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ARTICLE

High-throughput measurement of drug-cyclodextrin kinetic rate constants by small molecule microarray using surface plasmon resonance imaging

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Although applications of small molecule microarrays (SMMs) has been extensively studied but limited to the screening of small molecule inhibitors. Here for the first time, we conjugated SMMs with label free surface plasmon resonance imaging (SPRi) for measurement of kinetic parameters for drug-cyclodextrins interactions in high-throughput manner. A collection of insoluble drugs was immobilized onto biosensor surface by using photo-cross-linked technique as SMMs. A highly sensitive and recently reported surface chemistry based on surface initiated polymerization chemistry was used for SMMs fabrication. In total, 38 insoluble drugs were evaluated for their interaction profile and kinetic rate constants against 5 different types of cyclodextrins (CDs) including, α-CD, β-CD, γ-CD, 2-hydroxylpropyl β-CD (HP-β-CD) and sulphobutyl-ether -β-CD (SBE-β-CD). For the supramolecular drug-CD interaction kinetics, the response magnitude and detailed kinetic parameters were calculated and presented in the article. The presented method described a label free and high-throughput technique for real time measurement of kinetic constants for drug-CDs interactions which will assist the selection and use of different CDs in number of different applications.

Introduction

Aqueous solubility is one of the major roadblocks in developing novel small molecules into successful drugs.[1] A number of techniques were developed to increase the apparent solubility of lipophilic compounds without decreasing their optimized potency. These techniques include particle size reduction, pH adjustment, addition of solubilizing excipients, solid dispersion, micro-emulsification, nano crystallization, and inclusion complex formation.[2]

Cyclodextrins (CDs) have been playing a key role in pharmaceutical formulations to increase aqueous solubility and bioavailability of poorly soluble drugs through inclusion complex formation or solid dispersion.[3] The formation of inclusion complex totally depends upon the binding affinity of drug molecules with CDs and therefore, it is very important to study the affinity behaviour and kinetic parameters. It is assumed that the free and bound drugs exist in a state of equilibrium for the CD-drug supramolecular systems in aqueous solution, which is determined by the equilibrium binding constants (KA).

The KA is conventionally used to estimate the binding affinity between drugs and cyclodextrins. The KA values for a range of drugs and small molecules have been reported to be relatively weak in the milli to micromolar range.[4,5] The CDs are able to include wide range of organic and inorganic molecules within the hydrophobic cavity of inner surface through non-covalent interactions, explaining the high affinity towards drug molecules.

The details of CDs inclusion complex have been studied by molecular structural studies, providing information about stoichiometry, geometry, association sites and heterogeneity. Although the kinetic for the association and dissociation process are the base for this supramolecular system formation and *in vivo* functions of drug-CD complex,[6-8] quantitative measurement has proved challenging.[9-11] The short relaxation time (<1 s) and the requirement of high time resolution for is difficult to achieve. [12] To date, few studies have been reported focusing on the kinetic parameters (usually labelled as k_a and k_d for association and dissociation, sometimes as k_{on} and k_{off}) of drug-CD supramolecules.[13] Fluorescence correlation spectroscopy (FCS)[14] has been employed to compare the complexation kinetics of pyronines and analyze the individual steps during association and dissociation. However, this method is not applicable in the kinetic study of most drug molecules without fluorescence. The capillary electrophoresis (CE)[15] has also been employed to estimate the rate constants of drug-CD interactions. The relatively poor reproducibility for CE also limits their application in interactions with weak to moderate affinities. Recently we have reported the novel method based on high performance affinity chromatography (HPAC) for determination of kinetic rate constants.[16] Although the method was able to measure the weak affinities and the results were in agreement with capillary electrophoresis method, the HPAC method was also relatively laborious and time consuming. In HPAC, the modified mono-6A-N-propargylamino-6A-deoxy-CD was used as a stationary phase in silica column, which changed CDs chemically. Therefore, it is of special interest to establish high-throughput methodology to measure the kinetics of drug-CD supramolecular system with extensive, weak binding and fast dissociation.

Previously, two researcher reported the use of SPR system for the measurement of drug-CDs interaction, which described the interactions of β and γ-CD with selected drugs. The modified CD (6-monodeoxy-6-monoamino-γ-CD) was fixed onto gold sensor chip and drugs were flowed as analytes using Biacore SPR system.[17, 18] However, the methodology was not proved very productive due to non-highthroughput nature.

