers, with the exception of BICINE (**Figure 2C** and **D**). BICINE is a metal chelator, so it is possible it competes for the Ni^{2+} on the surface, and hence the observed drift. While it was not practicable to test a large number of buffer conditions, our results suggest the capture procedure has general buffer compatibility, but a proper assessment must be performed on a case-by-case basis, particularly for more atypical buffer compositions. Binding of a compound to captured target protein using both methods is nearly identical (**Figure 2E** and **F**, **Supplementary Table S1**).

While a surface with captured His-tagged Streptavidin stabilized quickly (within 5 minutes), the time for the surface to stabilize after target capture was target-dependent. For some targets it was as rapid as for Streptavidin, whilst for others it could take 30 minutes or longer. This could be problematic as any time gained in overcoming slow compound dissociation rates would be lost due to long surface stabilization times. It is worth noting that the biotinylated targets we have used also feature a His-tag, so it is possible that the Streptavidin becomes saturated and the target protein then binds to the Ni^{2+}-NTA surface via its His-tag. Here, the dissociation of mono His-tagged protein from the surface would dictate the stabilization time.

Despite clear advantages, all avidity-based solutions will suffer from a similar limitation, namely the difficulty of ensuring that all immobilized proteins are bound with maximal avidity. This will limit the immobilization levels possible, and for poorly active or particularly large targets may limit the utility of such approaches.

Switchavidin

In the original publication [17] it was shown that for preparation of a 2D surface, sequential capture of Switchavidin followed by a biotinylated target was possible. However, while Switchavidin alone can be immobilized to a high coverage on a biotin-derivatized 3D-matrix, the flexibility of the matrix results in saturation of biotin binding sites, thus rendering the Switchavidin unavailable for subsequent target binding (**Figure 3A**). Despite initiatives to lower the biotin density in the matrix (as low as <20 %), all attempts to sequentially immobilize biotinylated targets were highly inefficient.

To overcome this complication, Switchavidin can be pre-mixed with the biotinylated protein of interest and the Switchavidin-target complex is captured on the biotin surface (**Figure 3B**). While the capture of 6000RU of SIK3 to an SA surface, 6000RU to a surface pre-captured with His-SA, and 12000RU of the SIK3-SwA complex (**Figure 3C-E**) result in almost identical binding profiles for the test compound Bosutinib (**Figure 3F-H**), the max response is lower for the SwA surface compared to the other two surfaces. This illustrate one drawback of pre-

mixing the proteins in that the binding capacity of the surface cannot be properly determined and immobilization levels cannot be used directly to assess how much of a protein that has been immobilized.

For pre-mixing, the molar ratio between Switchavidin and biotinylated protein must be determined empirically and typically, a ratio of 1:2 between the tetrameric Switchavidin and single biotinylated target is optimal. The exact stoichiometry of the Switchavidin-target complex was assessed using Mass Photometry[19], a novel single molecule-based approach to determine the mass of protein complexes (**Figure S1**). Mixing tetrameric Switchavidin (64kDa) and biotinylated SIK3 (40kDa) in a 1:2 molar ratio results in a distribution of complexes with varying stoichiometry in which the 1:1-1:3 complexes, amenable to SPR immobilization, dominates.

On a typical commercial Biotin chip (like BD200M [20]) it is possible to capture high densities (~10,000 RU, **Figure 3**) of the Switchavidin-target complex, sufficient for small molecule binding characterization, with almost no observed baseline drift. In addition, the stabilization period for immobilization of the Switchavidin-target complex is typically very short in contrast to His-tagged Streptavidin where longer stabilization periods are more common. A further advantage of using Switchavidin is the possibility of using His-tagged protein as analytes, something that is essentially impossible when using the Dual His-tagged or His-tagged Streptavidin immobilization approaches on Ni^{2+}-NTA surfaces.

Surface regeneration

Ni^{2+}-NTA surfaces are typically regenerated using EDTA. While freshly captured His-SA or dual-His tagged target proteins are almost fully regenerated using 0.5 M EDTA, we have observed that the regeneration is not always complete and residual protein built up on the surface over time. In addition, capturing the protein and then not trying to regenerate it for hours makes EDTA regeneration even more inefficient. Hence, to be truly regenerable we needed to establish a more rigorous regeneration procedure. In our hands, the most effective regeneration protocol was a combination of short consecutive pulses of 0.5 M EDTA, a strong chaotropic agent with reducing agent (for example 5 M GuSCN, 5 mM TCEP), in-situ pepsin digest (1 mg/ml Pepsin in 1 M Glycine pH 2.5) and high pH with high ionic strength (50 mM NaOH, 1 M NaCl) (**Figure 4**). Using this procedure, inspired by Knoglinger *et al.* [21], on multiple proteins, has consistently resulted in >99 % regeneration efficiency with retained chip capacity. Some proteins only require certain steps in this procedure for complete regeneration and optimized protocols can be tested on a target by target basis, but the full protocol ensures broad applicability.

