Universal screening platform using three-dimensional small molecule microarray based on surface plasmon resonance imaging
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Vikramjeet Singh, Amita Nand and Sarita

Although much progress has been made in small molecule microarray in past decades, its potential has been limited by the lack of efficient methodology. Hence, we are reporting a potent methodology for the drug screening on a three-dimensional (3D) surface using carbene based photo-cross-linking reaction. The simultaneous display of a large number of small molecules on a single polymer chain in various orientations allows for the retention of their activity. The presented method was tested by using high-throughput surface plasmon resonance (SPRI) with the immunosuppressive drugs rapamycin and FK506. We showed that rapamycin and FK506 immobilized on the 3D surface, not the conventional 2D surface, bound to the FKBP with high affinity. Using FKBP-binding ligands and FKBP mutants with altered mutual binding affinities, we observed a strong correlation between the relative binding affinities determined by SPRi and those previously reported. In addition, other important parameters including, blocking, washing, robustness and surface reproducibility were also validated. Some well known kinase inhibitors of p38α, JNK and ERK2 proteins were also used to extend the method applications. All together, these results suggested that the newly developed 3D small molecule microarray in conjunction with SPRi can be a powerful platform for high throughput drug screening.
necessary parameters including, UV irradiation time, washing and regeneration processes.

**Results and Discussion**

**XPS analysis and comparison of 2D PEG and 3D SIP PCL surfaces by Fluorescence**

First of all, FTIR and XPS techniques were used for structure confirmation and the immobilization of photo-cross-linking moiety respectively. FTIR results (Fig. 2) confirmed the successful fabrication of PEG and SIP substrate onto gold surfaces. The obtained results from XPS indicated the presence of high amount of F and N elements on the surfaces which proved the presence of photo-cross-linking group (Table S-1). To check the high loading capacity of 3D SIP, a standard experiment was carried out to compare 3D SIP with 2D PCL surfaces using fluorescence detection technique. In order to compare the reactivity and capacity of both surfaces, rhodamine B, a well known fluorescence dye was used as a reference compound. Slides were evaluated in terms of signal intensity and spot morphology. Scanned images of 2D and 3D PCL surfaces by fluorescence microscope were shown in Fig. 3a. The background corrected rhodamine B intensity from 2D and 3D PCL surfaces were plotted against each other at 3 different concentrations (Fig. 3b). The 3D PCL slides produced on average approximately 5 times higher intensity over 2D PCL slides. As a negative control, rhodamine B showed very little non-specific adsorption on both surfaces. In addition with high loading capacity, the 3D SIP slide also produced more consistent spot morphology in terms of both size and shape of the spots when compared to the 2D PCL slide. In support, images from SPRi instrument (before washing procedure) were presented in Fig. S-1 for the comparison of spot morphologies over both the surfaces. A regular spot morphology is particularly important in the selection of the spots during the experiments and data analysis which affects the data quality and surface reproducibility.

**Surface evaluation**

To present an ideal methodology, it is very necessary to check the key parameters in each and every aspect. Washing of microarray and removal of bound proteins (regeneration) from the platform had major impact on data quality and microarray efficiency. Missing of any key point could be easily misleading and therefore, presented surface was evaluated very carefully in terms of UV irradiation time, washing and regeneration steps.

In previous reports, microarrays were irradiated under UV for 30 minutes which was very long and definitely not good for some sensitive small molecules structures. In order to validate, 5 chips were printed with rapamycin under identical conditions and irradiated for 0, 10, 20, 30 and 40 minutes. Interaction was measured by flowing FKBP12 (same concentration) and as expected, no signals were observed from 0 minutes and rest of slides showed not much difference (Fig. S-2) in signals intensities. Results suggested that, 10 minutes was enough to crosslink the small molecules onto SMMs platform.

Rapamycin and FK506 were choose to validate the washing step. As shown in Fig. S-3, FKBP12 protein flowed on improperly washed spots (30 minutes in DMF) of rapamycin and FK506, continuous losing of precipitated and physically adsorbed molecules resulted into subsequent decrease in baseline level which finally affect the kinetics of rapamycin (117 nM) and FK506 (9.3 nM). Same test was carried out on properly washed spots (30 minutes (ultrasonic) in DMDSO, DMF, ACN, ethanol, PBS and finally with distilled water) and found stable baseline and dramatic change in produced kinetics of rapamycin (1.19 nM) and FK506 (1.98 nM) showed good compatibility with original values.

