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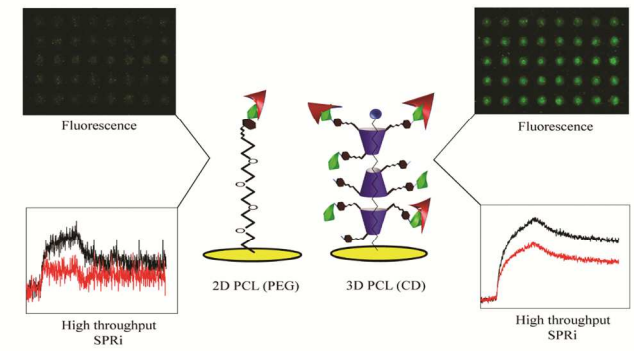

- 3D small molecule microarray based on α-cyclodextrin surface with photo-cross-linking strategy improved sensitivity and immobilization capacity over 2D platform
- Enable to screen deep binding pocket protein targets

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ARTICLE

3D Small molecule microarray with enhanced sensitivity and immobilization capacity monitored by surface plasmon resonance imaging

Cite this: DOI: 10.1039/x0xx00000x

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

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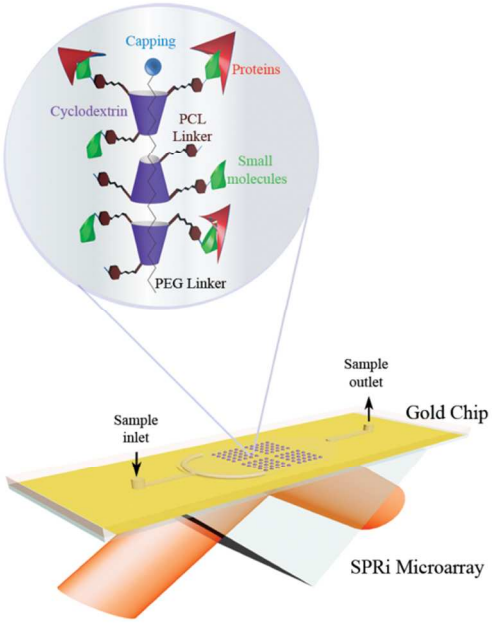
Here we report the 3D photo-cross-linking strategy based on the use of α-cyclodextrin as an alternative surface chemistry for the preparation of small molecule microarray (SMMs). Surface plasmon resonance imaging (SPRi) was used in combination with SMMs as a detection technology. Small molecules of diverse structure containing different functional groups can be covalently captured on a single slide and displayed in various orientations in a given spot. Several known small molecules-protein complexes were selected to validate this platform. Furthermore, a comparison between 2D PEG and 3D α-cyclodextrin photo-cross-linking surface was performed using fluorescence and SPRi techniques. Data produced from both type of surfaces strongly favors the better performance of 3D over 2D surface in terms of loading capacity, spot morphology, signal response, and kinetics values. We believe that this strategy will expand the utility of small molecule microarray for high throughput screening against a variety of target proteins.

Introduction

Small molecule microarrays (SMMs) have shown to be a valuable alternative for high throughput screening against protein targets in drug discovery research[1]. The field of SMMs is an exciting area of microarrays and enables the discovery of important and unexpected protein-ligand interaction that can result in therapeutic utility[2]. Small molecules can be further modified to become more efficacious and selective that can lead to therapeutic candidates. The biochemical evaluation of small molecules is an important first step in drug discovery pipeline[3]. Small molecule microarrays can provide such compounds or leads, yet better protocols are still in need for development in order for this platform to be more widely used in high throughput screenings. Several challenges include the functional immobilization of the diverse and complex structural properties of the compounds to screen and the density of these compounds on the arrays which affects sensitivity.

