protein interactions

The ProteOn™ HTE Sensor Chip: Novel Surface for Stable Capture of Histidine-Tagged Proteins for Protein–Small Molecule Interaction Analysis

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Introduction

The histidine tag is currently the most widely used tag in protein purification. It is typically composed of six or ten histidine residues fused at the amino or carboxyl terminus of a protein. Recombinant proteins containing a histidine tag are commonly purified on a matrix with nickel (II)-nitrilotriacetate (Ni-NTA) complexes that are prepared from nickel (II)-activation of nitrilotriacetic acid (NTA). In addition to protein purification, this technology has been used in label-free surface plasmon resonance (SPR) biosensors for biomolecular interaction analysis that involves histidine-tagged proteins. However, often in SPR experiments, the binding affinity of histidine-tagged proteins to the traditional mono-NTA surface (surface with single NTA groups) is not strong enough to achieve stable ligand immobilization. Thus, histidine-tagged ligand release from the mono-NTA surface is frequently observed, causing baseline drift. The kinetic analysis of the ligand-analyte interaction is consequently inaccurate or even impossible to perform in some cases.

In order to overcome binding instability, Bio-Rad developed a novel tris-NTA (3 x NTA) biosensor surface that uses a complex of three NTA groups to bind a histidine tag (Figure 1). The nickel (II)-activated complex tris-Ni-NTA (3 x Ni-NTA) interacts at three points with the histidine tag providing a higher binding stability compared to the mono-NTA surface (Lata and Piehler 2005). This results in a more stable baseline in SPR experiments, eliminating the need for irreversible cross-linking of the protein to the chip surface that is usually carried out with the mono-NTA surface. In addition, the tris-NTA surface is easily regenerated by chelating reagents such as EDTA. Because of these advantages, this novel technology is ideal for capturing histidine-tagged proteins for biomolecular interaction analysis.

Bio-Rad has launched ProteOn HTG and HTE sensor chips that feature the tris-NTA technology. The two types of sensor chips have identical surface chemistry but different binding capacity to achieve optimal performance in various applications. The low density surface of HTG chips is ideal for protein-protein and protein-peptide interaction analysis, and the high-density surface of HTE chips is better for protein–small molecule interaction analysis. Previously, we showed the high capturing stability of the HTG chip surface and its ability to capture non purified histidine-tagged proteins from crude samples (Rabkin et al. 2012).
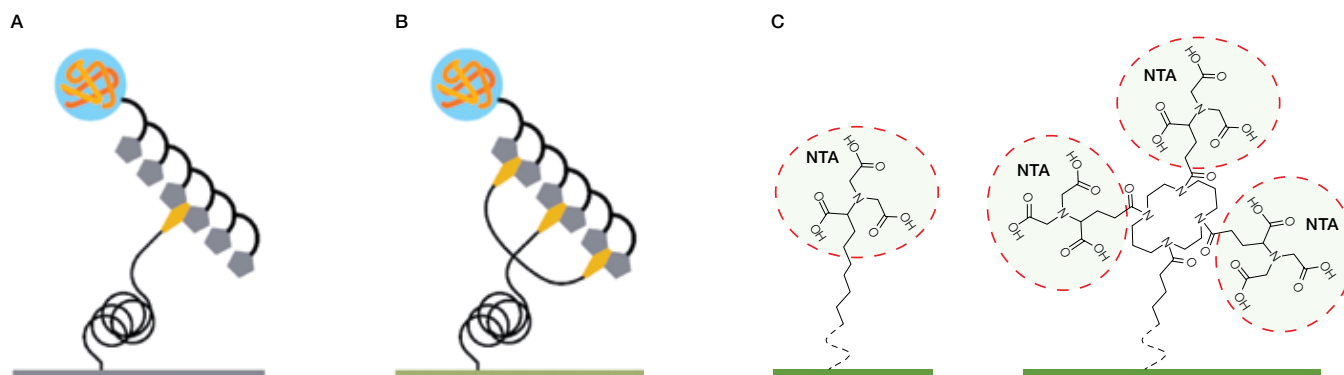