Regeneration is also a key step in SPRi experiments to reuse the chip for next cycles and to obtain accurate kinetics. To check the regeneration, FKBP12 and D37V mutant proteins (possessing different binding affinities with rapamycin) were flowed against immobilized rapamycin molecules and regenerated with 10 mM solution of NaOH at different time spans. Due to weak binding affinity, D37V mutant could be easily regenerated at short span of regeneration (600sec) and no difference was recorded when compared to 1000 sec. In reverse, short regeneration cycle was unable to remove the wild type of FKBP12 protein due to strong binding affinity with rapamycin but sucessfully removed by long regeneration step of 1000 sec (Fig. S-4). Although, the long regeneration steps also affects the surface efficiency but very necessary to produce quality data. The obtained data suggested that the same regeneration solution and time span cannot apply to present an ideal methodology, it is very necessary to check the key parameters in each and every aspect. Washing of microarray and removal of bound proteins (regeneration) from the platform had major impact on data quality and microarray efficiency. Missing of any key point could be easily misleading and therefore, presented surface was evaluated very carefully in terms of UV irradiation time, washing and regeneration steps.

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protein was subsequently flowed as analyte through the flow cell at single concentration of 100nM by setting 300sec association and 400sec dissociation phase separated by a single regeneration step. The response signals of biotin-streptavidin interaction (Fig. 4a) from the 3D PCL surface were significantly higher than from 2D PCL. This effect was observed even more clearly for rapamycin and FK506 (Fig. 4b). No signal was observed without UV irradiation (data not shown), suggested that obtained signals were originated from covalently linked small molecules.

**Fig. 4.** Comparison of (a) biotin-streptavidin and (b) FKBP12 binding ligand interactions over 2D PEG and 3D SIP surfaces by SPRi

During the analysis of above described interactions, an average 0.5 to 0.7 times for biotin and 3-4 times for rapa and FK506 of signal enhancement were recorded from 3D SIP over 2D PEG photo-cross-linked surface. A large difference in signal enhancement of streptavidin and FKBP12 protein might be due to the difference in molecular weights of proteins and binding ligands.

**Fig. 5.** Sensorgram showing interactions of six site directed mutants of FKBP12 with (a) rapamycin and (b) FK506

Furthermore, to prove the surface versatility, six site directed mutants (D37V, F48L, W59A, W59L, Y26F and Y26F82F) of FKBP12 protein binds to Rapa and FK506 with wide range of affinities were expressed and selected for testing on the reported surface. Each selected mutant possessing specific and different binding affinity with rapamycin and FK506 compounds. As shown in Fig. 5a and Fig. 5b, the binding behaviour and response signal of all six mutants clearly showing the differentiation in according to their original behaviour. Using FKBP12 mutants with altered mutual binding affinities, we observed a strong correlation (Fig. 7a and Fig. 7b) between the relative binding affinities determined by SPRi and those previously reported by fluorescence titration assay (Table 1). Due to very low binding affinities of some of the mutants with FKBP12 binding ligands, data from 2D PCL slide was not considered for comparison. The above discussed data provided the clear information about the super performance of proposed SMM platform.

**Table 1.** Kinetics parameters of all six mutants against rapamycin and FK506 obtained from SPRi (3D SIP) in comparison with fluorescence titration assay.

<table>
<thead>
<tr>
<th>Proteins</th>
<th>KD (nM) Rapa Fluorescence titration</th>
<th>KD (nM) Rapa SPRi assay</th>
<th>KD (nM) FK506 Fluorescence titration</th>
<th>KD (nM) FK506 SPRi assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>W59A</td>
<td>28±1.6</td>
<td>12±3.32</td>
<td>50±5.3</td>
<td>64±6.0</td>
</tr>
<tr>
<td>W59L</td>
<td>2±1</td>
<td>2.45±3.01</td>
<td>46.0±5.3</td>
<td>30.1±4.75</td>
</tr>
<tr>
<td>Y26F</td>
<td>4.5±2.5</td>
<td>8.32±3.61</td>
<td>22.0±5.6</td>
<td>21.2±8.1</td>
</tr>
<tr>
<td>Y26F82F</td>
<td>20.0±4.5</td>
<td>16.12±4.3</td>
<td>10.0±4.5</td>
<td>15.1±13.15</td>
</tr>
<tr>
<td>F48L</td>
<td>3.0±1.2</td>
<td>9.9±2.62</td>
<td>40±1.5</td>
<td>13.6±2.13</td>
</tr>
<tr>
<td>D37V</td>
<td>36.0±5.8</td>
<td>55.4±7.0</td>
<td>350±59</td>
<td>182.6±6.03</td>
</tr>
</tbody>
</table>