In the last decade, there has been significant progress in the surface chemistry side for immobilization of chemical compounds since its initiation by MacBeath et al in 1999[4]. Several immobilization strategies including covalent, non-covalent and photo-cross linking capturing onto glass and gold surface have been introduced[5-8]. Mainly 3-aryl-3-triflouromethyldiazarines has been used as photo affinity labeling group to immobilized small molecules by generating a highly reactive species carbene upon exposure to the UV radiation[9]. Previously several researchers including Kanoh et al reported non-selective method for immobilizing small molecules on 2D photo cross linked (PCL) surface (glass and gold)[10-11]. 2D surface was predominantly used for the preparation of SMMs provided satisfactory results for common inhibitors against their specific antibodies. Although, 2D PCL surface can be easily prepared, they have low capacity that results in sensitivity issues. To overcome these limitations, Marsden et al described a 3D hydrogel surface for selective immobilization that provided a higher capacity surface chemistry that enhances sensitivity and reduces non-specific adsorption of proteins[12]. However, due to selective nature of this surface and the use of fluorescently labeled proteins that can alter their functionality, more improvements are needed. Conventional HTS detection method such as TR-FRET, Fluorescence polarization and ALPHAscreen face equivalent challenges due to a number of limitations such as fluorescence interference, protein labeling, small molecule solubility, and lengthy analysis times. Therefore, an alternative label free detection technology can be significantly advantageous. A great advantage of SPRi over classical SPR technique[13] is throughput, allowing the parallel evaluation of hundreds or thousands of compounds simultaneously[14]. Moreover it provides a rapid identification of biomolecular interactions

along with their kinetic parameters in real time[15-17]. A variety of small molecules have been reported on SPRi for measuring protein-ligand interaction and protein-protein inhibition[18]. In this article, a combination of SMMs and SPRi has been used to detect ligand-protein interaction and benchmark them against those reported in the literature. We decided to use the 3D cyclodextrin (CD) PCL that was recently reported for detection of DNA molecules by He et al[19] (Schematic in Figure 1). According to the reported protocol, CDs were threadedinto linear PEG chain followed by capping with bulky functional group. In second step, a well known diazarine group added to CD which has been already proven in construction of small molecule microarray[10]. The attachment of the photo-cross-linker was confirmed by x-ray photoelectron microscopy. The performance of this reported platform was tested by fluorescence and surface plasmon resonance imaging. In this paper, we validate our system by using some well know small molecule-protein interactions. biotin-streptavidin complex, FKBP12 binding ligands, rapamycin and FK506 including rapafucins and some common kinase inhibitors of p38α, JNK1 and ERK2 with wide range of binding affinities were used to prove versatility of the surface in SMMs format.



Results and discussion

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

First of all, XPS experiments were performed to characterize the both (2D and 3D) photo-cross-linking surfaces to confirm the immobilization of photo-cross-linking moiety. The obtained results indicate the presence of high amount of F and N elements on surfaces which proved the presence of photo-cross-linking group (Table S-1). We envisaged that the high capacity of the 3D CD surface causes enhancement in SPR signal for small molecule-protein interactions. To confirm this, a standard experiment was carried out to compare 3D CD with 2D PCL surfaces using fluorescence detection. In order to compare the reactivity and capacity of both surfaces, rhodamine B, a well known fluorescence dye was used as a reference compound[20]. Slides were evaluated in terms of signal intensity and spot morphology. Scanned images of 2D and 3D PCL surfaces by fluorescence spectrometer were shown in Figure 2a. The background corrected Rhodamine B intensity from 2D and 3D PCL surfaces were plotted against each other at 3 different concentrations (Figure 2b). The 3D PCL slides produced on average approx 40% higher intensity over 2D PCL slides. As a negative control, Rhodamine B showed little non-specific adsorption on both surfaces. The 3D PCL slide also produced more consistent spot morphology in terms of both size and shape of spots in comparison of 2D PCL slide. A regular spot morphology is particularly important in the selection of the spots during the experiments and data analysis.