Fig. 1. Tris-NTA technology: comparison of mono-NTA and tris-NTA surfaces. The mono-NTA or tris-NTA groups are activated by nickel (II) to form Ni-NTA or 3 x Ni-NTA complexes, respectively. These complexes are able to capture histidine-tagged proteins. A, the mono-NTA surface is the traditional approach for capturing histidine-tagged proteins but achieves weak binding and results in ligand decay, baseline drift, distorted kinetic results, and inaccurate fitting to kinetic models; B, the ProteOn trivalent tris-NTA (3 x NTA) surface improves binding stability and increases binding selectivity to histidine-tagged proteins. Ni-NTA (◆) binding to histidine (⬟) occurs after activation with nickel (II); C, structure of surface bound mono-NTA molecule (left) and tris-NTA molecule (right). Each individual NTA group is circled.

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Here, we investigate the performance of the HTE chip in protein–small molecule interaction analyses using three model systems: ERK2 enzyme/inhibitors, cyclophilin-A/cyclosporin A, and human serum albumin/small molecule drugs. We also demonstrate the ability to regenerate the HTE tris-NTA surface and recapture proteins in a reproducible manner by performing 85 cycles of ligand capture and regeneration. Rapid protocol optimization using One-shot Kinetics™ technology, combined with the high stability for regeneration, makes the HTE chip ideal for high-throughput screening. In this work we demonstrate the great potential of the HTE chip in the small molecule drug discovery workflow.

Methods

Instrument and Reagents

Experiments were performed using the ProteOn XPR36 protein interaction system with HTE sensor chips. All experiments were performed at 25°C. Histidine-tagged protein A and protein A/G were from Biovision, histidine-tagged ubiquitin was from R&D Systems, histidine-tagged human serum albumin was from Abcam, and histidine-tagged human cyclophilin A was from Prospec. Histidine-tagged ERK2 was a gift from Professor David Engelberg, Hebrew University of Jerusalem. The small molecule analytes: cyclosporin A, quinidine, phenylbutazone, coumarin, warfarin, and ATP were from Sigma. The kinase inhibitors used for ERK2 binding experiments were part of the Calbiochem InhibitorSelect 96-Well Protein Kinase Inhibitor Library I (EMD Millipore) and the Tocriscreen Kinase Inhibitor Toolbox (R&D Systems).

Ligand Capture on HTE Chips — General Procedure

Unless otherwise stated, the running buffer was ProteOn PBS/Tween (phosphate buffered saline, pH 7.4 with 0.005% Tween 20). All ligand binding steps were performed at a flow rate of 30 µl/min. Before each experiment, the HTE chip surface was conditioned to provide rapid stabilization of the baseline and to verify that the tris-NTA surface was clean of metals. Chip conditioning was performed on all six channels according to the recommended HTG/HTE protocol, which includes horizontal and vertical injections of 0.5% SDS, 50 mM NaOH, 100 mM HCl, and 300 mM EDTA, pH 8.5. Chip activation was performed by injecting 10 mM $NiSO_4$ for 2 min prior to ligand injection.

Multiple Regeneration and Recapture Cycles on the HTE Chip

Three channels of an activated HTE chip were injected with either histidine-tagged protein A (50 µg/ml), histidine-tagged protein A/G (20 µg/ml), or histidine-tagged ubiquitin (40 µg/ml), all diluted in PBS/Tween running buffer. The injection volume was 150 µl at a flow rate of 30 µl/min. Chip regeneration was performed by injection of 400 µl EDTA 300 mM, pH 8.5, at a flow rate of 30 µl/min. Overall, 85 consecutive cycles of regeneration, activation, and ligand capture were performed on the three channels in vertical MCM orientation.

Capture of Histidine-Tagged ERK2 and Binding of Small Molecule Kinase Inhibitors

The running buffer for histidine-tagged ERK2 capturing was 50 mM Tris, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM $MnCl_2$, and 0.005% Tween 20, pH 7.4. A volume of 300 µl histidine-tagged ERK2 (100 µg/ml) was injected on an activated HTE chip at a flow rate of 30 µl/min. Before analyte injections, the running buffer was supplemented with 5% DMSO, and this buffer was used for analytes dilutions. For each of the analytes, five concentrations in twofold dilutions were injected in horizontal orientation (volume of 100 µl at a flow rate of 100 µl/min). In the sixth channel, running buffer was injected to enable double referencing. Analyte concentrations were as follows: ATP, 100–6.25 µm; JAK3 inhibitor VI, 16.6–0.2 µM; staurosporin, purvalanol B, aminopurvalanol A, and naphthyl PP1, 50–0.62 µM.