A great advantage of surface plasmon resonance imaging (SPRi) over classical SPR technique is high-throughput,[19] allowing the parallel evaluation of hundreds or thousands of compounds simultaneously.[20] Moreover, it provides a rapid identification of biomolecular interaction along with their kinetic parameters in real time.[21] A variety of bio-interactions have been reported on SPRi for measuring protein–ligand interaction and protein–

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protein inhibition.[22] The performance and efficiency of SPRi are mainly dependent upon the surface chemistry. We recently reported a small molecule microarray screening platform based on high sensitivity and immobilization capacity surface which is able to detect weak interactions of micromolar ranges.[23] The photo-cross-linked technique with surface initiated polymerization (SIP) chemistry proved to be highly efficient for screening of thousands of molecules on single platform.

In this article, a high-throughput SPRi method was established with employment of the same methodology by using SIP platform for measuring kinetic rate constant of drug-CD interactions. The schematic for the drug immobilization is presented in Fig. 1. The small molecule microarray of drugs in conjugation with SPRi has been proved as a breakthrough for providing high throughput screening and accurate kinetic measurements of drug-CD supramolecular systems. A small library of insoluble drugs was fabricated onto SIP surface by using photo-cross-linked chemistry and solutions of 5 different types of CDs (α-CD, β-CD, γ-CD, HP-β-CD and SBE-β-CD) were flowed at multiple concentrations as analytes. The photo-cross linking chemistry allowed non-selective and covalent immobilization of drugs which helped the retention in their activity. The data was evaluated for signal magnitude and kinetic rate constant of each interaction.

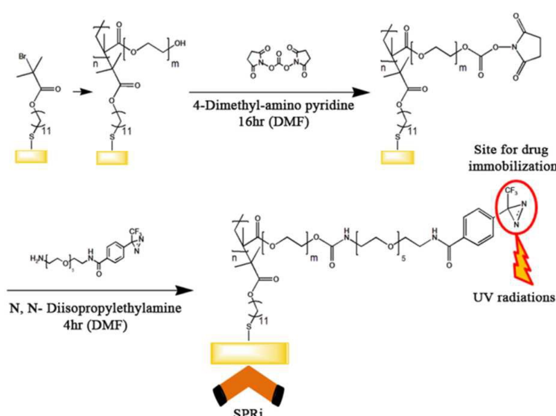

Fig. 1. A schematic representing the fabrication of 3D photo-cross-linking based small molecule microarray for the measurement of drug-CD interactions.

Experimental

Preparation of 3D polymer brush (SIP) surface:

The SIP surface was prepared according to our previous published work.[18] In brief, a mixed self-assembled (SAM) solution was prepared by initiators ω-mercaptoundecyl bromoisobutyrate (BrC(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 64mg 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 chip were incubated in a DMF solution containing 0.1M N,N'-Disuccinimidyl carbonate (DSC) and 0.1M Dimethyl amino pyridine (DMAP) for 16 hour for acidification.[24]

SMMs preparation

The photo-cross-linker moiety (3-Trifluoromethyle diazarine) was synthesized according to previous reported protocol by Kanoh et al.[25] 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 (20ul) 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 vaccum) 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 (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.

SPRi Method

All the experiments were carried out using the PlexArray® HT system (Plexera, LLC) which is based on surface plasmon resonance imaging.[23] All samples were injected at the rate of 2µL/s and 25oC. Oval regions of interests (ROIs) were set as 9 pixels × 7 pixels area in imaging area. ROIs of bovine serum albumin (BSA) were used as controls for measurement of specific signals. CDs solutions in PBST containing tween 20 (0.05%), pH 7.4 were used as analytes with an association and dissociation flow rate of 2ul/s at different concentrations by serial dilution. A solution of glycin·HCl (pH 4.2) 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 -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 2ul/s flow rate. Three different concentrations of each cyclodextrin (0.25, 0.5 and 1mM) 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.[23] For data analysis we choose two software packages, ORIGIN Lab and Data Analysis Module (DAM) of Plexera. All data from SPRi reported here are after subtraction of the background intensity/signal by DAM software. In short, the entire concentration of analyte was fitted with a 1:1 Langmuir interaction integrated rate equation to obtain the kinetic constants.

Results and Discussion

Identification and signal response

Small molecule microarrays of 38 insoluble drugs were constructed onto 3D SIP platform by using photo-cross-linked technique. All the solutions of 5 different types of CDs were flowed through flow cell separated by single regeneration step at multiple concentrations on single chip. A slightly acidic solution of Glycine·HCL (pH 4.2) was used as a regeneration solution to remove bound and physically adsorbed CDs from microarray region to flow next analyte cycle. Bovine serum albumin (BSA) of 1mM concentration was printed along with drugs and used as a negative control in experiments.