For Switchavidin, the published regeneration procedure comprising 2.5 % citric acid and 0.25 % SDS[17] worked well with almost all proteins tested (**Figure 3A** and **B**). In contrast to dual His-tagged ERK2 and His-Streptavidin regeneration described above, the regeneration of Switchavidin was >99 % efficient and did also not lose its efficiency when used repeatedly or when used on an older surface. However, for a few targets and settings we did observe minor accumulation (<5% of immobilized protein) over time on the biotin surfaces. Subjecting these surfaces to the regeneration protocol devised for the dual His-tagged protein or His-Streptavidin, described in Figure 4, did completely remove this accumulated

density. Hence it is recommended to use this more rigorous regeneration procedure prior to removing the sensor chip from the instrument or when changing to a different target protein.

Whilst these regeneration procedures might suggest an almost unlimited life time for a sensor chip, this is not the case. Most chips can be used repeatedly for weeks and even months, but they gradually lose capacity over time. Whether due to normal "wear and tear", accelerated degradation due to the regeneration solutions used or a result of repeated docking/undocked of the sensor chip (undocked sensor chips are typically stored at 4 °C in a humid environment) is still unclear. Consequentially, the capacity of re-used sensor chips must be monitored.

Examples of the benefits with regenerable sensor surfaces

Greatly increased throughput for kinetic characterization of very potent kinase inhibitors

Key benefits of the regeneration approach are clearly seen when looking at the profiling of a kinase inhibitor with long residence time (k_{off} from fit $\sim 3 \times 10^{-5}$ s^{-1}) (**Figure 5**). The cycle time for such a compound based solely on dissociation (traditional approach) would be >24 h/injection. As seen from Figure 4A, the cycle time using the Switchavidin approach is reduced to only around 40 min. increasing throughput to be >35-fold. Importantly, the kinetic parameters are consistent with data using traditional irreversible immobilization on a chip with covalently immobilized Streptavidin (**Supporting Table S2**).

Kinetic determination of the reversible- and irreversible step of covalent inhibitors

The possibility of repeated regeneration cycles furnishes the opportunity to study the kinetics of irreversible inhibitors—the most extreme case of long residence time—as shown in **Figure 6 (Supplemetary Figure S2)**. In this example, there is clear biphasic behaviour in the association and dissociation phases of the injections, consistent with the expected two-step binding interaction ($P + L \rightleftharpoons PL \longrightarrow PL^*$). Fitting these data to this model provides estimates for k_{on}, k_{off} and k_{inact}, that describe the non-covalent and covalent components of the interaction.

No single immobilization strategy will work for all situations (**Table 1**). They all come with advantages and limitations, but the three approaches investigated here add to the toolbox of available immobilization procedures. The requirements we listed come from a long history of measuring small molecule interactions to immobilized protein targets and as such are probably representative of what the general SPR experimentalists require. That said, it is not possible to cover all situations or requirements, but we hope this will aid in the selection of immobilization procedure.

It is important to point out that even though these immobilization procedures can increase throughput for slowly dissociating compounds, such measurements will still take time. In order to reliably quantify a dissociation rate, sufficient dissociation data must be collected (at least 5% dissociation, so 1.4 h for $k_{off} = 1 \times 10^{-5}$ s^{-1}), which limits how short these SPR

measurements can become. Nonetheless, the main benefit remains: the possibility to regenerate afterwards.

The Switchavidin and His-Streptavidin approaches share the common requirement of biotinylated target protein. This could be achieved via site-directed biotinylation (using e.g. an Avi-tag [22]) but can also be achieved chemically at neutral pH and ambient temperatures[9]. While the latter alternative provides less control of position and number of biotinylated residues, it offers an the option to modify target proteins to be compatible with regeneration assays post expression/construct design.

Whilst the immobilization strategies investigated in the current paper were chosen to increase the scope of SPR experiments that can be conducted, perhaps the ultimate gain in employing these approaches is cost saving. The SPR sensor chips are relatively expensive while one milligram of His-tagged Streptavidin or Switchavidin, enough for several hundreds of immobilizations, is relatively cheap to produce or purchase.