Capture of Histidine-Tagged Human Cyclophilin A and Binding of Cyclosporin A

The running buffer for histidine-tagged cyclophilin A (CypA) capturing and for cyclosporin A (CsA) binding was 10 mM HEPES, 150 mM NaCl, 2% ethanol, and 0.005% Tween 20, at pH 7.4. A volume of 100 µl histidine-tagged CypA (4 µg/ml) was injected on an activated HTE chip at a flow rate of 25 µl/min. For analyte injections, volumes of 300 µl CsA were injected at a flow rate of 150 µl/min in a horizontal MCM orientation using a five concentration series from 1000 nM to 12 nM in threefold dilutions (in running buffer). One channel was left with running buffer for double referencing.

Capture of Histidine-Tagged Human Serum Albumin and Binding of Small Molecules Analytes

A volume of 125 µl histidine-tagged human serum albumin (HSA) at 50 µg/ml in PBS/Tween running buffer was injected on an activated HTE channel at a flow rate of 25 µl/min. The ligand injection was followed by a 100 µl injection of 1 M ethanolamine pH 8.5 and 30 µl injection of 50 mM NaOH to remove electrostatically adsorbed ligand and stabilize the baseline. Before analyte injections, the running buffer was supplemented with 3% DMSO, and this buffer was used for analyte dilutions. The following small molecule analytes were tested: quinidine, phenylbutazone, coumarin, and warfarin. For each analyte, ten concentrations from 1000 µM to 26 µM in 1.5-fold dilutions were injected horizontally (volume of 50 µl at flow rate of 50 µl/min). Each injection consisted of five analyte concentrations and running buffer in the sixth channel to enable double referencing, and each series was repeated three times.

Sensorgram Acquisition and Data Analysis

For each of the kinetic models, a single injection of five analyte concentrations over the captured ligand surface allowed for the acquisition of the full kinetic profile, without the need for regeneration between different concentrations. Reference subtraction was done with an empty channel or interspots for analyte bulk subtraction and monitoring analyte nonspecific binding (NSB). Excluded volume correction was also applied when DMSO was used in the running buffer (Bio-Rad bulletin 5822). The analyte injections also included a blank sample (running buffer with no analyte) which allowed for real-time double referencing. In this manner, the slow dissociation of the captured ligand is measured at the same time as the interaction itself, allowing accurate and reliable double referencing without having to perform a separate blank injection.

Data were analyzed with ProteOn Manager™ software version 3.1. Each set of five reference-subtracted and aligned sensorgrams was fitted to curves describing a Langmuir 1:1 homogenous bimolecular reaction model. Global kinetic rate constants (k_a and k_d) were derived for each reaction. K_D was calculated from k_d/k_a ratio or by equilibrium analysis. The R_{max} value, the maximal analyte signal at ligand saturation, was also globally fitted from the sensorgrams.

Results and Discussion
Reproducible Surface Regeneration and Ligand Capture of the HTE Chip

The ability to regenerate the tris-NTA surface is an important feature of the HTE chip. It allows the reuse of the chip for multiple ligand capturing cycles. To test the regenerability and reproducibility of the HTE tris-NTA surface, 85 cycles of ligand capture and regeneration were applied to the chip with three different proteins as described in the Methods section. The regeneration efficiency was 100% and the captured ligands were completely removed by the EDTA injections. Figure 2 shows an overlay of the 85 ligand capture steps which exhibit high uniformity between all cycles with densities ranging from 3,500 to 3,000 RU (3.6% CV) for histidine-tagged protein A, densities of 1,600 to 1,400 RU (3.2% CV) for histidine-tagged

protein A/G, and densities of 3,150 to 2,800 RU (2.8% CV) for histidine-tagged ubiquitin. The data show that there is a general trend of decreased capturing capacity throughout the injections, but even after 85 cycles of regeneration/recapturing, the capacity remains at 85–90% of the initial capacity. These results demonstrate that the tris-NTA surface can be regenerated and used repeatedly many times without significantly losing its capturing capacity. This feature facilitates, a simple regeneration protocol of histidine-tagged proteins, which is especially useful when standard regeneration of covalently bound ligands decrease the ligand activity.