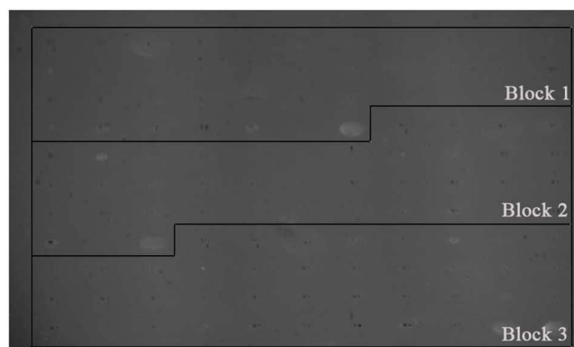

Fig. 2. Microarray image showing triplicate printing of each drug. The image was collected (14×14mm) after washing process and therefore the drug spots are almost invisible.

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The Figure 2 showing the microarray image directly captured from the SPRi instrument. all drug compounds were spotted in triplicate to ensure the uniformity of the measurement which are highlighted by Block 1, 2 and 3 with last spot of negative controls (BSA) in each block. The final signal responses showed in Figure 3 were obtained after subtraction of signal from BSA to get specific binding response. Response graphs plotted and shown in Figure 3 were obtained from single highest concentration (1mM) of CDs. The standard deviation was calculated from the triplicate spot of all drugs at single concentration of each CD which suggested that the surface was very uniform and suitable for the measurement of interactions on single platform. Each drug showed different response to different CDs especially parent CDs (α-CD, β-CD and γ-CD). It is very important to note that the β-CD and its two derivative forms, HP-β-CD and SBE-β-CD showed very similar trends of signal responses to large portion of the drugs. This is possibly due to the same inner cavity and the change in functional groups affects the interaction profile to little extent. Two drugs, Silymarin and Maloxicam showed no or negligible interaction to β-CD and HP-β-CD but in case of SBE-β-CD the interactions were detectable which could justify the effect of modified functional groups. Very recently, a dramatic improvement in the solubility of a natural active compound, namely, pseudolaric acid B (PAB), has been found with a 600 fold increase by HP-β-CD complexation. In addition, the solubility enhancement of PAB by HP-β-CD and SBE-β-CD were recorded significantly higher than β-CD by phase solubility studies. Interestingly, the signal response of PAB was also increased in case of HP-β-CD and SBE-β-CD when compared to β-CD and was in strong agreement with phase solubility data. In last, apart from the kinetic parameters, the response charts are quite useful to explain the binding profiles of each drug to different CDs.

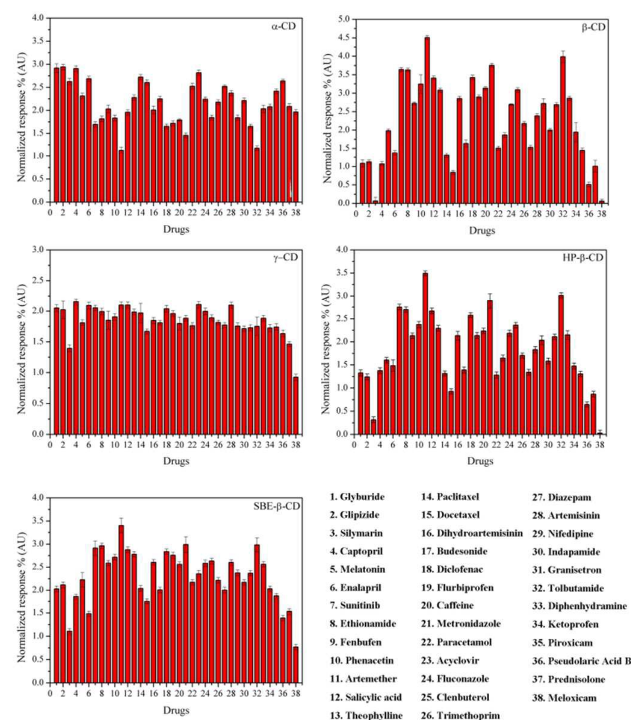

Fig. 3. A whole chart showing SPRi signal response for each drug against 5 different types of CDs (α-CD, β-CD and γ-CD, HP- β-CD and SBE-β-CD) with drug list. A standard deviation was calculated from triplicate spots of each drug from single slide.