To summarize, the three investigated regenerable SPR immobilization procedures (dual His-tag, His-tagged Streptavidin and Switchavidin) show great potential in their use for small molecule binding characterization. While there is no ultimate immobilization strategy, the addition of these to the immobilization tool box will enable the study of additional targets, compound modes-of-action (e.g. irreversible) and a higher throughput for slowly dissociating compounds, enabling improved data-driven decision making.

Conflicts of interest

All authors except V.H. are employees of AstraZeneca or were affiliated with AstraZeneca during this study.

Acknowledgements

We thank Dr Christopher Gray, Dr Andrea Gohlke, Dr Jennifer Konczal and Dr Justin Bower (Beatson Institute) for providing us with their double-his tag vector and insightful discussions of the use of double-his tags for protein immobilisation.

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Tables and table legends

Table 1: Summary of advantages and disadvantages for various immobilization strategies, including the ones examined in the current paper.

Requirements	Covalent[a]	His-tag capture[b]	Covalent His-tag capture[c]	Streptavidin capture[d]	Dual 8His-tag capture[e]	His-tagged Streptavidin[f]	Switchavidin[g]
Stable baseline	✓	✗	✓	✓	✓	✓	✓
Fully regenerable	✗	✓	✗	✗	✓	✓	✓
Mild immobilization conditions	✗	✓	✓	✓	✓	✓	✓
No genetically encoded protein tag required	✓	✗	✗	✓	✗	✓	✓
Immobilization level directly known	✓	✓	✓	✓	✓	✓	✗
Physico-chemical resistant immobilization	✓	✗	✓	✓	✗	✗	✓

a: Direct covalent immobilization to the sensor surface of the target, b: Capture of a mono-His-tagged target to a Ni-NTA surface, c: Covalent immobilization of a His-tagged target to an activated Ni-NTA surface, d: Capture of a biotinylated target to covalently immobilized Streptavidin, e: Capture of a dual 8His-tagged target to a Ni-NTA surface, f: Capture of His-tagged Streptavidin to a Ni^{2+}NTA surface and subsequent capture of biotinylated target to Streptavidin, g: Capture of Switchavidin- biotinylated target complex to a Biotin surface.

Figure legends

Figure 1: Schematic of the three regenerable immobilization strategies investigated in the current paper; Dual His-tagged target protein captured to Ni-NTA, Biotinylated target protein immobilized to His-tagged Streptavidin that is captured on a Ni-NTA surface, or biotinylated target protein immobilized to Switchavidin.

Figure 2. Capture levels using different concentrations of dual 8His-tagged ERK2 (**A**) and His-tagged Streptavidin (**B**) on a normal Ni^{2+}-NTA chip, showing which levels can be captured before significant drift is observable. The surfaces are stable using normal buffers with the exception of BICINE (**C** and **D**). The binding profile of a compound (SCH772984) to ERK2 is very similar irrespective of immobilisation procedure (**E** and **F, Supplementary Table S1**)

Figure 3. Sequential (**A**) or co-immobilization (**B**) of Switchavidin-SIK3. Only the later approach enabled sufficient surface coverage of SIK3 to enable monitoring of small molecule binding (insets). Different immobilization strategies does not affect affinity determination. SIK3 immobilization on three different surfaces, **C**) directly on SA-modified chip, **D**) via His-SA on Ni-NTA-modified chip or **E**) as Switchavidin complex on biotin-modified chip. **F**-**H**) Dose-dependent binding of the inhibitor Bosutinib (single cycle kinetic mode, red) with corresponding Langmuir fit (black).

Figure 4. Full regeneration of His-SA (red) and His-SA plus target protein (blue) from a Ni^{2+}-NTA surface requires a combination of 0.5 M EDTA, 5 M GuSCN with 5 mM TCEP, 1 mg/ml Pepsin in 1 M Glycine pH 2.5 and 50 mM NaOH with 1 M NaCl. Horizontal dashed black line indicates surface response prior to the capture of any protein.

Figure 5. (**A**). Repeatability of Switchavidin-mediated immobilization (blue). Overlay of 20 consecutive immobilizations and subsequent regenerations using 2.5 % Citric acid with 0.25 % SDS. (**B**). Dose dependent small molecule binding with extended residence time kinetic profile, sensorgrams in red and fitted 1:1 binding model in black.

