High Throughput, Label-free Screening Small Molecule Compound Libraries for Protein-Ligands using Combination of Small Molecule Microarrays and a Special Ellipsometry-based Optical Scanner

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Small-molecule compounds remain the major source of therapeutic and preventative drugs. Developing new drugs against a protein target often requires screening large collections of compounds with diverse structures for ligands or ligand fragments that exhibit sufficient affinity and desirable inhibition effect on the target before further optimization and development. Since the number of small molecule compounds is large, high-throughput screening (HTS) methods are needed. Small-molecule microarrays (SMM) on a solid support in combination with a suitable binding assay form a viable HTS platform. We demonstrate that by combining an oblique-incidence reflectivity difference optical scanner with SMM we can screen 10,000 small-molecule compounds on a single glass slide for protein ligands without fluorescence labeling. Furthermore, using such a label-free assay platform we can simultaneously acquire binding curves of a solution-phase protein to over 10,000 immobilized compounds, thus enabling full characterization of protein-ligand interactions over a wide range of affinity constants.

Introduction

In drug discovery, affinity and inhibition screening of thousands to millions of molecules against a protein target or a set of protein targets is routinely required to identify suitable ligands as candidates for further mechanistic and developmental studies. The number of biomolecular binding assays involved is further increased by post-translational modification of proteins such as glycosylation and phosphorylation. It has come to be expected that such a massive amount of screening assays should be done in a highly parallel manner so that endpoints or preferably kinetic constants of thousands of binding reactions are determined simultaneously. Over the last decade, microarrays [1-8] and micro-fluidics [9-12] have emerged as two major enabling platforms for high-throughput screening. They afford a large number of biochemical reactions and their detection to take place either concurrently (in cases of microarrays) or sequentially at a high rate (in cases of microfluidics). In addition to high throughputs and time saving, these platforms consume far less reagents than conventional assays. These attributes make them indispensable tools of drug discovery.

Identifying ligands with desirable affinity to a protein target from a large number of synthetic and natural compounds including aptamers and carbohydrates is facilitated by microarray-based binding assays where ligand candidates are immobilized on a solid support as an addressable array with thousands or tens of thousands of distinct features. By exposing the array to a solution-phase protein probe, as many binding reactions occur concurrently. It is advantageous to immobilize small molecules instead of protein targets for two reasons: (1) the number of small molecule compounds is much larger than that of protein targets, and thus, for high throughput consideration it is sensible to immobilize small molecules as large microarrays and keep relative few protein targets in solution phase; (2) small molecules are more rigid than proteins, and thus, less prone to conformational change when immobilized through a flexible linker to a solid support. One should expect the immobilized small molecules to better maintain their innate structural and chemical properties. Koehler and coworkers showed that ~ 70% of the compounds from NIH and commercial vendors without a "common" surface-anchoring residue can be immobilized on an isocyanate-functionalized solid support through non-specific reaction of nucleophile groups on these compounds [7,13]. As long as key structural residues on some (if not all) of

the "un-immobilizable" compounds are represented in one form or another by the "immobilizable" compounds, the "un-immobilized" compounds are in essence not excluded from screening.

Binding of a protein target to small molecule microarrays (SMM) has mostly been detected *ex situ* with fluorescence-based methods, by labeling either the protein with fluorescent tags (including incorporation of GFP) or a secondary probe that recognizes the primary protein [3,7,13]. The combination of SMM and fluorescence-based detection yields endpoints of binding reactions under a specific set of conditions including protein concentration, buffer, incubation time, and post-incubation processing before fluorescence readout. There are drawbacks in fluorescence-based SMM detection: (1) labeling protein targets with fluorescent tags requires extra reaction steps, and the efficiency of labeling varies from protein to protein; (2) directly labeling a protein alters the protein affinity profile, in ways often uncharacterized [14,15]; (3) *ex-situ* measurements renders the readout a function of assay conditions and limited to ligands with high affinity or small dissociation rates [9,14]; (4) end-point measurements yield significantly limited information on binding kinetics and as a result offer incomplete characterization of protein-ligand interactions [14].