Affinity profile based on signal response

As discussed above, the signal response generated from the drug-CD interactions provided the affinity information and varied with the types of CDs. However, if we look over the SPRi response graphs, it is hard to interpret the response of each interaction and the condition will be more worst with increase in the number of drugs. Therefore, it is very necessary to plot the response values in one easy readable graph and heat map or cluster analysis is the best way to represent these profiles. The signal responses from single highest concentration were averaged using statistics analysis and heat map (Figure 4) was generated using MAT LAB software. The heat map provides the range of affinity of all drugs towards each type of CD after automatic calculations and arranged the signal response in classified manner. Interestingly, the affinity profiles of β-CD, HP- β-CD and SBE-β-CD appeared in one class which is totally justified due to the presence of similar hydrophobic cavities and the contribution of additional functional groups to the affinities were also measured. The heat map is of quite importance and helps to interpret the data as it's difficult to compare the affinity response at a single platform in case of high-throughput measurement. In simple words, the affinity of interaction can be easily estimated from the signal magnitude and classification from the heat map.

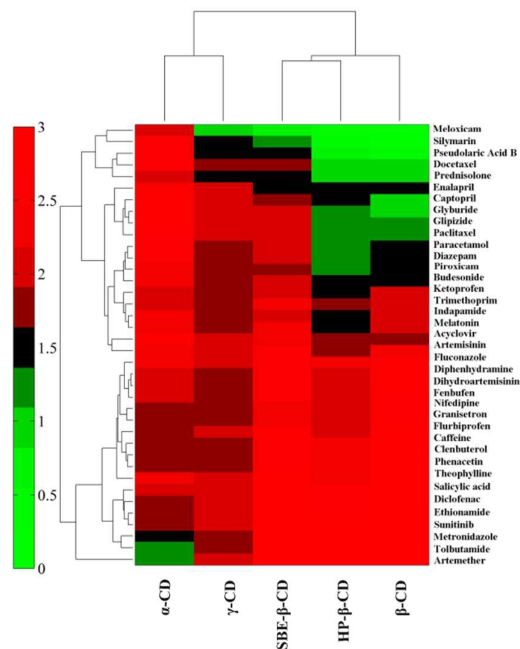

Fig. 4. Classification of drug-CD interaction responses by unsupervised cluster analysis. Small molecule microarray data of 38 drugs against 5 different types of CDs (α-CD, β-CD and γ-CD, HP- β-CD and SBE-β-CD) were mean normalized, log transformed, and analyzed by MAT LAB.

Kinetic analysis from SPRi

Apart from the signal response, our main motif of this study was to provide the accurate kinetic parameters for drug-CD interaction as it is quite important to evaluate the strength and behaviour of the interactions. For the same purpose, all 5 types of CDs were flowed at multiple concentrations (0.25, 0.5 and 1mM) over small molecule microarray on single slide. The average value of kinetics was calculated by using Plexera SPRi data module software.[23] The k_a of all drugs against each type of CDs were presented in Table 1. Detailed kinetic parameters including association rate constants (k_a), dissociation rate constants (k_d) and equilibrium association constant (KA) were calculated and presented in supplementary Table S-1 and Table S-2. If we roughly look on the kinetic data from SPRi, a general trend can be easily seen in kinetic values. With the fact we know that non-specific adsorption is a very common problem in SPRi based measurement which affects the kinetic parameters. The SIP has been proved most efficient in comparison to the other 3D surface chemistries to reduce non-specific bindings in recently published report.[26] A 1M solution of ethanolamine was used for surface blocking which is previously evaluated and found to be very efficient to reduce non-specific adsorption.[23] To minimize this problem, we have used advanced and newly developed surface chemistry with more efficient blocking agents. After this all, measurable signals were observed from non-specific adsorptions which were subtracted from desired spots to get specific interaction sensograms and kinetic calculations. Above all, it could

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be inferred that SPRi was able to measure the drug-CDs interactions and provide kinetic parameters in high-throughput manner.

