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A Method for Identifying Small Molecule Aggregators Using Photonic Crystal Biosensor Microplates

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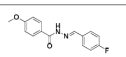
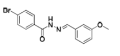
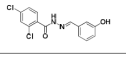
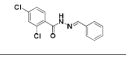
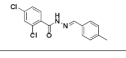
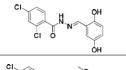
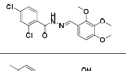
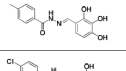
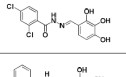
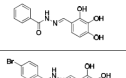
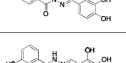
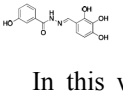
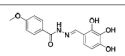
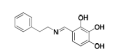
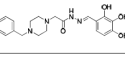
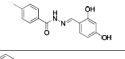
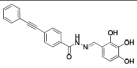
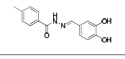
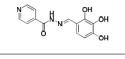
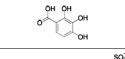
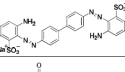
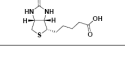
***Abstract* – Small molecules identified through high-throughput screens are an essential element in pharmaceutical discovery programs. It is now recognized that a substantial fraction of small molecules exhibit aggregating behavior leading to false positive results in many screening assays, typically due to nonspecific attachment to target proteins. Therefore, the ability to efficiently identify compounds within a screening library that aggregate can streamline the screening process by eliminating unsuitable molecules from further consideration. In this work we show that photonic crystal (PC) optical biosensor microplate technology can be utilized to identify and quantify small molecule aggregation. A group of aggregators and nonaggregators were tested using the PC technology, and measurements were compared with those gathered by three alternative methods: dynamic light scattering (DLS), an α-chymotrypsin colorimetric assay, and scanning electron microscopy (SEM). The PC biosensor measurements of aggregation were confirmed by visual observation using SEM, and were in general agreement with the α-chymotrypsin assay. As a label-free detection method, the PC biosensor aggregation assay is simple to implement and provides a quantitative direct measurement of the mass density of material adsorbed to the transducer surface, while the microplate-based sensor format enables compatibility with high-throughput automated liquid handling methods used in pharmaceutical screening.**

I. INTRODUCTION

Pharmaceutical drug discovery programs employ a wide variety of high-throughput screening (HTS) methods to identify lead compounds for further development [1]. However, some compounds within small molecule libraries can form multimeric aggregates, and such aggregates are known to result in nonspecific interactions with many proteins [1], leading to unreliable outputs from several types of screening assays [2, 4]. Compounds that can form large aggregates and inhibit the interactions with the target protein are often referred to as "promiscuous inhibitors" [1, 3] due to their ability to alter the function of many different proteins in a nonspecific manner. Previous studies have shown that high percentages (21-36%) of small molecule library members can form aggregates at screening concentrations, thereby overwhelming valid hits from the screen and drastically affecting the hit rate from a HTS assay [3]. Therefore, HTS methods can be improved if aggregating compounds in a given library can be identified and eliminated before screening is performed [1, 2].

TABLE I
SMALL MOLECULE LIBRARY

Chemical Structure	Name & MW
	**1** 272.3 g/mol
	**2** 333.2 g/mol
	**3** 309.1 g/mol
	**4** 293.1 g/mol
	**5** 307.2 g/mol
	**6** 325.1 g/mol
	**7** 383.2 g/mol
	**8** 286.3 g/mol
	**9** 341.1 g/mol
	**10** 271.2 g/mol
	**11** 352.2 g/mol
	**12** 288.3 g/mol

Chemical Structure	Name & MW
	**13** 302.3 g/mol
	**14** 257.3 g/mol
	**15** 372.4 g/mol
	**16** 271.3 g/mol
	**17** 372.4 g/mol
	**18** 270.3 g/mol
	**19** 273.2 g/mol
	**20** 170.1 g/mol
	**CR** 696.7 g/mol
	**Biotin** 242.3 g/mol

In this work, we demonstrate the use of photonic crystal (PC) biosensor microplates as a label-free detection method for quantifying small molecule aggregation in a high-throughput fashion. PC biosensors have been demonstrated as a highly sensitive method for performing a wide variety of biochemical and cell-based assays [8].

Two types of detection instruments are used in this study; a kinetic detection instrument (Figure 1) and an imaging instrument. Due to page limitations, we recommend [8] for more information on the PC device and detection instruments.

