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

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## Keywords:

high-throughput screening, small molecule, drug like, drug screening, aggregation, aggregator, photonic crystal, biosensor

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 used 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  $\alpha$ -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  $\alpha$ -chymotrypsin assay. DLS measurements, in contrast, demonstrated inconsistent readings for many compounds that are found to form aggregates in shapes, very different from the classical spherical particles assumed in DLS modeling. 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, whereas the microplate-based sensor format enables compatibility with high-throughput automated liquid-handling methods used in pharmaceutical screening. (JALA 2009;14:348–59)

## INTRODUCTION

Pharmaceutical drug discovery programs use a wide variety of high-throughput screening (HTS) methods to identify lead compounds for further development.<sup>1–6</sup> However, some compounds within small molecule libraries can form multimeric aggregates, and such aggregates are known to result in nonspecific interactions with many proteins,<sup>1,7–12</sup> leading to unreliable outputs from several types of screening assays.<sup>7,13</sup> Compounds that can form large aggregates and inhibit the interactions with the target protein are

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often referred to as “promiscuous inhibitors”<sup>1,11,12</sup> due to their ability to alter the function of many different proteins in a non-specific manner. In screens that measure inhibitory activity, such compounds are a primary source of false positive hits that must subsequently be identified by lower throughput secondary screening methods; recent studies have also shown that in some cases aggregation can lead to nonspecific enzymatic activation.<sup>14</sup> 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.<sup>3</sup> Therefore, HTS methods can be improved if aggregating compounds in a given library can be identified, characterized, and eliminated before screening is performed.<sup>1–3,7,11</sup> Although recent studies have shown that addition of detergent to assay buffers can minimize the aggregating effects of certain small molecules, high-detergent concentration can also have deleterious effects upon the biomolecular interactions being studied, with such effects varying from compound to compound.<sup>1–3</sup> Additionally, detergent adds several layers of complexity to the investigation of such interactions, as the sequestration of small molecule aggregates within detergent micelles is subject to increasingly elusive and more complex kinetics than is the specific activity of the small molecules.

There are currently several detection methods used to quantitatively measure small molecule aggregation. One of the most common methods, dynamic light scattering (DLS), is used to quantify the size of small-molecule aggregates by measuring the time-dependent fluctuation of scattering intensity of a coherent light source illuminating particles suspended in solution.<sup>1,3,7</sup> The light scattered from two or more particles constructively or destructively interferes at the detector, and by calculating the autocorrelation function of the light intensity and assuming a particle distribution, it is possible to determine particle size. DLS is a low throughput and time-intensive detection technique that can produce measurements that are difficult to interpret when evaluating insoluble (precipitating) compounds that typically give large fit errors.<sup>1–3,11,15,16</sup> The DLS fit errors originate from the DLS particle-sizing model, which assumes scattering from uniformly sized spherical particles, whereas aggregates can, in fact, form in shapes including irregular nonuniform clumps, thin sheets, and fibrous tendrils.<sup>1,3,15,16</sup> Other common methods for identification of aggregating compounds include enzyme-based inhibition assays involving AmpC,  $\beta$ -lactamase, or chymotrypsin.<sup>1,11</sup> These assays measure the absorbance of enzymatic reaction products that produce a detectable colorimetric change in solution. When aggregation and nonspecific enzyme inhibition occur, the enzymatic reaction rate is altered and a change in reaction kinetics can be observed. Furthermore, the colorimetric readout is a direct readout of enzymatic activity, but small molecules with significant absorbance in the range of the enzymatic product can affect the absorbance output signal without respect for their potential for aggregation. Direct detection of small-

molecule aggregation on an optical biosensor surface has been demonstrated using surface plasmon resonance (SPR), in which the kinetic reaction rates of target protein interaction with small molecules are observed and used to differentiate between compound/target affinity binding and compound aggregation.<sup>7</sup> SPR is a more reliable method than either DLS or enzymatic inhibition because it directly quantifies the affinities of the molecules observed, but is typically implemented in a serial flow-cell detection format. In addition to severely limiting throughput, the serial flow-cell format is subject to the accumulation of larger aggregating molecules. Sufficient removal is often impossible, necessitating the costly replacement of the chip to achieve true reproducibility.<sup>7,17–19</sup>

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, with a mass density sensitivity resolution less than 0.1 pg/mm<sup>2</sup> and a large dynamic range.<sup>20,21</sup> As described in previous publications, the PC biosensor comprises of a subwavelength periodic surface structure that resonantly reflects a narrow band of wavelengths when illuminated with a broadband collimated light source.<sup>19–21</sup> The wavelength reflected from the PC surface is modulated by changes in the refractive index of material within an evanescent field region that extends  $\sim$ 300–500 nm from the PC surface into the adjacent liquid media. The PC sensor is fabricated upon flexible plastic substrates using a nanoreplica molding process, and incorporated into the bottom surface of standard 96-, 384-, and 1536-well microplates. Adsorption of biomaterial on the PC surface is monitored by a detection instrument (BIND Reader; SRU Biosystems, Woburn MA, USA) that illuminates the PC microplate from below with a broadband light source, and uses a spectrometer to measure changes in the peak wavelength value (PWV) of the resonantly reflected light. Two types of detection instruments for PC biosensors are used in this work. First, an optical fiber-based system illuminates and collects light from a  $\sim$ 2-mm diameter region of the biosensor to report PWV shifts that represent the averaged shift over the illuminated area. The optical fiber instrument incorporates 8 multiplexed illumination/detection reading heads, for gathering measurements from 8 biosensor regions in parallel, and is capable of scanning an entire 384-well microplate in  $\sim$ 20 s. The scanning may be repeated to gather kinetic binding information.<sup>21,22</sup> A second instrument uses free space optics to illuminate the sensor and an imaging spectrometer to produce measurements of the spatial distribution of PWV across the PC surface with a resolution of  $\sim$ 22.3  $\times$  22.3  $\mu$ m<sup>2</sup>/pixel.<sup>23,24</sup>