Binding Interaction Between Histidine-Tagged ERK2 and Small Molecule Kinase Inhibitors

ERK2 is a mitogen-activated protein kinase (MAPK) involved in cell proliferation, differentiation, survival control, and embryonic development. The importance of MAP kinases (such as ERK2) in cell signaling makes them particularly interesting as drug targets and inhibitors of kinases are part of many drug screens used to identify new drugs.

Histidine-tagged ERK2 ligand was captured on an HTE chip and used to screen for binding of small molecules from two commercial kinase inhibitor libraries using a single concentration of each small molecule inhibitor. The binding response of each inhibitor was normalized by the molecular weight (MW) of the molecule and the hits with the highest binding were then subjected to detailed kinetic and equilibrium analysis. For those experiments, histidine-tagged ERK2 was captured on an HTE chip to a saturation density of 12,800 RU. The ligand capturing stability was very good, with a baseline drift less than 5 RU/min (0.04% of the ligand density). This enabled the characterization of the binding of six analytes on the same ERK2 surface without the need to covalently attach the captured protein to the tris-NTA surface. Six analytes were each tested at five concentrations in a single injection using the One-shot Kinetics protocol of the ProteOn system. Each such injection also included a blank buffer injection in the sixth channel that enabled real-time double referencing to correct for the minor ligand drift. With
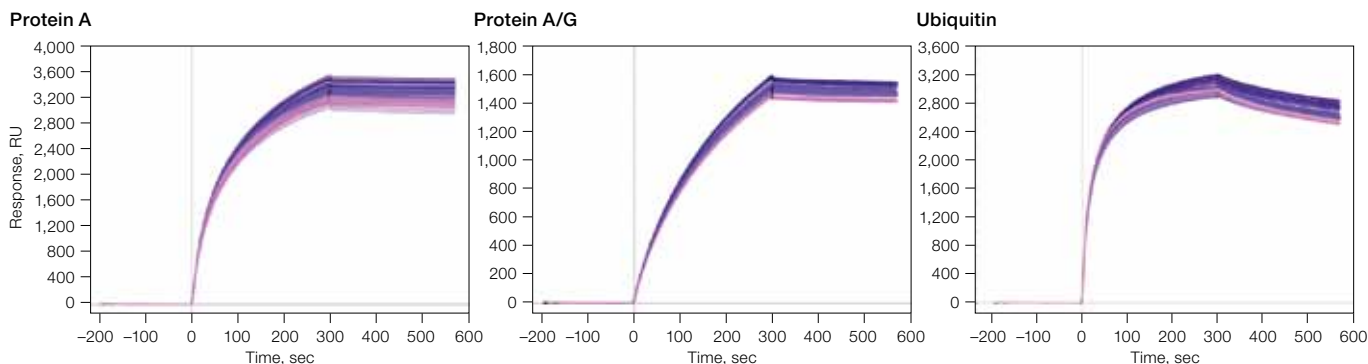

Fig. 2. HTE chip regeneration and recapturing performance. Overlay of 85 histidine-tagged protein A, protein A/G, and ubiquitin ligand capturing steps. Each capturing and regeneration cycle included activation with NiSO$_4$, histidine-tagged ligand capture, and EDTA regeneration. For simplicity, only one interaction spot from each ligand channel is presented; similar results were obtained for all other ligand channels. Each ten consecutive ligand injections are colored in the same color, starting from dark purple to pink.