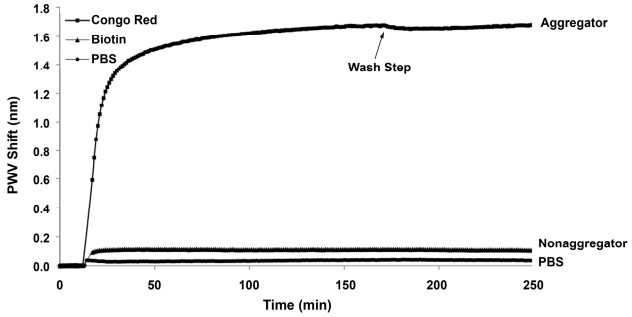


Fig. 1. Kinetic plot of PC biosensor PWV shift as a function of time for a typical aggregating compound (Congo Red) and a nonaggregating compound (biotin). Phosphate-buffered saline (PBS) was run as a negative control.

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To study aggregation detection using a PC device, we selected a group of 22 compounds including known aggregators, known nonaggregators, and previously uncharacterized compounds that were suspected of aggregation. The results of comparison experiments between PC biosensor aggregation measurements (collected in a 384-well microplate format) and measurements obtained by dynamic light scattering (DLS), enzyme-based inhibition assays, and physical observation using scanning electron microscopy (SEM) are reported herein.

II. RESULTS

A. Experimental Approach

To demonstrate the validity of our aggregation detection assay, we screened the compound library (Table 1) with a number of detection methods currently used to detect promiscuous inhibitors and molecules that exhibit aggregating behavior. The positive control, Congo Red (CR), has consistently been shown to be prone to aggregation. DLS was used to measure the ability of each molecule to aggregate as a result of the scattering intensity of solutions of each molecule [6, 7]. Nonspecific inhibition of enzyme-based reactions is a hallmark of promiscuous inhibitors, and so an α-chymotryspin-based enzyme inhibition assay was also used across the small molecule library [3]. The PC biosensor assay was then used in both high-throughput kinetic and imaging modes to detect aggregation on the PC-biosensor surface. To further confirm the presence of small molecule aggregates, the PC device was visually inspected by SEM.

B. Estimated Diameter Using DLS

The compounds evaluated in this study are listed in Table 1. Library compounds 1-20 are previously uncharacterized as aggregators, while negative controls (DMSO, biotin, buffer) and the positive control (CR) were also utilized. The small molecules showed diameters greater than 100 nm and large standard deviations (for N=3 independent measurements) in the DLS measurements. The scattering intensity ranged from 10 to 500 Hz for compounds tested. Increased scattering intensity correlates with increased size of the particles formed in solution and, therefore, aggregation. While the 100nm bead positive control gave results consistent for 100nm diameter particles, the results for DMSO only and biotin (nonaggregator control) were within the same range (data not shown). This provides evidence that DLS may be limited in the detection of the types of aggregates formed by drug-like compounds.

C. α-Chymotrypsin Assay Analysis

Inhibition of α-chymotrypsin was quantified by the slope of the data generated from the increase in absorbance at a wavelength of 405 nm over time when succinyl-AAPF-PNA is cleaved by α-chymotrypsin. The linear portion of the graph (the first 15 minutes) was used for slope calculation and comparison to DMSO and other compounds. Depicted in Figure 2 are the highest concentrations (250 μM) of compounds 1-20 and CR that were utilized, and their inhibitory/activating properties in this assay.

All percent activities were normalized to the slope of the line generated from DMSO treated α-chymotrypsin + substrate. Note that several apparent increases in activity occur with compounds previously described as promiscuous inhibitors (CR). We believe this discrepancy to be attributed to the fact that the colored nature of the compounds may skew results obtained by the spectrophotometer.

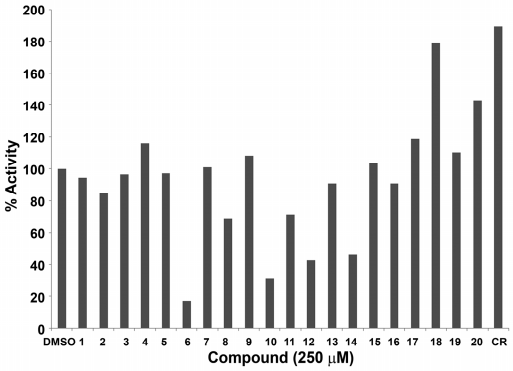

Fig. 2. The percent activity (normalized to DMSO control) of α-chymotrypsin in the presence of test compounds at 250 μM. Data is representative of three independent experiments.