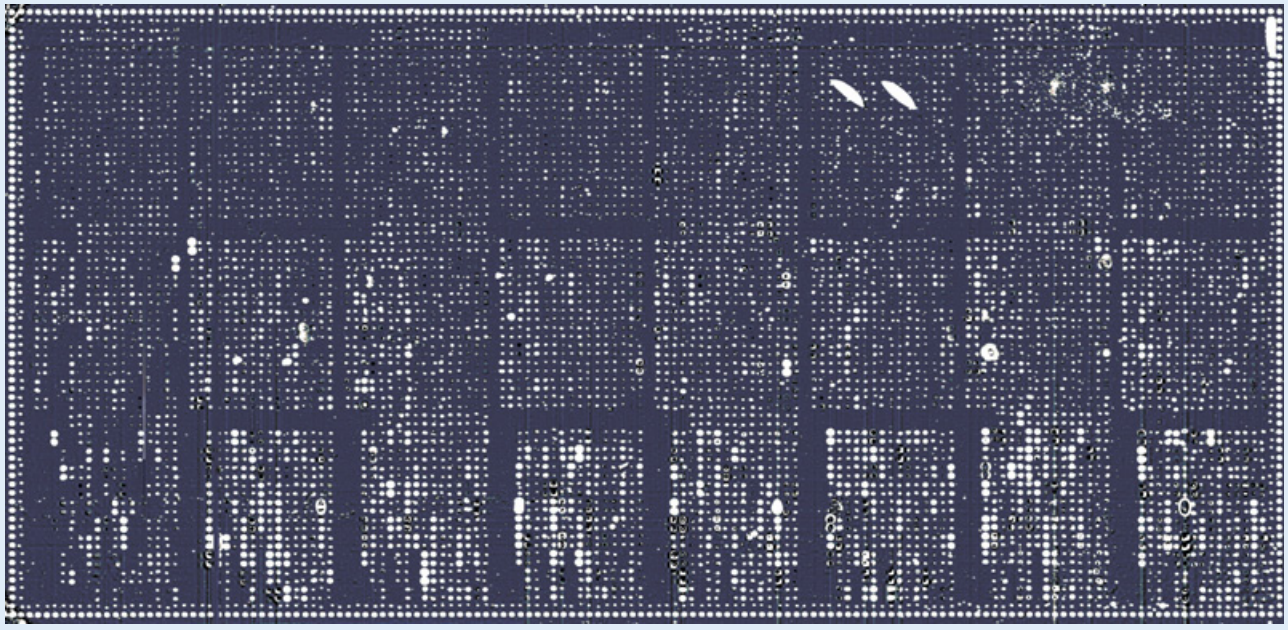
We describe a combination of an ellipsometry-based optical scanner [16,17] and small-molecule microarrays on isocyanate-functionalized glass surfaces as a non-fluorescence-based assay platform in high-throughput screening of small molecule libraries for protein ligands. This platform avoids the drawbacks associated with the fluorescence-based detection. The *in-situ* nature of the ellipsometry detection enables measurements of binding curves and in turn determination of binding kinetic constants over a wide dynamic range [17,18]

Small molecule microarray of a NCI/DTP compound library. For small molecule compounds, we use five compound sets from NCI/DTP Open Repository: (1) Challenge set with 57 compounds (10 mM in DMSO, 20 µL); (2) Natural Products set with 235 compounds (10 mM in DMSO, 20 µL); (3) Structural Diversity set with 1,990 compounds (10 mM in DMSO, 20 µL); (4) Mechanistic Diversity set with 879 compounds (1 mM in DMSO, 20 µL); (5) an Open set with 4,800 compounds (1 mM in DMSO, 20 µL). There are a total of 7,761 compounds that come in 96-well plates. Directly from the stock solutions and using an microarrayer with 8 printing tips, we immobilized these compounds into two small-molecule microarrays on separate isocyanate-functionalized glass slides, prepared with a protocol developed by Bradner and coworkers [13]. We printed one half of the NCI compounds in duplicate on one slide along with a set of control compounds, and the other half also in duplicate on the second slide along with the same set of control compounds. Each slide contained a total of ~ 10,000 printed features over an area of 2 cm × 4 cm. A printed glass slide was assembled with a fluid chamber so that the printed microarray could be processed while being imaged or read out in real time with an ellipsometry-based scanner [16,17].