In the course of applying our recently developed PC biosensor assay for detection of inhibitors of protein–DNA interactions, we noted several compounds in our compound collection that gave substantially larger shifts in the reflected wavelength signals than could be explained solely by

stoichiometric binding of the molecule to the immobilized target.<sup>25</sup> As an example, representative data for Congo Red (CR) are displayed in Figure 1. Similar results were obtained for several documented aggregating compounds, including Rose Bengal<sup>11</sup> and Indigo<sup>12</sup> (data not shown). These large binding signals were consistently several times the binding signal of a nonaggregating negative control compound (biotin in Fig. 1), and would occur even upon surfaces that were blocked against biochemical binding.

To study these phenomena in greater detail, 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 DLS, enzyme-based inhibition assays, and physical observation using scanning electron microscopy (SEM) are reported herein.

## MATERIALS AND METHODS

### Dynamic Light Scattering

Each of the 22 small molecules (Table 1, maintained as 10 mM stock solutions in DMSO) was diluted to 50  $\mu$ M in deionized (DI) water (0.5% DMSO v/v) to a total volume of 800  $\mu$ L in a 1-mL glass cuvette. Data were collected using a NICOMP 380 ZLS Particle Sizer (Agilent Technologies, Inc., Santa Clara, USA). The instrument was adjusted to measure the optimal light scattering intensity according to the manufacturer's instructions.

### Alpha-Chymotrypsin Enzymatic Assay

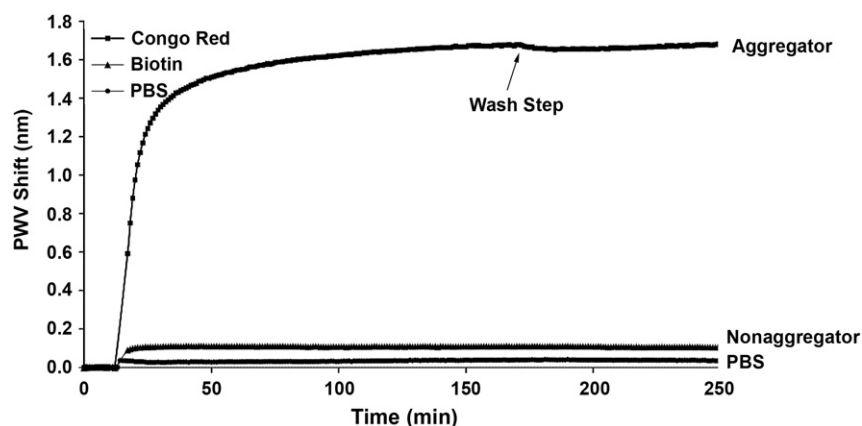
A SpectraMax Plus (Molecular Devices, Sunnyvale, CA, USA) spectrophotometer (96- or 384-well microplate reader) was first calibrated with a concentration series of the  $\alpha$ -chymotrypsin (Sigma-Aldrich, St. Louis, MO, USA) from 10 ng/

mL to 100  $\mu$ g/mL to determine a fixed enzyme concentration, and the substrate succinyl-AAPF-PNA (Sigma-Aldrich) concentration was fixed at 200  $\mu$ M in assay buffer (100 mM Tris, 20 mM CaCl<sub>2</sub>, pH 7.8). The selected enzyme concentration was set at 300 ng/mL to give approximately 10–15 min of linear kinetic optical density unit (OD) output, offering enough time to pipette the substrate into all the wells. After the calibration, each small molecule was diluted to 1, 2.5, 5, 10, 25, 50, 100, and 250  $\mu$ M in assay buffer, and incubated with the enzyme for 30 min at room temperature. Finally, the substrate was added to the mixture (final volume of 50  $\mu$ L), and the kinetic OD output was recorded for 30 min at a wavelength of 405 nm.