Figure 6. Model and SPR data of the binding of an irreversible PAD4 inhibitor (**Supplementary Figure S2**). Global fitting of the depicted model to the data (black lines) resulted in k_{on} of 3700 $M^{-1}s^{-1}$, k_{off} of 0.21 s^{-1} and a k_{inact} of 0.017 s^{-1}. Local Rmax was used in the fitting but no RI/bulk compensation was used.

Figure 1

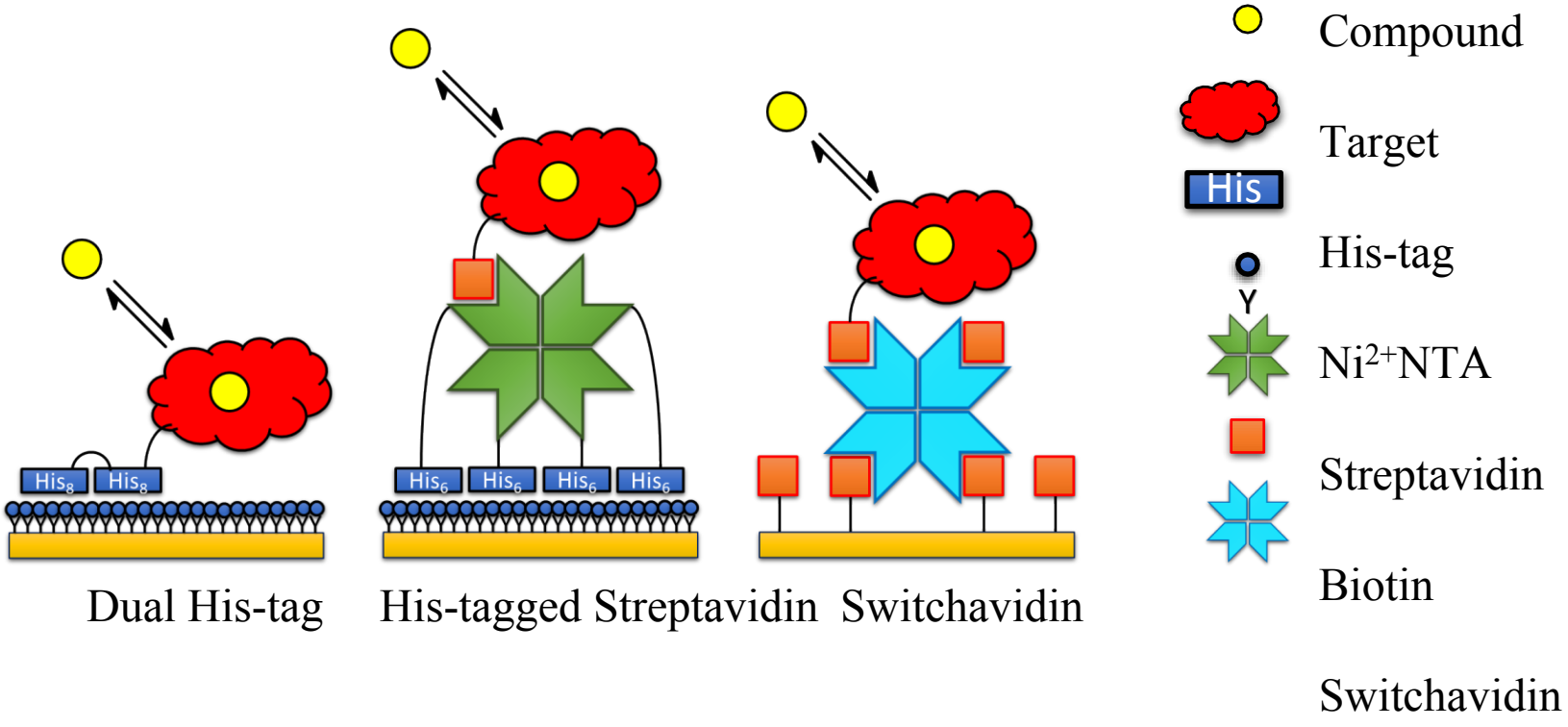
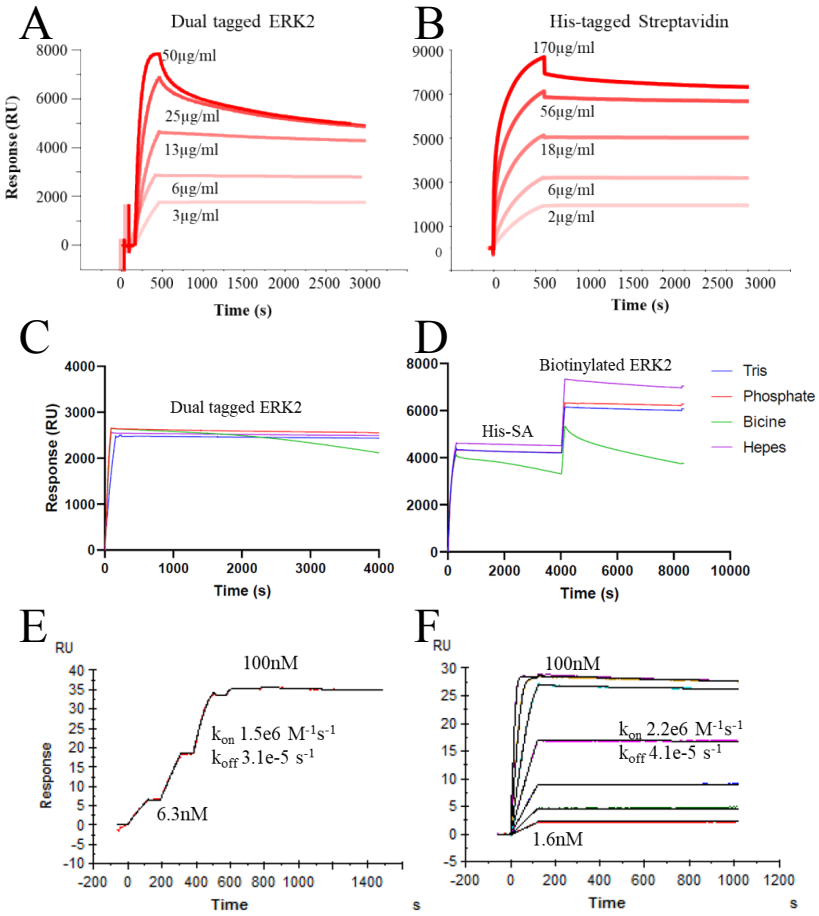