Optical image of NCI compound microarrays on functionalized glass slides acquired with an ellipsometry-based optical scanner. We detected printed NCI microarrays and subsequent reactions of these microarrays with solution-phase protein probes using an oblique-incidence reflectivity difference scanning microscope - a special form of scanning ellipsometry [16,17] The oblique-incidence reflectivity difference (OI-RD) signal is defined as the difference of fractional reflectivity changes between the p-polarized and s-polarized components of a monochromatic light off a solid surface when it is covered with an immobilized molecular layer, $(r_p - r_{p0})/r_{p0} - (r_s - r_{s0})/r_{s0} \equiv \Delta_p - \Delta_s$ [19,20]. r_{p0} and r_{s0} are the complex reflectivities of the bare surface; r_p and r_s are the reflectivities of the surface when it is covered with the immobilized molecular layer. Δ_p - Δ_s is proportional to the surface mass per unit area Γ of the immobilized molecular layer just as surface-plasmon resonance (SPR) responses [16,17,19,21] Using an OI-RD scanning microscope, we directly measure Δ_p - Δ_s from immobilized NCI compound microarrays on functionalized glass slides.

Figure 1 shows the OI-RD image (equivalent to an image of the surface

FIGURE 1



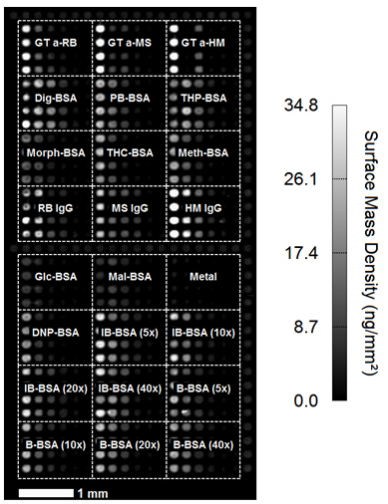
OI-RD image of a printed NCI compound microarray with ~ 10,800 spots immobilized on isocyanate-functionalized glass slide before further processing. The microarray contains 3,900 NCI/DTP compounds printed in vertical duplicate that form 24 blocks. The double borders (BSA and biotin-conjugated BSA) and spots as double lines between the blocks are control features. The image covers an area of 2 cm x 4 cm.

mass density Γ [16,17,19] of an NCI compound microarray taken before the array was further processed. The features along the perimeter are bovine serum albumin (BSA); the features immediately inside are biotin-conjugated BSA; both BSA and biotin-BSA are control features. Further inside are ~ 3,900 NCI compounds printed in vertical pairs. They form 24 rectangular blocks. Printed horizontally between blocks of the NCI compounds are other control compounds.

Binding reaction of human kinase insertion domain receptor (KDR) with 7,761 NCI compounds on microarrays – label-free endpoint measurement. Human kinase insertion domain receptor (KDR or VEGFR2) fused to the Fc fragment of rabbit IgG was purchased as the protein probe. On fresh NCI compound microarrays, we performed reactions of KDR with 7,761 compounds by incubating the microarrays in a KDR solution at 0.00068mg/ml or 60nM in 1× PBS for 60 minutes and then washing the microarrays with 1× PBS. Figure 2 shows the change in OI-RD image of one NCI compound microarray after the reaction. The distinguishable pairs in the vertical direction reveal the few compounds that captured KDR. Using this method, we identified 27 "hits" from a total of 7,761 NCI compounds that reacted with KDR with high affinity.