### PC Aggregation Detection

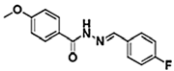
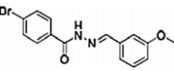
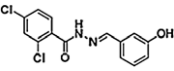
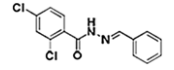
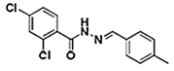
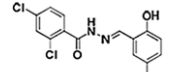
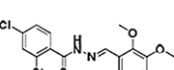
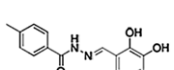
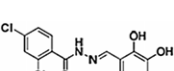
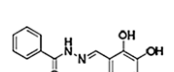
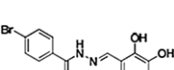
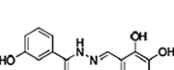
The 20 small molecules were obtained from an in-house library<sup>25,26</sup> and stored at 4 °C in DMSO at a concentration of 10 mM. The chemical structure, molecular weight (MW, g/mol), and CLogP values of each compound are presented in Table 1. The CLogP values were computed with Chem-Draw software (CambridgeSoft, Cambridge, USA). CR was purchased from the Agfa-Gevaert Group (AGFA, Mortsel, BEL). Biotin, phosphate-buffered saline (PBS) solution, and Tween-20 were purchased from Sigma-Aldrich Co. Triton X-100 was purchased from Union Carbide Corporation (Houston, TX, USA). TiO<sub>2</sub>-coated, 384-well BIND sensor microplates and streptavidin (SA2)-coated 384-well BIND sensor plates were purchased from SRU Biosystems, Inc. (Woburn, MA, USA).

The TiO<sub>2</sub> and streptavidin-coated 384-well sensor plates were stabilized with a well volume of 15  $\mu$ L with 4 different buffers: DI, PBS, PBS with 0.1% (v/v) Tween-20, and PBS with 0.1% (v/v) Triton X-100. The analyte concentration used for the PC assay was determined using a series of concentrations of the positive control CR. The minimum and maximum



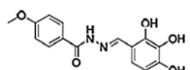
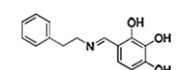
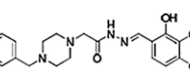
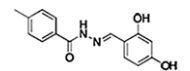
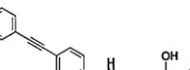
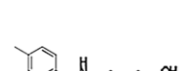

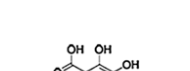
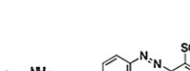

**Figure 1.** Kinetic plot of photonic crystal biosensor peak wavelength value shift as a function of time for a typical aggregating compound (Congo Red) and a nonaggregating compound (biotin). Both molecules were introduced in phosphate-buffered saline (PBS) buffer with 0.05% DMSO to separate biosensor microplate wells treated with streptavidin. Aggregation is characterized by a large positive wavelength shift that does not reach a stable value, even after > 2 h of exposure to the sensor surface. A buffer wash step (three times with PBS) does not remove the aggregated material from the sensor surface.

**Table I.** Summary of the chemical structure, name, and molecular weight (MW) of each compound

Chemical structure	Name, MW, and CLogP
	1 272.3 g/mol 3.07
	2 333.2 g/mol 3.77
	3 309.1 g/mol 3.29
	4 293.1 g/mol 3.32
	5 307.2 g/mol 3.82
	6 325.1 g/mol 3.44
	7 383.2 g/mol 2.45
	8 286.3 g/mol 2.67
	9 341.1 g/mol 2.90
	10 271.2 g/mol 2.17
	11 352.2 g/mol 3.13
	12 288.3 g/mol 1.63

(continued)

Table I (continued)

Chemical structure	Name, MW, and CLogP
	13 302.3 g/mol 2.16
	14 257.3 g/mol 2.91
	15 372.4 g/mol 3.17
	16 271.3 g/mol 3.21
	17 372.4 g/mol 4.81
	18 270.3 g/mol 2.65
	19 273.2 g/mol 0.99
	20 170.1 g/mol 1.06
	Congo Red 696.7 g/mol -3.05
	Biotin 242.3 g/mol -1.33

concentrations detected were 2.5 and 50  $\mu\text{M}$ , respectively. The assay appeared to demonstrate decreased sensitivity at concentrations lower than 50  $\mu\text{M}$ , with a marked decrease in PWV at concentrations less than 12.5  $\mu\text{M}$  (data not shown). In deference to these data, the small molecules were prepared at 50  $\mu\text{M}$  with the corresponding buffers and 15  $\mu\text{L}$  were added to the stabilized plate. The PWV shift was then recorded as a function of time for approximately 5–6 h. The plate was

rinsed with the corresponding buffer and the PWV shift was monitored before and after the final wash step. Endpoint readings were established after the wash step.

### PC Aggregation Imaging

The PWV shifts of the small molecules at a concentration of 50  $\mu\text{M}$  were monitored using an uncoated PC biosensor microplate with an exposed  $\text{TiO}_2$  surface and DI as a solvent. Images were scanned at a pixel resolution of  $22.3 \times 22.3 \mu\text{m}^2/\text{pixel}$  after stabilization with DI water (baseline image) and again after the aggregation period (aggregation image). To record the PWV shift due to aggregation, the baseline image was subtracted from the aggregation image on a pixel-by-pixel basis, resulting in PWV shift image. For purposes of display, the range of PWV shift depicted in the image was set from  $-1.40$  to  $2.10 \text{ nm}$  with a user-selected color map.