His8
His8
His6
His6
His6
His6
Dual His-tag
His-tagged Streptavidin
Switchavidin
Compound
Target
His
His-tag
Ni2+NTA
Streptavidin
Biotin
Switchavidin

Figure 2


A
Dual tagged ERK2
50µg/ml
25µg/ml
13µg/ml
6µg/ml
3µg/ml
Response (RU)
Time (s)
B
His-tagged Streptavidin
170µg/ml
56µg/ml
18µg/ml
6µg/ml
2µg/ml
Time (s)
C
Dual tagged ERK2
Response (RU)
Time (s)
D
Biotinylated ERK2
His-SA
Tris
Phosphate
Bicine
Hepes
Time (s)
E
RU
100nM
k_{on} 1.5e6 $M^{-1}s^{-1}$
k_{off} 3.1e-5 s^{-1}
6.3nM
Response
Time
s
F
RU
100nM
k_{on} 2.2e6 $M^{-1}s^{-1}$
k_{off} 4.1e-5 s^{-1}
1.6nM
Time
s

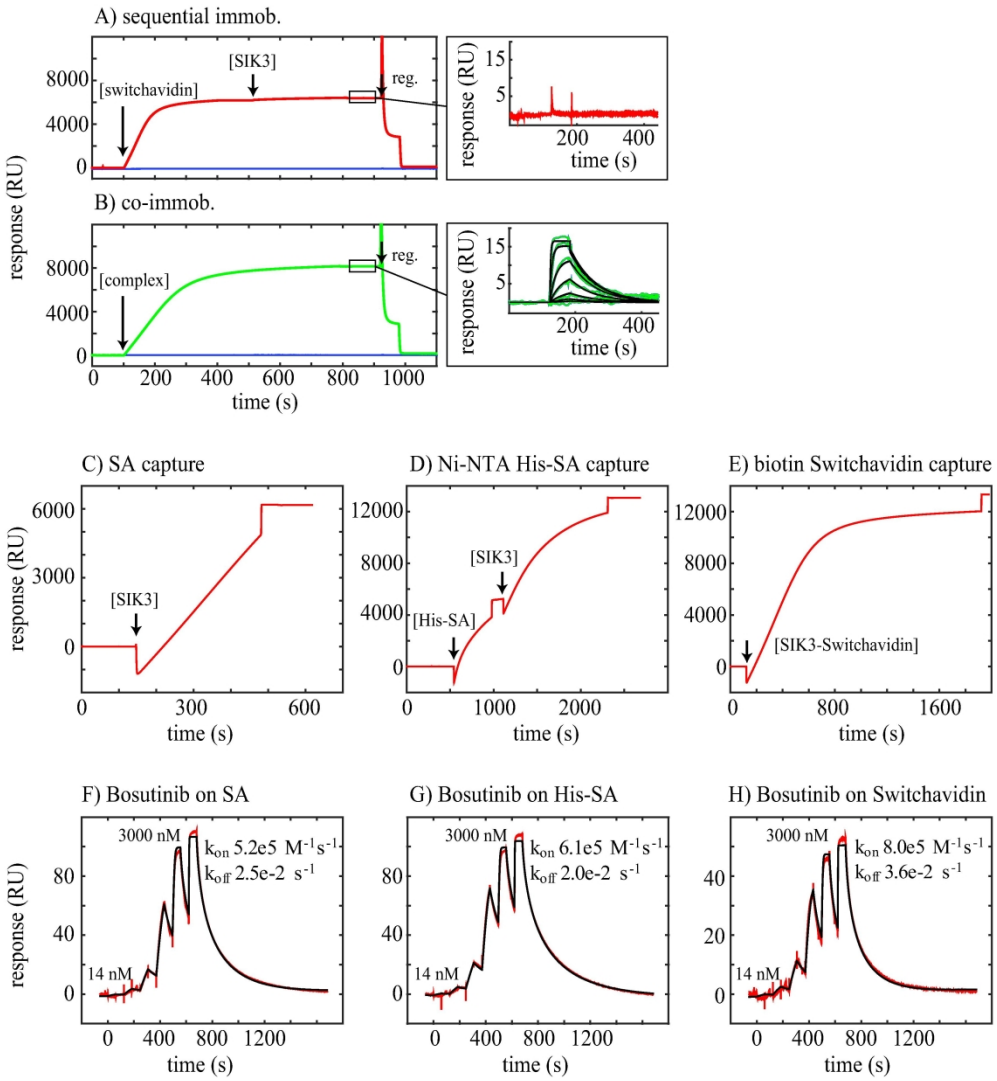
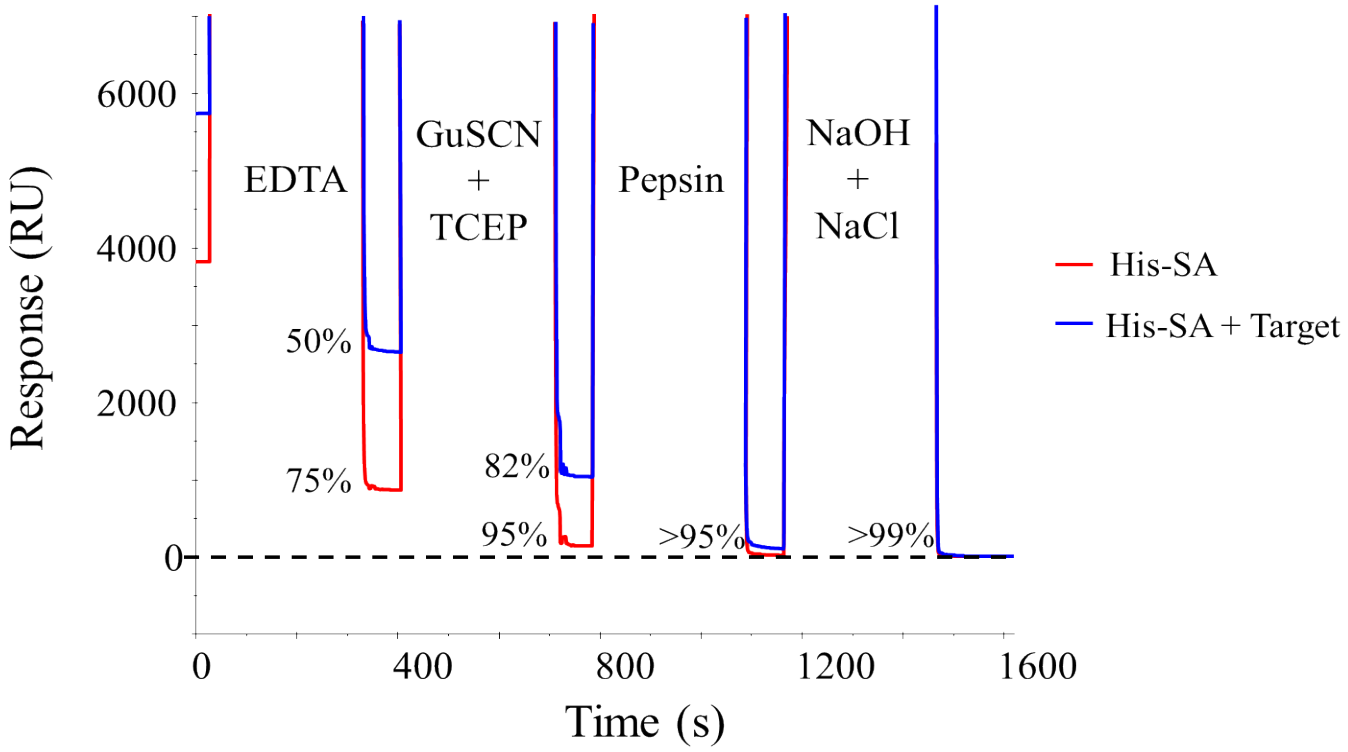