Comparing 2D PEG and 3D CD surface by SPRi

To prepare 2D and 3D structure respectively, Chips with both surfaces (poly-ethylene-glycol and cyclodextrin) were fabricated with the photo-cross-linked group to capture different small molecules. FKBP12 and streptavidin were used subsequently as analytes through the flow cell at single concentration of 100nM by setting 300sec association and 400 sec dissociation phase separated by a single regeneration step. The response signals from the interaction between biotin and streptavidin (Figure 3a) using the 3D PCL surface is significantly higher than from 2D PCL. This effect was observed even more clearly for rapamycin and FK506 (Figure 3b). No signal was observed without UV irradiation (data not shown), suggested that obtained signal was originated from covalently linked small molecules. During analysis of above described interactions, an average 0.5 to 0.7 times for biotin and 3-4 times for rapa-FKBP12 and FK506 of signal enhancement were recorded from 3D CD over 2D PEG photo-cross-linked surface. A large difference in signal enhancement of streptavidin and FKBP12 protein could be due to difference in molecular weight of proteins and binding ligands.

Comparison of rapafucins interactions

In addition to FK506 and rapamycin, we also measured the interaction of six new FKBP-binding macrocyclic compounds called rapafucins (Figure S-2) with FKBP12 protein on 3D CD and compared with 2D PEG surface. The binding affinity of these six rapafucins had been determined by an FKBP-denaturation assay with apparent KD values ranging from 19 to 1,440 nM[21]. Similar to what was seen with FK506 and rapamycin, the binding response of FKBP12 to the five rapafucins immobilized on the 2D surface were significantly weaker (Figure 3c) with the sixth failing to show measurable binding. But the binding response of the six rapafucins on the 3D surface was significant (Figure 3d) show high performance of reported surface.

Identification and comparison of kinase inhibitors interactions

After evaluating FKBP12 binding ligands, rapafucins, rapa and FK506[22] and biotin-streptavidin[23], we aimed to expand the scope of this methodology to including some more complex interactions. We were interested to know whether this technique was applicable for measuring the interactions of proteins containing deep binding pockets. In order to do this, we focused on kinases which represent interesting targets for many therapeutic areas. Kinases have emerged as key component in most signal transduction cascades that researcher are constantly screening for better inhibitors[24]. Until now, only a small number of kinases have been targeted by small molecule inhibitors and there is urgent need to develop strategies for efficient screening of new inhibitors[25]. The majority of these molecules bind to the highly conserved ATP pocket or other specified pockets which are deeper than the previous tested FKBP12 and streptavidin proteins[26, 27]. In this direction, three well characterized protein kinases, p38α, ERK2 and JNK1 were chosen for this study. Four well known inhibitors of p38α (SB 202190, SB 239063, SB 203580 and EO 1428), two ERK2 inhibitors (TCS erk 11e and Kenpaullone), and two JNK1 inhibitors (BI 78D3 and SP 600125) were printed on the sensor chips in multiplex as described in methods[28-35]. All of three relative proteins were flowed in sequence through the flow cell at a single concentration (2uM) on the same chip. The binding signal observed when the p38α inhibitors were tested using the 2D PCL were significantly lower or negligible than when using the 3D CD surface (compare Figure 4a with Figure 4b). This same behavior was also observed with the other kinase-inhibitor combinations (Fig 4c and 4d) from 2D than 3D PCL surface. As shown in Figure 5, all kinase interactions were successfully identified on 3D PCL surface. All 4 p38α inhibitors interacted exclusively with p38α, while the ERK2 inhibitors only interacted with ERK2. The same was observed with the 2 JNK1 inhibitors that specifically interacted with JNK1. The dramatic difference of signal intensities between 2D and 3D PCL surface can be well explained. Very low or negligible signal response was observed from 2D PCL surface for every kinase interaction could be due to low immobilization capacity and deep binding pocket of target proteins. Due to which, molecules linked to PEG chain are not able to get inside into deep binding pockets of kinases. In other hand, very clear signal was obtained for each 3D PCL surface could be due to the free rotary and flexible nature of α-cyclodextrin around PEG axix[36]. Above all, 3D PCL surface inherited the high immobilization capacity from 2D PCL surface but with one great advantage of screening of proteins with deep binding pocket in highthroughput manner. A whole screening chart comparing 2D and 3D PCL surfaces presented in Figure S-3.