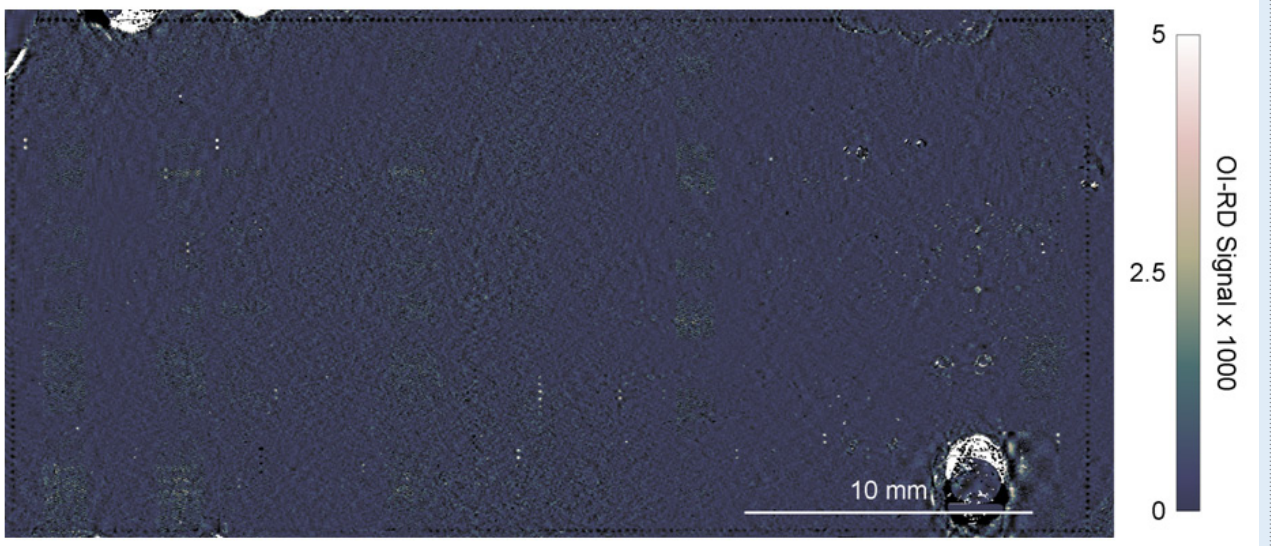
Simultaneous measurement of 10,000 protein-ligand binding curves, – reaction of a glycan-binding protein with a host of glycans and protein targets. The change in optical image as displayed in Figure 2, albeit acquired label-free, is an endpoint measurement of protein-compound reactions after the solution of the protein probe has been replaced with 1× PBS for a period of time (~ 30 minutes). As a result, the image only reports binding reactions with dissociation times τ_d being comparable or longer than 30 minutes [14], and thus, with equilibrium dissociation constant K_d (inversely proportional to τ_d) in the range of nM. In order to capture binding reactions with K_d ranging from sub-mM to pM and fully characterize reaction kinetics, it is necessary to record binding curves of protein-ligand reactions. To respond to such a need, we developed the capability of our OI-RD scanning microscope to simultaneously record binding curves of a protein probe with 10,000 immobilized ligand candidates. To illustrate the instrument capability, we fabricated a large microarray with 10,880 printed features printed on an epoxy-coated glass slide. The microarray consists of 16 "identically" printed sub-arrays. Each sub-array (Figure 3) consists of 24 distinct protein targets that are printed in quadruplet at 6 different target concentrations (0.5 μM to 16 μM) [16,19,22] These 24 protein targets are: (1) bovine serum albumin (BSA); (2) human IgG (HM); (3) mouse IgG (MS); (3) rabbit IgG (RB); (4-6) polyclonal goat IgG against human/mouse/rabbit IgG (GT anti-HM, GT anti-MS, GT anti-RB); (7) Methamphetamine-BSA (Meth-BSA); (8) tetrahydrocannabinol-BSA (THC-BSA); (9) morphine-BSA (Morph-BSA); (10) Theophylline-BSA (THP-BSA); (11) phenobarbital-BSA (PB-BSA); (12) digoxin-BSA (Dig-BSA); (13) Metallothionein (Metal); (14-17) biotin-BSA (B-BSA) with the loading molar ratio of the NHS-biotin ester to BSA at 5x, 10x, 20x, and 40x; (18-21) iminobiotin-BSA (IB-BSA) with the loading molar ratio of the NHS-biotin ester to BSA at 5x, 10x, 20x, and