Figure 3. Sequential (A) or co-immobilization (B) of Switchavidin-SIK3. Only the later approach enabled sufficient surface coverage of SIK3 to enable monitoring of small molecule binding (insets). Different immobilization strategies does not affect affinity determination. SIK3 immobilization on three different surfaces, C) directly on SA-modified chip, D) via His-SA on Ni-NTA-modified chip or E) as Switchavidin complex on biotin-modified chip. F-H) Dose-dependent binding of the inhibitor Bosutinib (single cycle kinetic mode, red) with corresponding Langmuir fit (black).

153x164mm (600 x 600 DPI)

Figure 4


Response (RU)
6000
4000
2000
0
EDTA
GuSCN
+
TCEP
Pepsin
NaOH
+
NaCl
50%
75%
82%
95%
>95%
>99%
0
400
800
1200
1600
Time (s)
His-SA
His-SA + Target

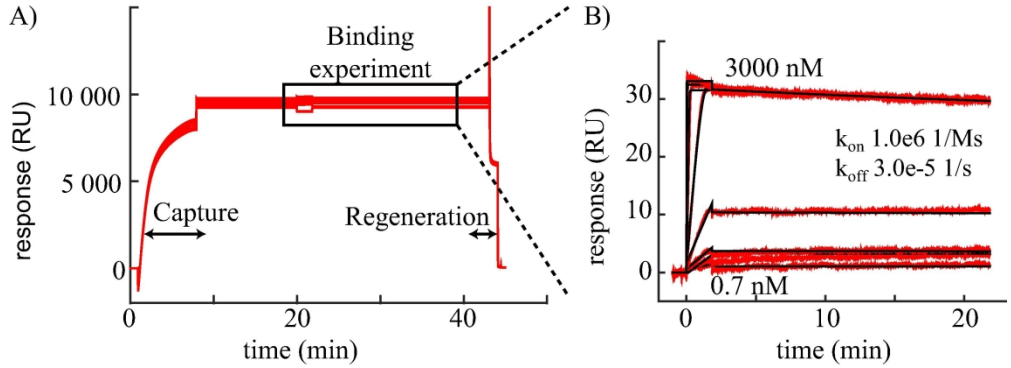
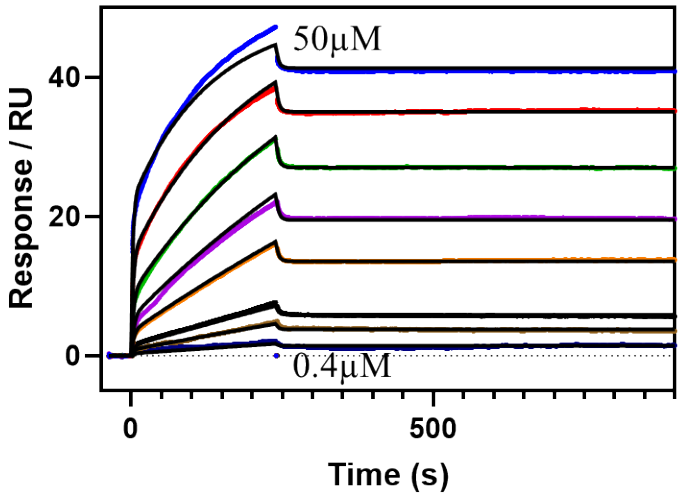


Figure 5. (A). Repeatability of Switchavidin-mediated immobilization (blue). Overlay of 20 consecutive immobilizations and subsequent regenerations using 2.5 % Citric acid with 0.25 % SDS. (B). Dose dependent small molecule binding with extended residence time kinetic profile, sensorgrams in red and fitted 1:1 binding model in black.