### Scanning Electron Microscopy

Uncoated PC biosensor microplate wells with an exposed  $\text{TiO}_2$  surface were incubated with 3  $\mu\text{L}$  of each small molecule (50  $\mu\text{M}$  in 0.5% DMSO). The sensors were then rinsed with the following sequence of solvents: DI, acetone, DI, isopropyl alcohol, and DI. Samples were mounted on an aluminum stage with carbon tape adhesive and sputter coated with  $\sim 50 \text{ \AA}$  gold. Images were obtained with a Hitachi SE UHR FE-4800 scanning electron microscope (Hitachi, Tokyo, JP) at 15 kV. Micrographs were recorded at 2  $\mu\text{m}$  under focus at varying magnifications.

### Measuring the Effects of Detergent Using PC Biosensors

The streptavidin-coated 384-well sensor plates were stabilized in 15- $\mu\text{L}$  PBS or PBS + 0.05% Tween-20. The small molecules were diluted to 50  $\mu\text{M}$  in PBS or PBS + 0.05% Tween-20, and 15  $\mu\text{L}$  of each sample was incubated with the stabilized biosensor for approximately 5–6 h, and scanned with the PC readout instrument. Next, PBS was prepared with varying percentages of Tween-20: 0%, 0.01%, 0.05%, 0.1%, 0.5%, 1%, and 5%. CR was prepared with each buffer in a dilution series ranging from 0.4 to 50  $\mu\text{M}$ , and 15  $\mu\text{L}$  of each dilution was added to the stabilized plate. The PWV shift was then recorded for approximately 5–6 h. Plates were then rinsed with the corresponding buffer, and PWV shift was monitored before and after the final wash step. Endpoint readings were established after the wash step.

## RESULTS

### Estimated Diameter Using DLS

The compounds evaluated in this study are listed in Table 1. Library compounds 1–20 are previously uncharacterized as aggregators, whereas negative controls (DMSO, biotin, buffer) and the positive control (CR) were also used. Results of the DLS measurements are shown in Figure 2a. 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. The small molecules with high scattering intensity are shown in black ( $\sim 300 \text{ Hz}$ ), those with low intensity are shown in gray ( $\sim 30 \text{ Hz}$ ). The DLS instrument provided a fit error value for each compound (Fig. 2b), and compounds with high scattering intensity showed low fit error, and 9 of the low intensity compounds showed large fit error relative to the 100-nm bead control sample. Additionally, although the 100-nm bead positive control gave results consistent for 100-nm diameter particles, the results for DMSO only and biotin (non-aggregator control) were within the same range. This provides further evidence that DLS may be limited in the detection of the types of aggregates formed by drug-like compounds.