FIGURE 3



The surface mass density of a subarray that consists of 24 distinct molecular targets printed in quadruplet and in 6 target concentrations (16 μM, 8 μM, 4 μM, 2 μM, 1 μM, 0.5 μM). 16 such subarrays along with the control features around the perimeters of these subarrays form a 10,880-feature molecular target microarray that is fabricated on an epoxy-functionalized glass slide over an area of ~ 2 cm × 4 cm. The surface mass density map is determined from the OI-RD images of the microarray [16,19,22] taken before it reacts with protein probes of interest such as ConA.

FIGURE 2



Change in OI-RD image of the NCI compound microarray acquired in 1× PBS after incubation in a solution of KDR 0.0068mg/ml or 60nM in 1× PBS for 60 minutes. The distinguishable doublets in vertical directions reveal the compounds that have captured KDR from the solution. We can identify 14 "hits" out of ~ 3,900 immobilized NCI compounds on this microarray.

40x; (22) 2,4-dinitrophenol-BSA (DNP-BSA); (23) glucose-BSA (Glc-BSA); and finally (24) maltose-BSA (Mal-BSA). The remaining 1664 features are printed for controls.

We used *concanavalin* A (ConA) as the protein probe. ConA is known to react specifically with glucose. Unlike monoclonal antibodies, ConA is also expected to react with other molecular motifs as we will reveal here. Instead of acquiring OI-RD images before and after incubation with a ConA solution at a concentration of 300 nM in 1x PBS, we measured the difference between the optical signal from one pixel inside the target area and the average of the optical signals from two pixels in the neighboring *unprinted* area as a background-corrected readout. It took ~ 20 seconds to sequentially read from 10,880 immobilized targets for one time point. By repeating the time-point measurement during incubation phase (for 30 minutes after the protein probe solution replaces 1× PBS) and subsequent dissociation phase (for 90 minutes after the protein probe solution is replaced by 1× PBS again), we acquired 10,880 ConA binding curves in one experiment. Figure 4 displays 576 out of 10,880 binding curves of ConA to the protein target microarray. As expected, ConA reacted with glucose-BSA and yet not with maltose-BSA, indicating the specific affinity to glucose. However, it is clear that ConA reacted non-specifically with all IgG molecules. There is also evidence of non-specific reaction with drug-BSA conjugates. We note that even though the density of the immobilized glucose-BSA and the amount of the captured ConA by glucose-BSA changed by more than a factor of 10, the temporal behaviors of the binding curves remained unchanged as one should expect. As a result the binding kinetics extracted from these binding curve sets do not depend on which pixel inside the target area is used to yield the binding curve [17].

Our present microarray-based binding curve detection technology is moving microarrays from a highly parallel end-point assay platform to an equally parallel kinetic constant assay platform that directly yields equilibrium dissociation constants. It is an effective tool for affinity ranking ligands of a protein probe with K_d ranging from pM to sub-mM. It enables simultaneous characterization of both specific and non-specific reactions of a protein probe with a wide variety of immobilized targets as we illustrated here. With 2 ~ 3 such OI-RD optical scanners simultaneously in operation, we should be able to measure equilibrium dissociation constants of a protein probe against 100,000 compounds in a couple of days.