ATP

Staurosporin

JAK3 Inhibitor VI

Purvalanol B

Aminopurvalanol A
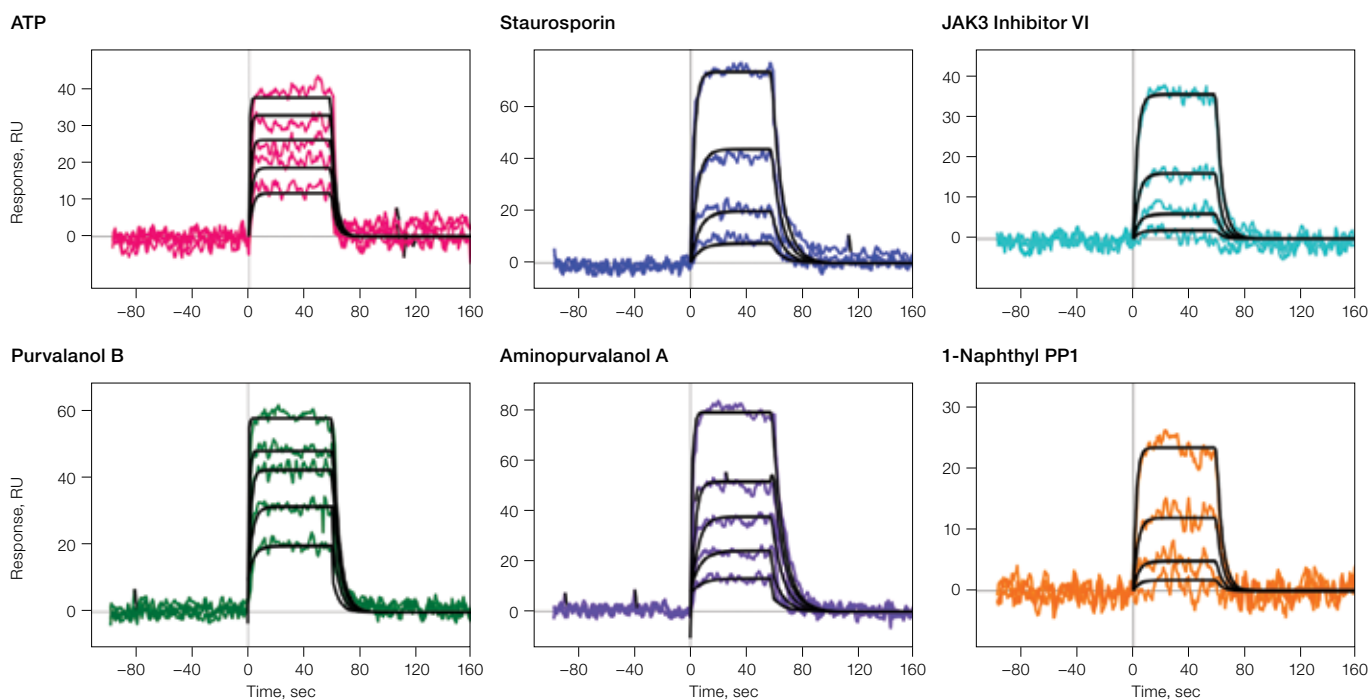
1-Naphthyl PP1

Equilibrium Analysis

Fig. 3. Detailed equilibrium analysis of several small molecule kinase inhibitors to histidine-tagged ERK2 enzyme. ERK2 was captured on an HTE chip to a density of 12,800 RU. Capture was followed by injections of the six analytes. The top panel shows the sensorgram for different concentrations of each of the six analytes with the overlaid 1:1 model fit in black. The bottom panel shows the plots of the equilibrium response versus analyte concentrations.
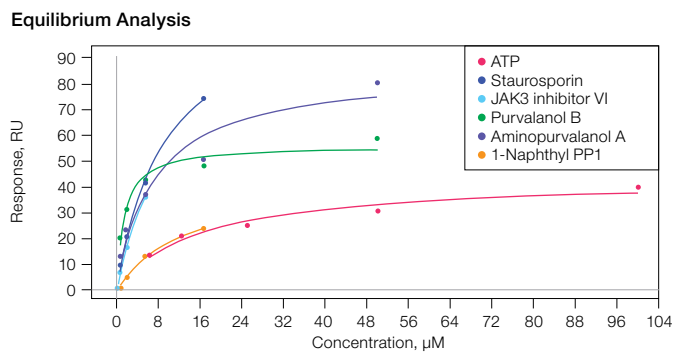
Table 1. Equilibrium and kinetic constants derived from the plots in Figure 3.