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**Bio-kinetic analysis of small molecules-protein interactions**

For every screening, it is important to determine the weak as well as the strong binding interactions. We checked the 3D PCL surface performance in terms of uniformity and kinetics deviations from different experiments with multiple concentrations of each target protein. Surface reproducibility was also checked by measuring the kinetics of rapamycin and FK506 against FKBP12 protein. Rapamycin and FK506 were spotted (20 spots of each) over whole print area and measured affinities (Table S-2) suggested the high reproducible nature of the surface. It was observed that the dissociation rates from the 3D PCL slides are much slower especially in the case of rapa-FKBP12 and FK506-FKBP12 with KD values much closer to the literature (Table 2). The dissociation constants for FK506 and rapamycin obtained from different methods (literatures) were compared with those obtained from SPRi assay, a strong correlation can be observed between affinities.
obtained from the 3D surface, but not the 2D surface (Table 2). The produced data together suggested that, potential of the reported surface is not only limited to the interaction identification but also can provide accurate kinetics parameters.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Target protein</th>
<th>KD (nM) Literature</th>
<th>KD (nM) 2D (SPRi)</th>
<th>KD (nM) 3D (SPRi)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rapamycin</td>
<td>FKBP12</td>
<td>0.2</td>
<td>17.5±4.2</td>
<td>1.6±1.3</td>
</tr>
<tr>
<td>FK506</td>
<td>FKBP12</td>
<td>0.4</td>
<td>26.6±4.2</td>
<td>1.7±1.24</td>
</tr>
</tbody>
</table>

Table 2. Kinetic parameters of FKBP binding ligands obtained from SPRi (2D and 3D PCL) in comparison with chemical denaturation assay.

For kinase interactions, we choose not to measure kinetics for 2D PCL surface due to very low or negligible signal responses. The kinetic data for all kinase inhibitors obtained by using SPRi was also compared to the Ki/KD/IC50 values reported from the literature in Table 3. The difference between SPRi and literature values might be due to the nature of binding constants compared, as affinity values of all these inhibitors were measured by using different in vitro and in vivo assays. In some cases, this difference is significant and might be due to high density of immobilized molecules and dense structure of the SIP surface.

Detailed kinetic parameters (avg. of 3 conc.) of FKBP12 and kinase interactions were presented in Table S-3, S-4, S-5, S-6 and S-7. Taking all this into consideration, we could infer that the 3D SIP PCL surface in conjunction with SPRi seems very suitable to screen wide range of interactions in high throughput manner.

- **Experimental**
  - **Preparation of 3D polymer brush (SIP) surface**
    - The SIP surface was prepared according to our previous published work.² In brief, a mixed SAM solution was prepared by initiators ω-mercaptopundecyl bromoisobutyrate (Br(CH3)2COO(CH2)11SH) and EG3-thiol in 1:99 ratio. The chips were immersed in this mixture (1mM total concentration) for 16 hours at room temperature, and then thoroughly washed by ethanol and Milli-Q water and dried in a nitrogen stream. Polymerization solution was prepared by 64µg Bipy, 10ml 0.04M CuCl2, 2.6g HEMA, 7.2g OEGMA, 20ml Milli-Q water and 20ml methanol. After 30min deoxygenation, 10ml of AsCA (0.04M) were added to the solution and the chips were immersed in this solution for 16 hours at room temperature under an atmosphere of nitrogen. After being thoroughly washed with methanol and Milli-Q water, the chips were incubated in a DMF solution containing 0.1M DSC and 0.1M DMAP for 16 hours for acetylation.
  - All kinase inhibitors were purchased from TOCRIS Bioscience. FKBP12 plasmid was a kind gift from Prof. Jun O. Liu from John Hopkins university.