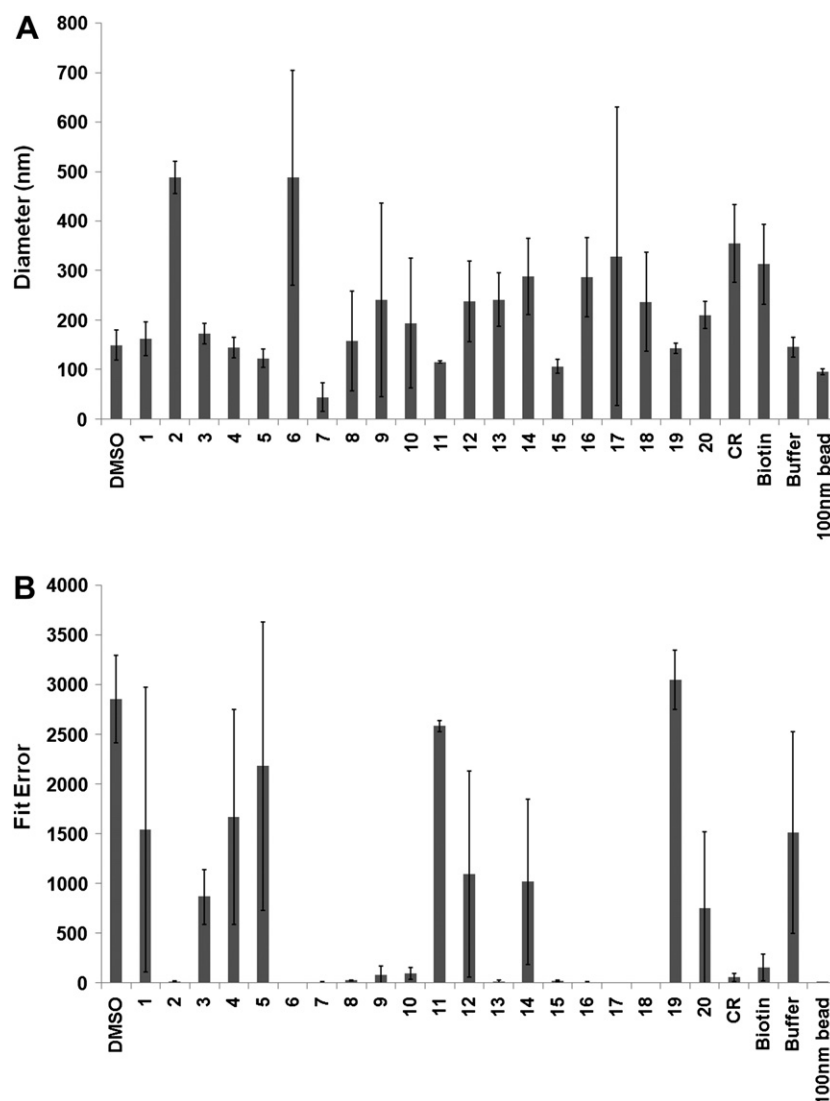
### Alpha-Chymotrypsin Assay Analysis

Inhibition of  $\alpha$ -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 the  $\alpha$ -chymotrypsin. The linear portion of the graph (the first 15 min) was used for slope calculation and comparison to DMSO and other compounds. To highlight the inhibitory/activating properties of these compounds in this assay, the highest concentrations (250  $\mu\text{M}$ ) of compounds 1–20 and CR that were used are depicted in Figure 3. All % activities were normalized to the slope of the line generated from DMSO treated  $\alpha$ -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.

### PC Aggregation Detection and Imaging

The PWV shifts recorded for each of the 22 compounds are shown in Figure 4. The PC biosensor recorded an increase in the PWV for several of the compounds. Although the sensor surface was washed rigorously with buffer three 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.

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 was analyzed further both with the PC biosensor method and SEM. Specifically, based on the PC biosensor data in Figure 4, compounds 1 and 2 were selected as nonaggregators, 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 5. The PWV shift image shows a large PWV shift for the two putative aggregating small molecules



**Figure 2.** (A) Dynamic light scattering (DLS) particle diameter measurements of the 22 compounds and 100-nm bead control. Error bars represent one standard deviation for  $N = 3$  independent measurements. Compounds with black bars represent those with  $> 300$  Hz scattering intensity. (B) DLS fit error for the size measurements obtained in (A). Note that large standard deviations in diameter measurements do not correlate with large fit errors.

(8, 19), whereas 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.

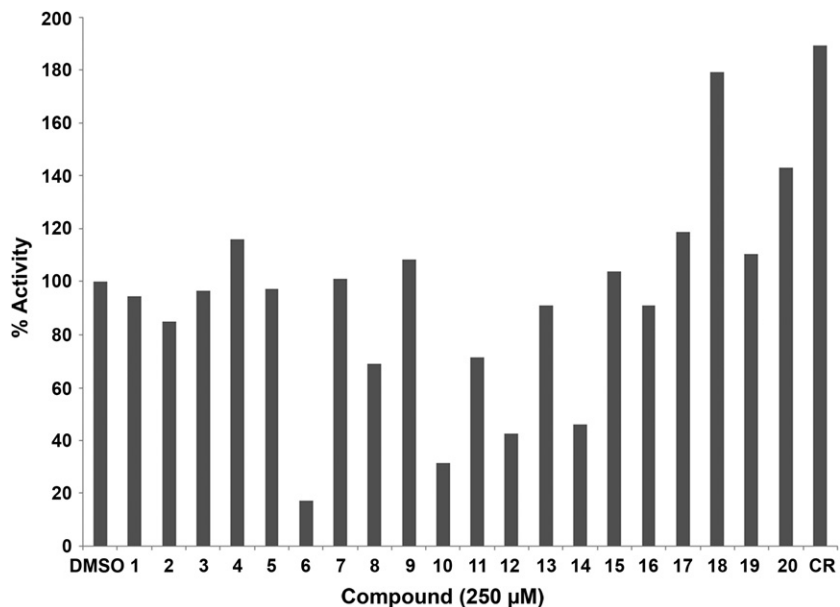
### SEM Aggregation Confirmation

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 (Fig. 6a–c). In contrast, the sensor surface

of compounds 8 and 9 (that had registered a positive signal in the PC aggregation assay) has a gel-like substance attached on the surface as visualized by SEM (Fig. 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.

### Detergent Effect

As a positive control for aggregation, CR was used to examine the effect of detergent on nonspecific aggregation,

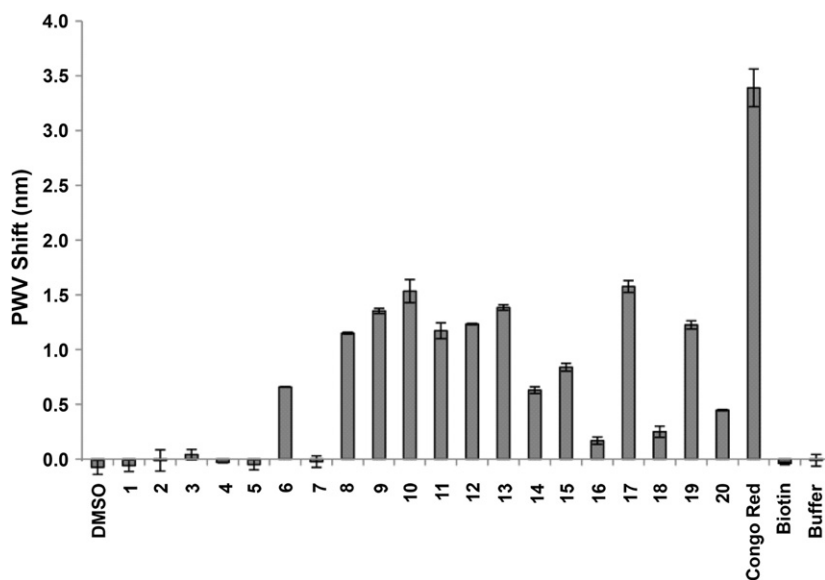


**Figure 3.** The % activity (normalized to DMSO control) of  $\alpha$ -chymotrypsin in the presence of test compounds at 250  $\mu$ M. Many compounds did not adversely affect  $\alpha$ -chymotrypsin activity, although some inhibited the enzymatic activity, whereas others increased enzyme activity, which is attributed to the colored nature of these compounds. Data are representative of three independent experiments.