| Analyte | MW | Equilibrium | | Kinetic | | | |
		K_D, µM	R_{max}, RU	k_a, 1/Ms	K_d, 1/s	K_D, µM	R_{max}, RU
ATP (positive control)	507.2	15.6	43.3	1.79×10^4	3.06×10^{-1}	17.1	43.5
Staurosporine	466.5	8.8	112.1	1.58×10^4	1.33×10^{-1}	8.4	110.9
JAK3 inhibitor VI	383.4	8.2	89.7	2.50×10^4	2.20×10^{-1}	8.8	93.3
Purvalanol B	432.9	1.4	55.7	6.27×10^4	2.07×10^{-1}	3.3	61.5
Aminopurvalanol A	403.9	7.2	85.2	1.40×10^4	1.27×10^{-1}	9.1	95.9
1-Naphthyl PP1	317.4	12.9	41.3	1.52×10^4	2.30×10^{-1}	15.2	45.0

three of the inhibitors, staurosporin, JAK3 inhibitor VI, and 1-naphthyl PP1, the highest concentration signals were more than the theoretical calculated R_{max} values. This could be a result of aggregate formation at high analyte concentrations; therefore, those concentrations were omitted from the analysis. The resulting data fit well with a 1:1 binding model and the K_D values calculated from kinetic and equilibrium analyses were consistent (Figure 3 and Table 1).

Binding Interaction Between Histidine-Tagged Human Cyclophilin-A (CypA) and Cyclosporin A (CsA)

CypA is a peptidylprolyl cis-trans isomerase which accelerates the folding of proteins. The cyclophilins are involved in different inflammatory conditions and pathogenesis of several diseases. CsA is a 1202.12 Da cyclic peptide, widely used for preventing organ rejection following transplants. The potent immunosuppressive activity of CsA is mediated by high affinity binding to cytosolic CypA, which subsequently blocks the signal transduction pathway for immunostimulation. The binding kinetics of the CypA-CsA interaction was previously measured using SPR, but the very unstable capturing on a mono-NTA chip surface required covalent fixation of the histidine-captured CypA in order to eliminate the extensive baseline drift and allow kinetic analysis (Wear et al. 2005).

Here, by using the HTE sensor chip, it was possible to capture histidine-tagged CypA on the chip surface with no drift at all, so cross-linking of the CypA to chip surface was not necessary. Conditioning and activation followed by histidine-tagged CypA capturing yielded a ligand density of 1,800 RU. The stable ligand capture allowed performing three consecutive injections of the CsA analyte without the need for ligand recapture. The three injections completely overlap and the data obtained fit well with a 1:1 binding with mass transport limitation model (Figure 4 and Table 2). The histidine captured CypA protein retained high levels of activity and the ratio between the experimental R_{max} and the theoretical R_{max} was 77%.
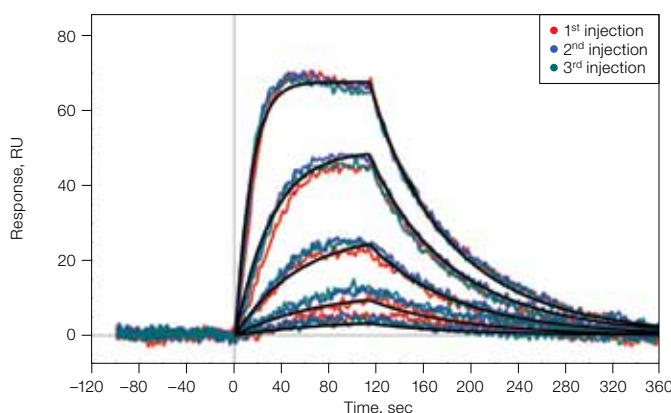
Fig. 4. Detailed kinetic analysis of CsA binding to histidine-tagged CypA. CypA was captured on an HTE chip to a final density of 1,800 RU. Capture was followed by three injections of CsA analyte. The chart shows an overlay of the sensorgrams corresponding to the three injections (each repeat in a different color) with the overlaid 1:1 model fit in black.