Bio-kinetic analysis of 3D PCL surface

For every screening, it is important to determine the weak as well as the stronger binders. We checked the 3D PCL surface performance in terms of uniformitivity and kinetics deviation from different experiments with multiple concentrations of FKBP12 (50, 100 and 200nM) and kinases (500, 1000 and 2000nM). After this, we compare the kinetic values from both 2D and 3D surface against the literature. It observed that the dissociation rates from the 3D slides are much slower especially in the case of rapa-FKBP12 and FK506-FKBP12 with KD values much closer to the literature (Table 1). In case of rapafucins, KD values from 3D and 2D PCL surfaces were compared with those obtained from chemical denaturation assay and a satisfactory correlation was observed from 3D surface while, 2D shows a very weak correlation in addition with high deviations (Table 1). When the dissociation constants for FK506, rapamycin and the rapafucins obtained from different methods, a strong correlation was seen between affinity obtained from the 3D surface, but not the 2D surface in comparison with chemical denaturation assay as shown in Figure 5a-b. These produced data together suggested that, reported surface is suitable for measuring weak interaction of micromolar ranges.

For kinase interactions, we choose not to measure kinetics for 2D PCL surface due to very low or negligible signal response. The kinetic data for all kinase inhibitors obtained by using SPRi was also compared to the Ki/KD/IC50 values reported from the literature (Table 2). Detailed kinetic parameters (avg of 3 conc.) of FKBP12 and kinase ligands are presented in Table S-2, S-3 and S-4. Taking all this data into consideration, we can say that the 3D CD PCL surface linked with SPRi seems very suitable to screen wide range interactions with different affinities in highthroughput manner.

Experimental

Materials: Unless otherwise noted, material and solvents were obtained from commercial suppliers and used without further purification. Gold coated slides (Plexera), SH-(PEG)n-COOH (M.W. 2000) and SH-(PEG)n-OCH3 (M.W. 1000) (Shanghai Yan Yi biotech.). α-cyclodextrin (M.W. 1000), EDC-HCl (1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride) and NHS (N-hydroxy succinimide) (Aladdin Chemistry). succinic anhydride, 2,2 ethylenedioxy-bis (ethylamine), and 4-(dimethylamino) pyridine (DMAP) were purchased from Aldrich. [3-(trifluoromethyl)-3H-diazirin-3-yl] benzoic acid was purchased from J&K chemicals (Beijing, China). N-tert-butoxycarbonyl-2,2'-ethylenedioxy-bis(ethylamine) was prepared according to previous published procedure[37]. All chemical reaction was monitored by thin layer chromatography (TLC). Streptavidin (Santa Cruz Biotech.), DMSO, DMF, ethanol, ethanolamine, ACN and biotin (Aldrich). SB 202190, SB 239063, SB 203580, EO1428, SP 600125, BI 78D3, TCS erk 11e and Kenpaullone purchased from TOCRIS Bioscience. FKBP12 protein was purchased from Sinobiological Inc.

rapamycin, FK506 and six rapafucin compounds were provided by Prof. Jun O. Liu from John Hopkins University. The protein kinases p38α, JNK1 and EKR2 were expressed and purified in Prof. Jia-Wei Wu's lab, Tsinghua University.

α-cyclodextrin surface assembly: After extensive cleaning of gold chips with ethanol for 30 minutes and in plasma for 5

minutes, the chips were immersed in 1mM ethanol solution of carboxy terminated PEG and methoxy terminated PEG used as spacer in 1:10 ratio overnight at 4°C . Upto this step, 2D surface is assembled and used to prepare 2D PCL surface for comparison purpose in Fluorescence and SPRi study. For the preparation of 3D cyclodextrin matrix, PEG assembled slides were treated with aqueous α-cyclodextrin solution for two hours at room temperature. After threading the cyclodextrins to PEG chain, the terminal carboxy group was activated by EDC (0.39M) and NHS (0.2M) mixture followed by capping of chain by Z-Tyr-OH group. Finally, the cyclodextrins hydroxy groups were converted into carboxy by DMF solution of succinic anhydride and DMAP for 16 hours at room temperature under shaking.