Comparison with other methods

Due to remarkable importance, a number of different methods were reported for the measurement of kinetic rate constants for drug-CD interactions including, fluorescence correlation spectroscopy (FCS), capillary electrophoresis (CE), phase solubility, phase distribution, HPAC and SPR method. However, the fluorescence limitations, large sample amount, time consuming and laborious procedure limits their application for drug-CD interaction measurement. In comparison, with the superiority of rapid and real time kinetics measurement in high throughput manner, the SPRi method could be the best method for measurement of multiple drug-CD interactions. Very recently, we have published several articles focused on the SPRi applications including, biomarker identification, in situ protein synthesis and SMMs for screening of small molecules inhibitors with very advanced surface chemistries.[27-30] It is concluded that SPRi was able to generate significant signals for weak interactions from high sensitive surface chemistries. In general, a notable difference can be always found when the kinetic values from SPRi were compared with other reported methods. But this difference or the non-correlation between different methods can be explained by difference in methodology used. For example, in SPRi, the drug molecules were immobilized over sensor surface and CDs were used as a mobile phase. While, the conditions were totally reversed in HPAC and SPR, where the drugs were used as a mobile phase and CDs were modified and fixed onto the surface. Even they (SPR and HPAC) shared the same strategy, a significant deviation in the magnitude of kinetics parameters were observed, which might be due the different environment and instrument itself. However in this report, photo-cross-linking technique was used to immobilize the drug molecules which allowed one molecule to display in various orientations. And each orientation may have different binding affinity to the specific CD and therefore, the kinetic constant we have obtained from our method is probably the average of different multiple orientations. The other more important factor which is difficult to ignore even after so much cure is the non-specific bindings which is presented in all systems but quite different and reverse to the each other and might be contributed in kinetic parameters.

Table 1. The calculated association rate constants (k_a) obtained from SPRi.

Drugs	α-CD	β-CD	γ-CD	HP-β-CD	SBE-β-CD
Glyburide	1790	1590	1800	6530	943
Glipizide	1860	601	4460	7170	4070
Silymarin	4990	--	73.6	6540	2660
Captopril	360	2880	2840	9210	2950
Melatonin	5660	6990	512	7370	2030
Enalapril	1650	78700	6560	5180	1580
Sunitinib	2660	1150	4200	1900	4620
Ethionamide	185	6000	615	7930	308
Fenbufen	15.2	39	588	1330	2480
Phenacetin	758	1730	4500	1760	2140
Artemether	1950	1020	1770	4000	2350
Salicylic acid	1620	2060	1010	5750	2340
Theophylline	57.5	3640	4680	8020	1790
Paclitaxel	1910	1000	734	2370	1990
Doctaxel	7840	1000	5900	6800	5160
Dihydroartemisinin	4020	4600	3290	2460	1630
Budesonide	2660	6270	200	604	2040
Diclofenac	1750	3540	620	143	397
Flurbiprofen	3140	2270	1680	2690	1780
Caffeine	1900	4590	1480	4510	2360
Metronidazole	1900	8.54	658	543	1090
Paracetamol	3990	6730	6090	2180	668
Acyclovir	619	86900	2020	8870	2250
Fluconazole	4530	55800	2800	1390	6530
Clenbuterol	1880	3190	3400	5720	1660
Trimethoprim	1540	969	518	2210	886
Diazepam	398	1480	1450	361	1580
Artemisinin	9990	6610	2050	535	1590
Nifedipine	1170	4420	1230	88.8	162
Indapamide	6920	6070	2650	1130	1270
Granisetron	1750	2660	1670	2120	807
Tolbutamide	1000	3140	137	1980	4220
Diphenhydramine	5570	6260	3780	1230	3430
ketoprofen	1840	1710	1500	4650	6730
Piroxicam	6470	8760	3830	1780	2330
Psudolaric acid B	2950	1800	5160	1930	380
Prednisolone	881	1660	3060	2010	1640
Meloxicam	3520	--	1940	913	1520

Conclusions

The novel high-throughput method to measure kinetic rate constants of drug-CD interactions based on the SPRi was presented and thoroughly discussed here. Apart from the all, thousands of drugs can be easily fabricated into small microarray format for kinetic measurement against desired types of cyclodextrins. In addition with kinetic values, the strength of interaction can be directly evaluated from the signal magnitude for a huge drug library and affinity profile could be designed to arrange in affinity based order. This affinity based profile can be very helpful for selection of cyclodextrin as solubility enhancer and excipient in drug delivery purpose.

Acknowledgements

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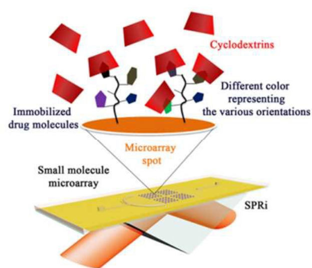

A high-throughput methodology for measurement of drug-CD kinetic rate constants

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