Table 2. Kinetic constants derived from the plots in Figure 4.

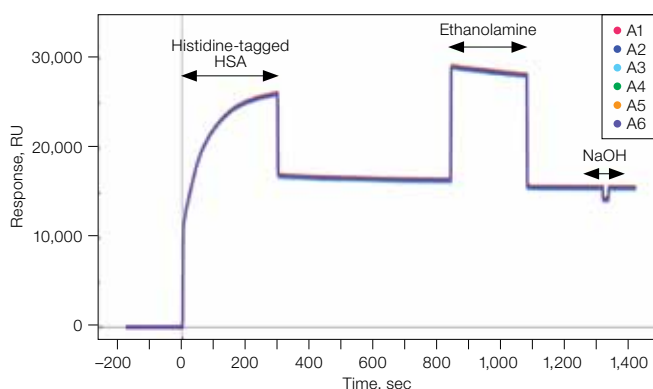| k_a (1/Ms)	K_d (1/s)	K_D (μM)	R_{max} (RU)
7.74×10^4	1.76×10^{-2}	0.23	83.1

Fig. 5. Capture and baseline stabilization of histidine-tagged HSA on the HTE sensor chip. An overlay of six spots within a single channel is shown. The baseline drift due to ligand dissociation was reduced from ~50 RU/min after the ligand capturing to less than 1 RU/min after the ethanolamine and NaOH injections.

Binding Interaction Between Human Serum Albumin (HSA) and Small Molecule Drugs

HSA is a protein that plays an important role in drug distribution. It reversibly binds to most drugs and decreases their availability in circulation. Therefore, it is critical to study interactions between drug candidates and HSA in the drug discovery workflow. SPR biosensors have been applied to analyze drug-HSA interactions since the early 2000s. It is reported that for this particular application, SPR technology showed benefits by allowing real-time label-free interaction analysis, giving high-quality results compared to other methods (Day and Myszka 2003).

The HTE chip is an effective tool for analyzing drug-HSA interactions. It allows high capturing stability of histidine-tagged HSA on the chip surface for high quality results with minimum ligand sample consumption. In this study, four small molecule drugs were analyzed to show the performance of the HTE chip in this application. Conditioning and activation followed by histidine-tagged HSA capturing yielded a ligand density of 16,900 RU with a baseline drift of ~50 RU/min (Figure 5). Washing the surface with 1 M ethanolamine and 50 mM NaOH have reduced the baseline drift to less than 1 RU/min, indicating that the drift resulted from electrostatically adsorbed HSA protein on the surface and not from dissociation of the tris-NTA captured protein.