SMMs preparation: The photo-cross-linker moiety (3-Trifluoromethyle diazarine) was synthesized according to previous reported protocol by Kanoh et al[10]. PEG and cyclodextrin assembled slides were activated by freshly prepared aqueous mixture of EDC/NHS solution for 20 minutes. Slides were then incubated with 100mM base added (500mM DIPEA) solution (DMF) of photo-cross-linker (20ul) and covered with cover slips and placed in the dark for 4 hours at room temperature17. 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 at room temperature. 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 (PBST) 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.

X-ray photoelectron spectroscopy (XPS) and fluorescence method: Surfaces for XPS and fluorescence were fabricated according to above described fabrication procedure. Element analysis by XPS (ESCALAB 250Xi spectrometer, Thermo Fisher Scientific Co.) with the monochromatic Al Kα X-rays source (1486.6 ev) was carried out to confirm and check the immobilization of photo-cross-linker moiety on surface. The spectrum was acquired at a takeoff angle of 0O with a 0.78mm2 spot size at a pressure of less than 3×10-9 mbar. Further to compare the spot morphology and immobilization capacity by fluorescence method, Different concentrations of rhodamine B (2mM, 6mM and 10mM) were spotted on area fabricated with and without linker as negative control on both 2D and 3D PCL surfaces under the same conditions in DMSO. Printed slides were washed subsequently with DMSO and ethanol in ultrasonic for 30 minutes each to remove physically adsorbed compounds and scanned for fluorescence (GenePix 4000B microarray scanner) at 532 PMT gain.

SPRi Method: All the experiments were carried out using the PlexArray® HT system (Plexera, LLC) which is based on surface plasmon resonance imaging according to our previous work[38]. All samples were injected at the rate of 3μL/s and 25oC. 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 recombinant proteins diluted in PBST containing tween 20 (0.05%), pH 7.4 were used as analytes with an association and dissociation flow rate of 3ul/s at different concentrations by serial dilution. A solution of NaOH (10mM) was used to regenerate the surface and remove bound proteins from the small molecules enabling the sensor chip to be reused for additional analyte injections.

Binding experiments and data analysis: All small molecules were stored as stock solution in 100% dimethyl sulphoxide (DMSO) at -20oC. Protein samples were stored in PBST at -80oC. PBST was used as both binding 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 (Figure S-1). 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. Data was analyzed according to our previous work[38].

Conclusions

A novel platform for small molecule microarray with enhanced sensitivity and immobilization capacity is reported. Small molecule microarray using a photo-cross-linking technology allows covalent capture and random immobilization of the molecules on the surface of the biosensor chips. This immobilization strategy allows the generation of microarray with molecule derived from natural resources, solid-phase synthesis, chemical synthesis and FDA approved compound libraries. Molecules containing multiple reaction sites give the opportunity to display themselves in various orientations in a given spot area. The multiple orientations are greatly beneficial for microarray to expose the same molecule with different sites to a target protein to increase the probability of interaction. However, the photo-cross-linker can also generate a chromophore that can lead to false positives. The SPRi technology allows rapid screening in microarray format and provides real time kinetics gives a significant advantage to this platform. The diverse nature of the libraries is more suitable for screening against various protein targets. Potential hits from this first screening can be validated by using some specified in vitro or in vivo assay and help researchers to select libraries for further screenings. This approach will provide the efficient system for high throughput screening of small molecule against protein of interest.

Acknowledgements

We specially thank Prof. Jun O. Liu for providing FKBP12 binding ligands and Kd's of chemical denaturation assay for rapafucins. We also would like to thank Prof. Jia-Wei Wu for providing high quality kinase proteins for validation of surface and Dr. Alejandro Merino for his scientific advice and editing this manuscript. We gratefully acknowledge the financial

support from 973 Program (2009CB930702), NSFC Grant (61077064/60921001) and Program of International S&T Cooperation (2010DFB33880).

Notes and references

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

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