D. PC Aggregation Detection and Imaging

The PWV shifts recorded for each of the 22 compounds are shown in Figure 3. The PC biosensor recorded an increase in the PWV for several of the compounds. Although the sensor surface was washed rigorously with buffer 3 times the wavelength shift signal remained. We interpret these results as nonspecific attachment of material to the sensor surface as a result of compound aggregation.

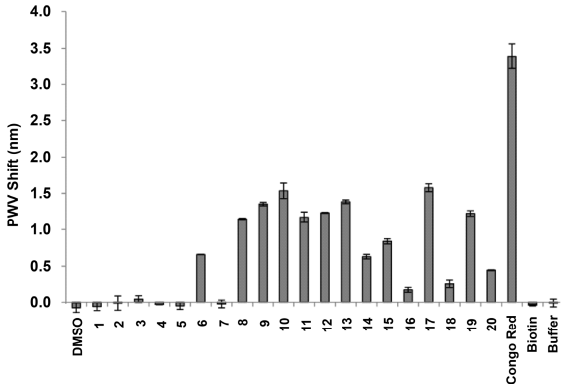

Fig. 3. The PWV shifts recorded (using the fiber probe detection instrument) for a PC biosensor coated with streptavidin and then treated with the indicated compound.

Compounds 1-20 provided a trial set for the ability of PC biosensors to detect aggregators. As this assay implicated several of these small molecules as aggregators, a subset were analyzed further both with the PC biosensor method and SEM. Specifically, based on the PC biosensor data in Figure 3 compounds 1 and 2 were selected as non-aggregators, and compounds 8 and 19 were selected as aggregators. Sensor surfaces treated with these four compounds were scanned using the PC imaging instrument, and the results are displayed in Figure 4A.

The PWV shift image shows a large PWV shift for the two putative aggregating small molecules (8, 19) while the two reference compounds (1, 2) and the vehicle control showed no noticeable binding signal. The PWV shifts recorded by the imaging detection instrument are consistent with those measured using the optical fiber probe detection instrument. The imaging detection method shows that aggregation for compounds 8 and 19 appears to occur uniformly across the biosensor surface at the bottom of the well, and not in sparsely isolated clusters.

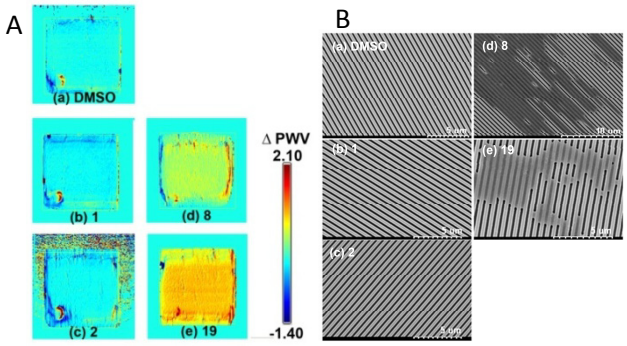

Fig. 4. A: PWV shift images gathered with the PC imaging detection instrument, demonstrating uniformly high levels of aggregation distributed across the biosensor surface for aggregators ((d) 8, (e) 19) and lack of PWV shift for two non-aggregator compounds ((b) 1, (c) 2) and (a) DMSO control. B: SEM images of (a) DMSO, compound (b) 1, (c) 2, (d) 8, and (e) 19. The two aggregators (8, 19) showed gel-like substance attached on the sensor surface, while such substance could not be located for the non-aggregator and DMSO control.

E. SEM Aggregation Confirmation

The same four small molecules from the PC imaging experiment were examined using SEM. Experiments using sensor surfaces treated with the vehicle (DMSO) and compounds 1 and 2 resulted in a "clean" grating surface when examined by SEM, in which no particulates or other deposits could be observed (Figure 6a, b, c). In contrast, the sensor surface of compounds 8 and 9 (that had registered a positive signal in the PC aggregation assay) have a gel-like substance attached on the surface as visualized by SEM (Figure 6d, e). The material is observed to attach to the sensor in irregularly-shaped clusters that fill in the grating grooves and extend for several grating periods (a single grating period is 550 nm). Although isolated clusters are shown in Figure 6, clusters could be found distributed uniformly across the sensor region as suggested by the PC imaging measurements shown in Figure 5. The material has the appearance of a thick film with undefined shape and in no case did we observe spherical particles or particle-like precipitates.

III. DISCUSSION

The goal of the work presented in this paper was to determine whether PC biosensor assays may be used as a direct means for detecting aggregation of small molecules. In liquid media exposed to the PC biosensors, compounds that aggregate appear to result in deposition of material upon the sensor surface; this manifests itself as a large increase in the PWV, making it easy to identify such nuisance aggregating compounds.