129x47mm (600 x 600 DPI)

Figure 6

$$P + I \underset{k_d}{\overset{k_a}{\rightleftharpoons}} PI \xrightarrow{k_{inact}} PI*$$



Regenerable biosensors for small molecule kinetic characterization using SPR

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Supplemental figures and legends

Figure S1

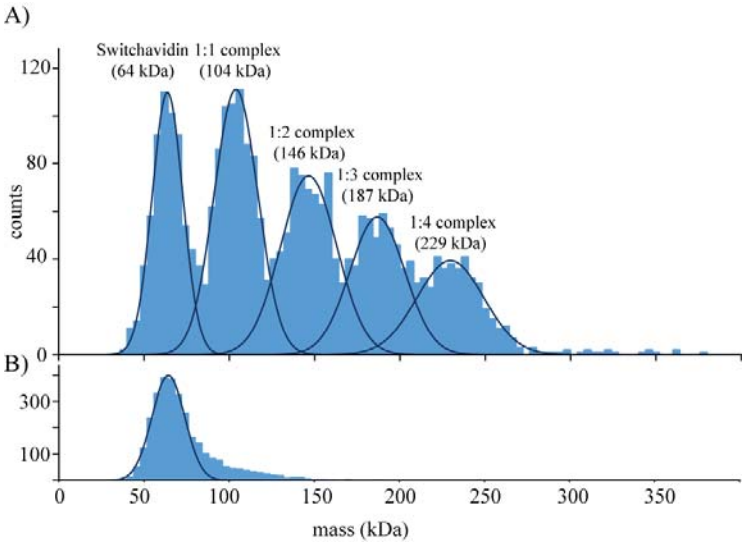

Figure S2

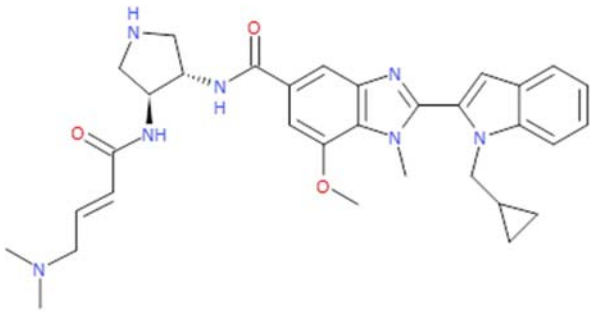

Figure S1. (A) Mass Photometry reveal distribution of pre-mixed switchavidin-target complex. (B) tetrameric Switchavidin reference.

Figure S2. Structure of the irreversible PAD4 compound used in Figure 6.

Table S1. Affinity and kinetic parameters for the ERK2 inhibitor, SCH772984.

	k_{on} mean ± SD (10^5 $M^{-1}s^{-1}$)	k_{off} mean ± SD (s^{-1})	pKD mean ± SD	n
NTA capture-coupling	1.1	4.32E-05	9.4	1
Biotin-streptavidin	9.4 ± 5.5	4.4 ± 1.7 E-05	10.3 ± 0.5	3
dual-8His ERK2	22	4.1E-05	10.7	1
6His-SA	15	3.1E-05	10.7	1

Table S2. Affinity and kinetic parameters for SIK3 inhibitors.

	SA immobilization				SwA immobilization			
	k_{on} (10^5 $M^{-1}s^{-1}$)	k_{off} (s^{-1})	pK_d (-log M)	n	k_{on} (10^5 $M^{-1}s^{-1}$)	k_{off} (s^{-1})	pK_d (-log M)	n
SIK3 inhibitor 1 (Fig. 3B, inset)	5.0±2	0.04±0.01	7.1±0.1	3	3.0±1	0.04±0.02	6.9±0.1	3
SIK3 inhibitor Bosutinib (Fig 3F-H)	5.2±0.5	2.5±0.2	7.3±0.1	3	8.0±0.4	3.6±0.4	7.4±0.1	3
SIK3 inhibitor 2 (Fig. 5)	16	0.00013	10.2	1	10±12	3E-5±3E-5	10.5±0.2	2