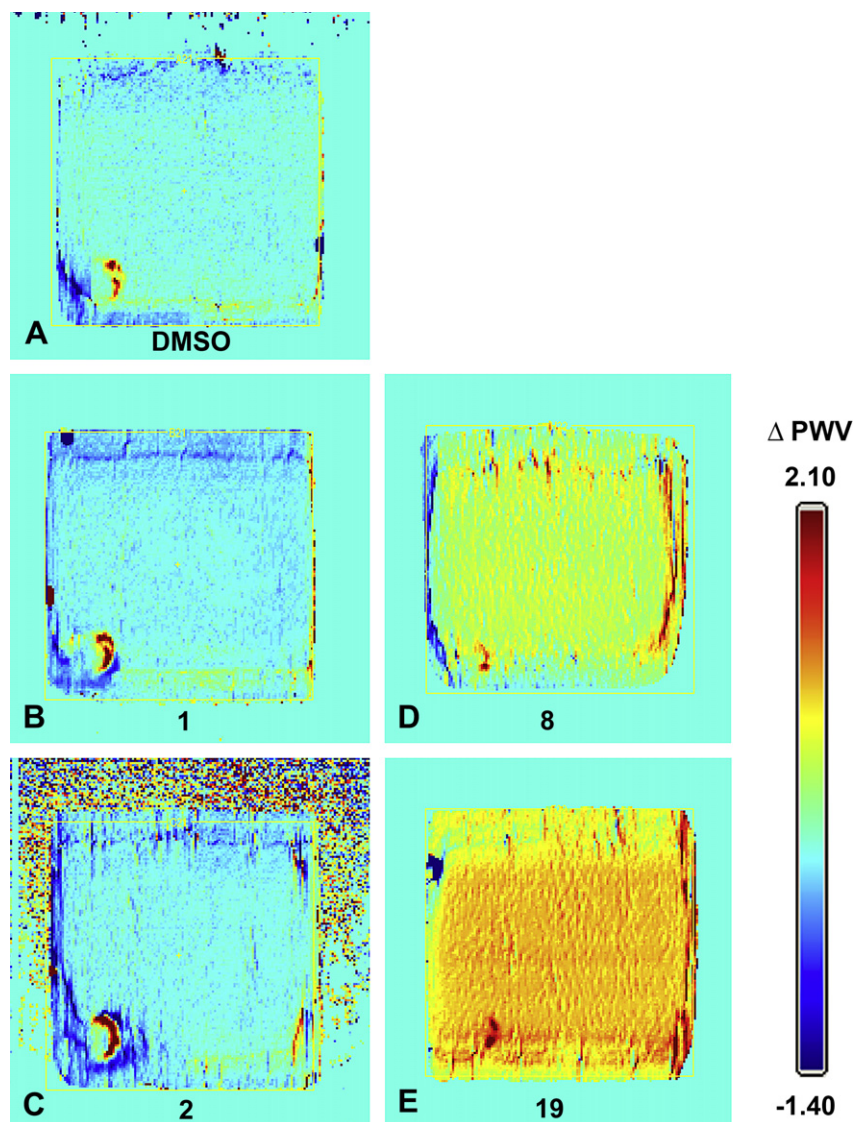
as CR displayed the largest aggregation signal with the PC biosensor assay. As shown in Figure 7a, the aggregation signal for CR was reduced 50% by adding a small percentage (0.05% v/v) of Tween in the PBS buffer. The effect of detergent upon aggregation was found to be concentration dependent, as shown in Figure 7b, confirming the results stated by previous studies.<sup>1,3</sup>

## DISCUSSION

The goal of the work presented in this article 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



**Figure 4.** The peak wavelength value (PWW) shifts recorded (using the fiber probe detection instrument) for a photonic crystal biosensor coated with streptavidin and then treated with the indicated compound. Samples were incubated with compound at a concentration of 25  $\mu$ M in phosphate-buffered saline for 60 min before reading. The positive control compound Congo Red showed the largest PWW shift, whereas the negative controls (DMSO, biotin, and buffer) showed small PWW shifts.