Of the 22 compounds tested, the PC biosensor assay measured no aggregation for several compounds (1, 2, 3, 4, 5, 6, 7, 14, 16, 17, 18) in addition to three negative controls (DMSO, biotin, and buffer). Several compounds resulted in measured aggregation with the PC assay (8, 9, 10, 11, 12, 13, 15, 19, 20), in addition to the positive control (CR) (Figure 4). The material deposited upon the sensor surface attributed to aggregation remained even after rigorous washes with buffer, suggesting that the aggregation was not a loose precipitate and that the measured signals were not caused by effects such as bulk refractive index of the small molecule buffer.

Although DLS is often used to measure the size of dispersed particles in solution, the method was not useful for characterizing the aggregations of the compounds in our panel. Multiple readings showed disparities among the results for each small molecule. Particle diameter measurements of all 22 samples were obtained (including the negative controls), but with large standard deviations and particle size readings of ~100 nm particle diameter for the negative controls (DMSO, biotin, and buffer) severely limited the

utility of the data obtained. DLS measurements of scattering intensity can be used as a means for estimating particle diameter based upon Mie-theory calculations that assume uniform spherical particles [5, 7]. However, if the particles do not fit this model, the results are inconsistent, as shown by our results. We note that most of the compounds that register high scattering intensity were also aggregators identified by the PC biosensor. The DLS measurements could not be performed in a high throughput fashion, as the detection instrument could only measure one sample at a time with each measurement taking 30-60 min.

While DLS is widely used to characterize particle aggregation, enzyme-based assays are a common HTS for promiscuous inhibitors. The α-chymotrypsin-based enzyme inhibition assay uses a colorimetric reaction to measure the reaction rate for each compound as a function of concentration, requiring a concentration series for each molecule under study and a calibration standard for comparison. Several compounds were identified as promiscuous inhibitors identified using this method (6, 10, 12, 14). These results are mostly consistent with those obtained with the PC biosensor detection method. Colored compounds and those subject to precipitation, including several of the small molecules evaluated here, can affect absorbance measurements as a result of physical characteristics unrelated to their propensity for aggregation. As a result, enzymatic inhibition assays can identify potential promiscuous inhibitors that inhibit the particular enzyme-substrate interaction used, but they remain incapable of identifying all the aggregators because not all aggregating compounds are promiscuous inhibitors[6]. Therefore, this detection method presents several challenges to accurately identifying possible aggregators within a small molecule library that limit reliability and throughput.

Because DLS and the α-chymotrypsin colorimetric methods were inconsistent in confirming aggregation of the compounds in the panel, physical inspection was required using SEM to examine the PC biosensor surface. Two aggregators (8, 19), two non-aggregators (1, 2), and one reference sample (DMSO) were examined under SEM. Surprisingly, islands of thick films were found on the surface of the PC sensors exposed to the potential aggregators (Figure 4B) and it is likely that these deposits caused the large measured PWV shifts. The deposits were absent from sensors exposed to non-aggregators, as well as from the sensors exposed to DMSO only. The same samples were scanned using the PC imaging instrument, showing that the deposits are uniformly present over the entire sensor surface area and that the deposits cause a large positive in shift in PWV (Figure 4A).

IV. CONCLUSION

We describe a method for using PC optical biosensors in a 384-well microplate format as a means for identification and quantitative characterization of small molecule aggregation effects. The sensor measures the optical density of material deposited upon its surface, and therefore directly measures aggregating material that forms on the sensor surface from the liquid media within each well of the biosensor microplate. A small panel of chemical compounds, negative controls, and positive controls were characterized by the PC biosensor method, DLS, a chymotrypsin enzyme assay, and direct visual observation with an electron microscope. SEM observation showed that aggregation deposits on the sensor were found to form clusters of dense material with irregular shapes that are not easily fit with standard spherical particle models used in DLS, resulting in large fit errors and standard deviations obtained by that method. The aggregates were found to persistently attach uniformly to the entire sensor surface area and were not removable by vigorous washing. Aggregation detection with the PC biosensor assay agreed with measurements gathered by the chymotrypsin assay, but the PC biosensor method proved to be more amenable to higher measurement throughput and a simpler procedure.

V. ACKNOWLEDGMENT

We are grateful to the National Institutes of Health (R01 CA118562) for funding this work. The authors thankfully acknowledge SRU Biosystems for providing the photonic crystal biosensor microplates. B.T.C. is a founder of SRU Biosystems. Co-authors L. L. Chan and E. A. Lidstone contributed equally to this work.

VI. REFERENCES

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