- **SMMS preparation**
  - The photo-cross-linker moiety (3-Trifluoromethyl diazirine) was synthesized according to previous reported protocol by Kanoh et al.² PEG and SIP assembled slides were activated by freshly prepared aqueous mixture (1:1) of EDC/NHS solution for 20 minutes. Slides were then incubated with 100mM base added (500mM DIPEA) solution (DMF) of photo-cross-linker (20µl) and covered with cover slips and placed in the dark for 4 hours at room temperature. Slides were then extensively washed with DMF for 30 minutes and blocked with 1M solution of ethanolamine in DMF. After washed with DMF and ethanol (10 minutes) and dried with N2, slides were ready for printing. Stock solutions (10mM) in 100% DMSO were spotted in multiplex using a Genetix QArray 2 spotter (produced 300µm features) and left for complete evaporation of DMSO (under vacuum) at room temperature for 2 hrs. After printing, the slides were exposed to UV irradiation 2.4 J/cm2 (365 nm) in a UV chamber (Amersham life science). The slides were subsequently washed with DMSO, DMF, ACN, ethanol, phosphate buffered saline (PBS) and finally with distilled water for 30 minutes (ultrasonically) respectively, to remove non-covalently bound compounds. Dried slides were assembled with flow cell and then mounted on SPRi instrument for measurement.

- **FTIR, X-ray photoelectron spectroscopy (XPS) and fluorescence method**
  - Surfaces for XPS and fluorescence were fabricated according to above described procedure. Fourier transform infrared (FTIR) spectrophotometer (GX-Perkin Elmer, USA) was used at reflection mode at a resolution of 4 cm⁻¹ over the 4000–400 cm⁻¹ spectral region to reveal the chemical bond and functional groups such as carbonyl (C=O) and hydroxyl (OH) groups belonging to COOH-terminated alkanethiol SAMs. Element sensitivity, specificity, regeneration and washing parameters. The ability to differentiate the specificity between same proteins with altered binding pockets made it more special and superior over all previously reported SMMs. Presented SMMs technology can be used for screening of thousands of small molecules against any protein targets, including cancer-driving mutants identified from cancer genome sequencing. This new methodology will provide an ideal screening platform that is capable of screening small molecule libraries against targets of interest. On the base of above discussed data we strongly believe that presented methodology will start a new revolution in small molecule microarray field.

**Fig. 7.** Graph showing kinetics parameters correlation of FKBP12 mutants against (a) rapamycin and (b) FK506 obtained from 3D SIP surfaces with fluorescence titration assay

**Conclusions**

A high-throughput method for rapid detection of small molecule-protein interaction is a missing link for the exploitation of the drug screening against various protein targets. We present a 3D photo-cross-linking methodology for high-throughput screening of small molecule inhibitors to fill this technology gap. Several well known small molecule-protein interactions including kinase inhibitors were successfully examined. The reported platform was compared with 2D surface in every aspect and significant improvement in immobilization, sensitivity and kinetics parameters was recorded. The platform was carefully validated amid reproducibility, specificity, regeneration and washing parameters. The ability to differentiate the specificity between same proteins with altered binding pockets made it more special and superior over all previously reported SMMs.
samples were injected at the rate of 3µL/s and 25
Binding experiments and data analysis
injections.

PBST was used as both analyte and running buffer. A typical sample injection
SPRI Method
All the experiments were carried out using the PlexArray® HT system
images.18 All samples were injected at the rate of 3µl/s and 25°C. Oval regions of
interests (ROIs) were set as 12 pixels × 9 pixels area in imaging area. ROIs of
biotin were used as controls for measurement of specific signals. Purified

Kinase (500, 1000 and 2000nM) were used to flow onto the microarray to
Three different concentrations of FKBP12 (50, 100 and 200nM) and each
and 400 seconds dissociation phase with running buffer at 3ul/s flow rate.

PBST was used as both analyte and running buffer. A typical sample injection cycle consists of 300 seconds association phase with the analyte solution
and 3D PCL surfaces under the same conditions in DMSO. Printed slides were
washed subsequently with DMSO and ethanol in ultrasonic for 30 minutes to ensure the data repeatability. Data was analyzed according to our
previous work.18

Binding experiments and data analysis
All small molecules were stored as stock solution in 100% dimethyl sulfoxide (DMSO) at -20°C. Protein samples were stored in PBST at -80°C. PBST was used as both analyte and running buffer. A typical sample injection cycle consists of 300 seconds association phase with the analyte solution and 400 seconds dissociation phase with running buffer at 3ul/s flow rate. Three different concentrations of FKBP12 (50, 100 and 200nM) and each kinase (500, 1000 and 2000nM) were used to flow onto the microarray to ensure accurate kinetics. All the experiments were repeated at least three times to ensure the data repeatability. Data was analyzed according to our previous work.18

References
18 V. Singh, A. Nand, Sarita, J. Zhang and J. Zhu, Arab. J. Chem. doi:10.1016/j.arabjc.2015.06.037