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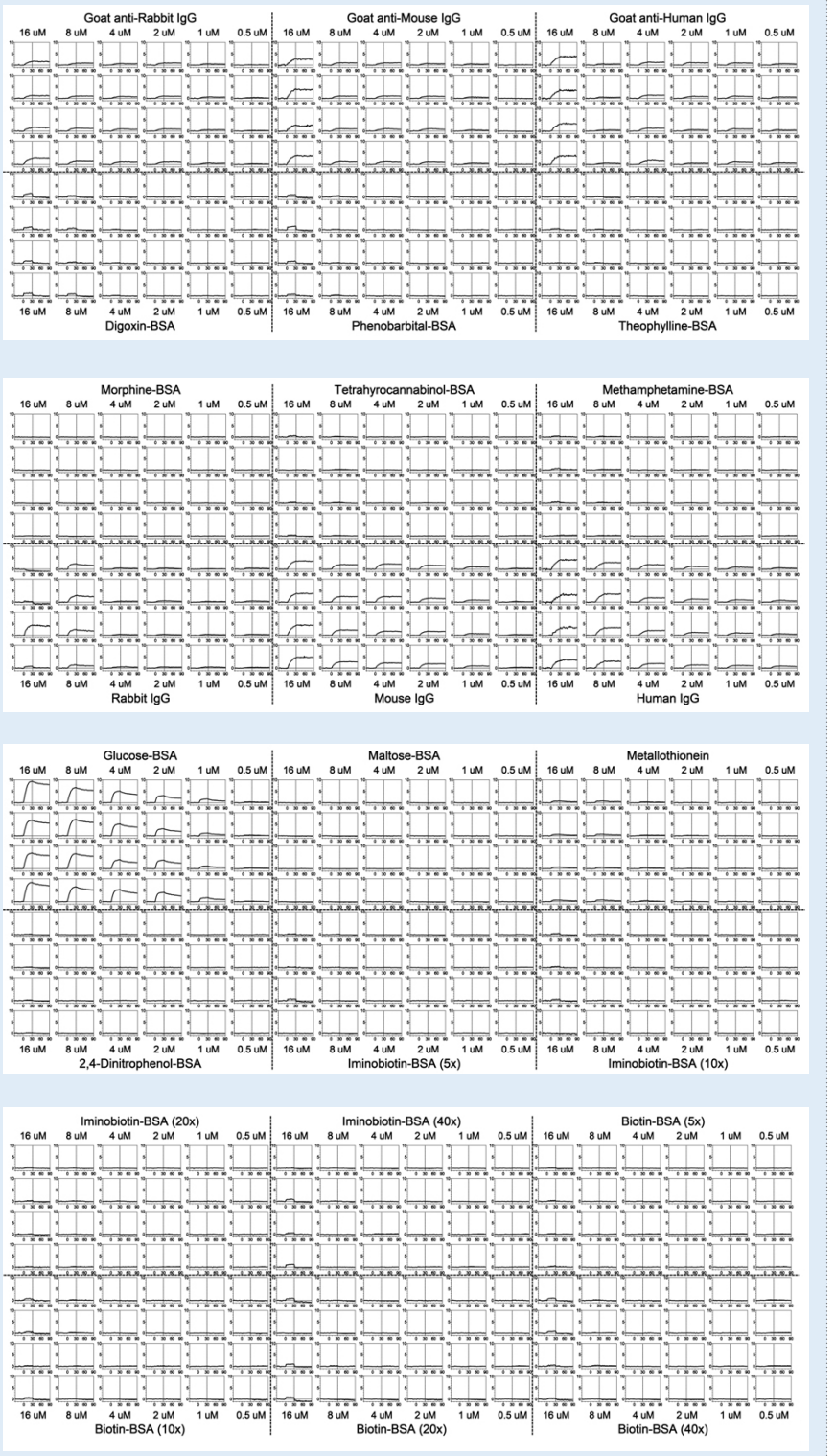
FIGURE 4



A small portion of 10,880 simultaneously acquired binding curves of ConA to as many immobilized targets on an epoxy-functionalized glass slide surface at ConA concentration of 300 nM. In each panel, the vertical axis is the surface mass density of the captured ConA and has the full scale of 10 ng/mm². The horizontal axis is in minutes and has a full scale of 120 minutes. The incubation phase starts at t = 0 and lasts for 30 minutes. The dissociation phase starts at t = 30 minutes and lasts for 60 minutes. In addition to specific reactions with glucose, ConA also reacted with the immobilized IgG molecules.

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