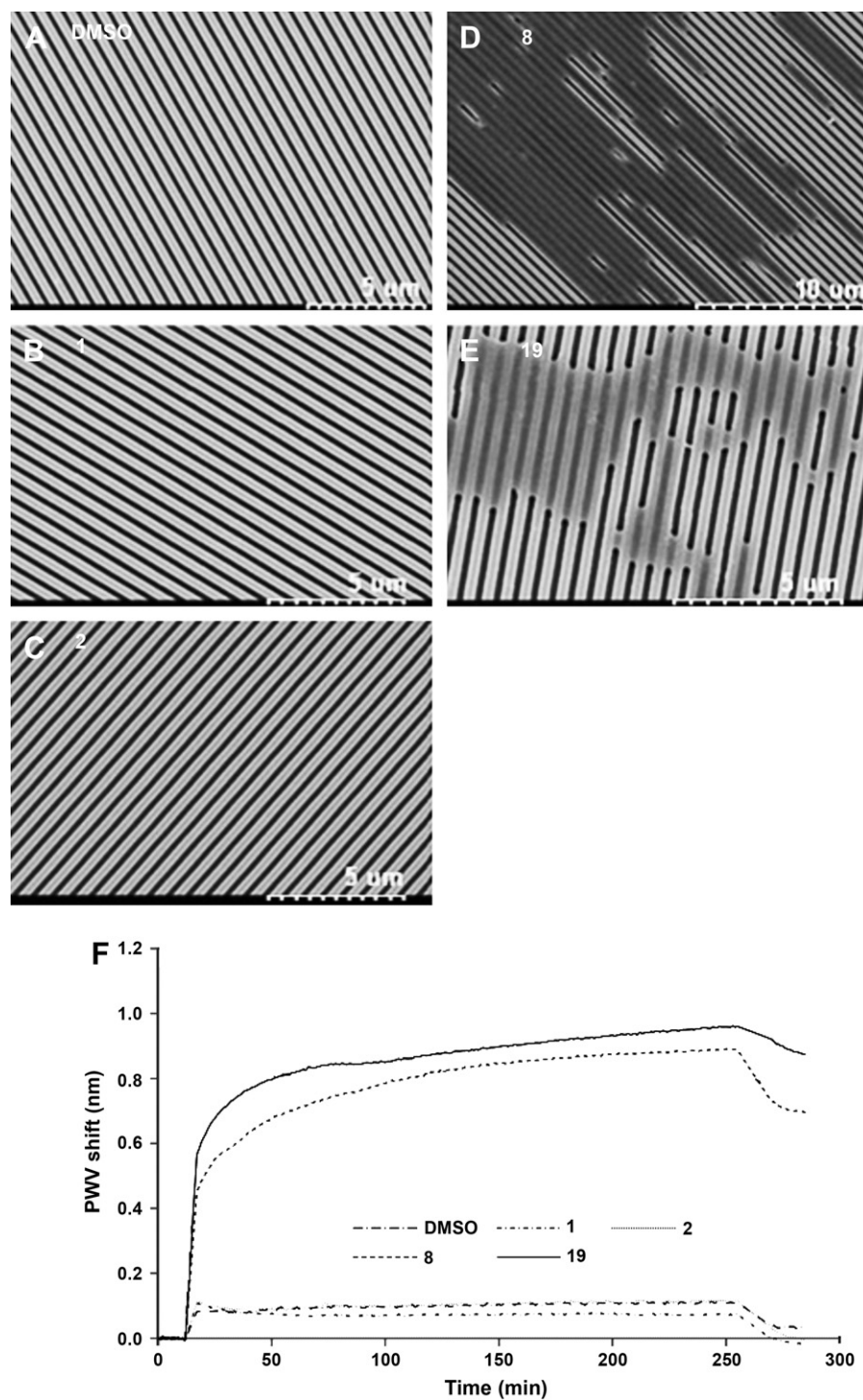


**Figure 5.** The peak wavelength value shift images of selected individual 384-well microplate wells, gathered with the photonic crystal imaging detection instrument, demonstrating uniformly high levels of aggregation distributed across the biosensor surface for several compounds ((D) 8, (E) 19) and lack of wavelength shift for two nonaggregator compounds ((B) 1, (C) 2) and (A) DMSO reference solution.

sensor surface; this manifests itself as a large increase in the PWV, making it easy to identify such nuisance-aggregating compounds. To demonstrate the potential utility of this method for prescreening aggregators from a compound library, we sought to validate the PC biosensor aggregation method against other commonly used techniques. We also sought to validate whether commonly used strategies for reducing aggregation, namely the addition of detergent to the compound buffer, would result in modulation of the aggregation signal measured by the biosensor, and to demonstrate characterization of the concentration dependence of compound aggregation.