The high ligand density and capturing stability allowed for multiple analyte injections without needing to recapture the ligand. Figure 6 shows the responses for interactions between the captured HSA and four small molecule drugs with MW ranging from 146 to 324. Using the One-shot Kinetics technique, it was possible to measure the response of ten concentrations of each drug in only two injections. Superimposing sensorgrams of three triplicate injections demonstrates the high reproducibility and stability of the ligand surface. HSA was previously shown to have multiple binding sites for small molecules (Day and Myszka 2003), and indeed, the responses obtained here did not fit a simple single binding site model.
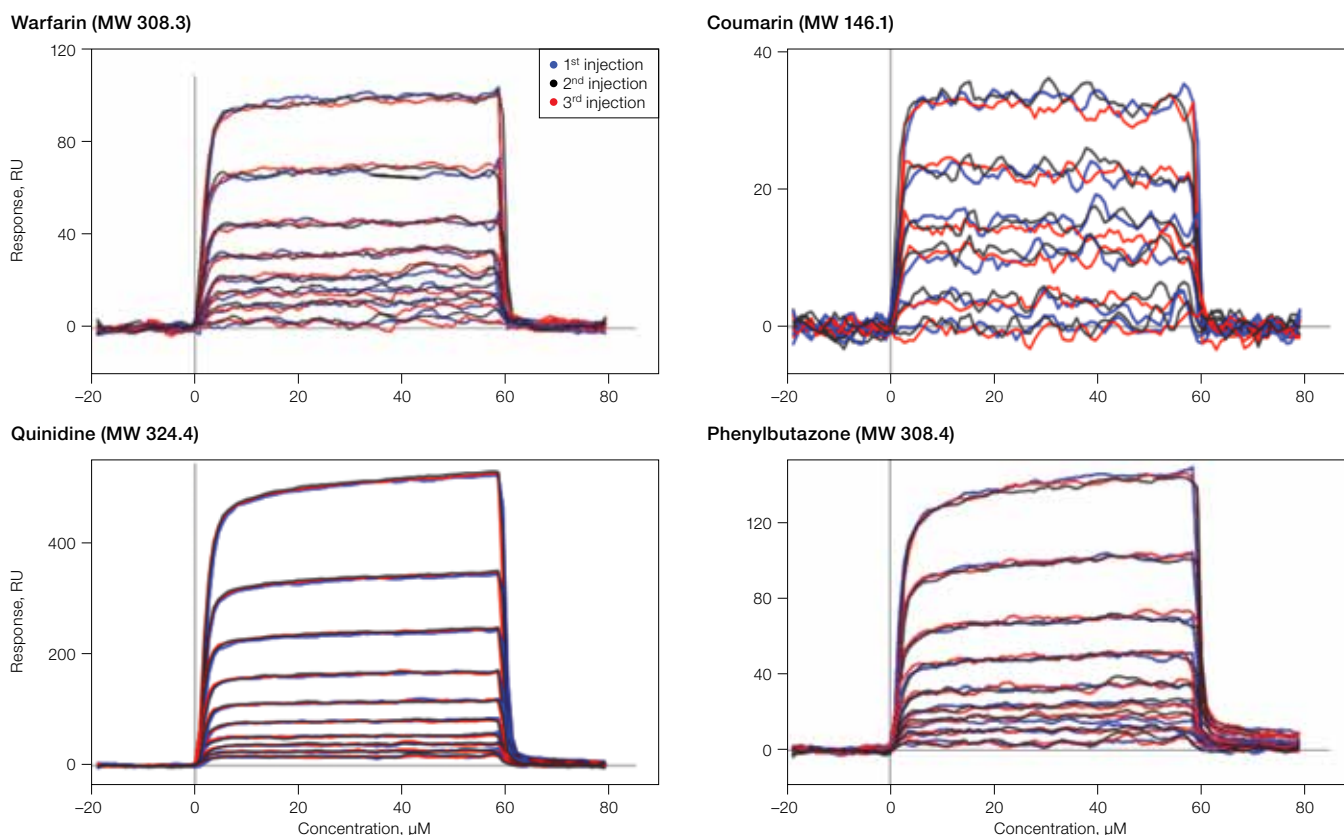
Warfarin (MW 308.3)

Coumarin (MW 146.1)

Quinidine (MW 324.4)

Phenylbutazone (MW 308.4)

Fig. 6. Binding of four small molecules to histidine-tagged HSA. HSA was captured on the the chip to a final density of 15,600 RU. Capture was followed by two injections of each analyte to create ten concentration response series. The charts show overlays of the sensorgrams corresponding to the three injections (each repeat in a different color). With some of the analytes, the responses of several concentrations were too close, so not all concentrations are shown.

Conclusions

In this study, the HTE sensor chip was used for three cases of protein–small molecule interaction analysis. The very stable capturing of histidine-tagged ligand proteins allows for accurate determination of kinetic and equilibrium constants for various interactions. Small molecules with molecular masses as low as 146 Da were successfully measured. In addition, it was proven that the tris-NTA surface has exceptional regeneration capability after as many as 85 regeneration and recapture cycles of histidine-tagged proteins.

When used with the patented One-shot Kinetics approach, the HTE chip offers unprecedented throughput in biomolecular interaction analysis. As was shown previously with the HTG chip, the tris-NTA surface can also be used to capture histidine-tagged proteins directly from crude medium — an option that saves a great deal of effort on pre-purification of target proteins (Rabkin et al. 2012). These valuable features make the HTE chip an ideal screening tool in drug discovery applications.

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