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) (Fig. 4). Despite the elevated concentrations used in the PC detection and enzymatic inhibition assays, only a small fraction of the compounds (1, 2, and 7) showed evidence of precipitation. It is worth noting that these results show little correlation with estimated hydrophilicity/solubility, as both CR and biotin possess hydrophilic CLogP values ( $-3.05$  and  $-1.33$ , respectively). The test compounds had a broad distribution of hydrophobic CLogP values ( $0.99$ – $4.81$ ), with neither more nor less hydrophilic molecules, showing preferential detection of aggregation. Furthermore, the material deposited upon the sensor surface attributed to aggregation remained even after rigorous washes with buffer. These data suggest that the aggregation was not a loose precipitate and that the measured signals

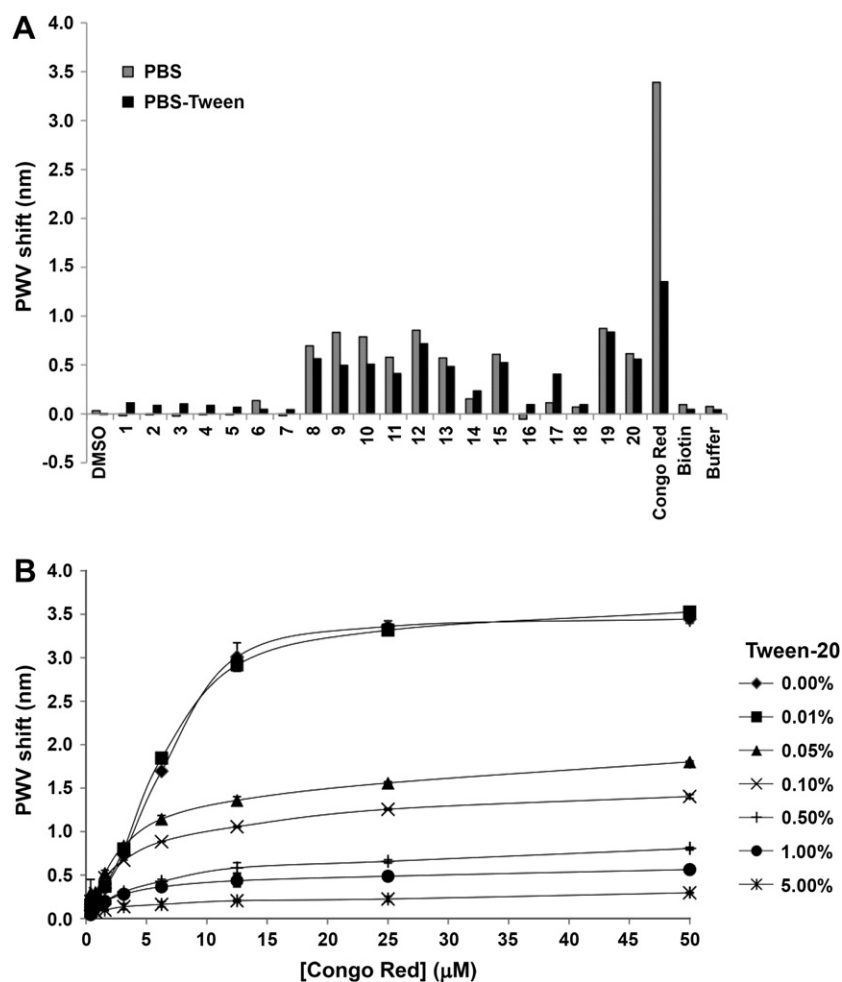


**Figure 6.** Scanning electron microscopy images of (A) DMSO, and compounds (B) 1, (C) 2, (D) 8, and (E) 19. The two aggregators (8, 19) showed gel-like substance attached on the sensor surface, whereas such substance could not be located on the nonaggregator and DMSO surfaces. In the kinetic plot (F), the aggregators slowly increased to a high peak wavelength value shift signal even after a rigorous washing step.

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 (Fig. 2) and particle size readings of  $\sim 100$  nm particle diameter for the negative controls (DMSO, biotin, and buffer) severely limited the utility of the data obtained. DLS measurements of



**Figure 7.** (A) Photonic crystal (PC) biosensor aggregation measurements showing the effect of 0.05% Tween-20 added to the buffer as a method for reducing compound aggregation. (B) A plot of PC biosensor aggregation signal as a function of aggregator (Congo Red) concentration for Tween percentages of 0–5%, demonstrating that as detergent percentage increases, the aggregation signal decreases, and that aggregation is also dependent on the concentration of the compound.

scattering intensity can be used as a means for estimating particle diameter based on Mie-theory calculations that assume uniform spherical particles.<sup>14,15</sup> 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 (shown in black bars in Fig. 2) 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.

Although DLS is widely used to characterize particle aggregation, enzyme-based assays are a common HTS for promiscuous inhibitors. The  $\alpha$ -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 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.<sup>16</sup> Therefore, this detection method presents several challenges to accurately identify possible aggregators within a small molecule library that limit reliability and throughput.

Because DLS and the  $\alpha$ -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 nonaggregators (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 (Fig. 6) and it is likely that these deposits caused the large measured PWV shifts. The deposits were absent from sensors exposed to nonaggregators and 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 shift in PWV (Fig. 5).

As stated previously, the use of detergent has been reported as a means to reduce the compound aggregation, and a quantitative method for measuring aggregation should ideally be able to measure the effects of detergent and the effects of the compound concentration. Such measurements may prove useful in the identification of marginal compounds, for which aggregation effects may be avoided under the correct conditions. To this end, we conducted an experiment to confirm that the PC biosensor can be used to observe the effect of detergent on possible aggregators. Initially, PBS + Tween-20 (0.05% v/v) was mixed with the compounds and a decrease in the aggregation signal occurred for most of the small molecules. CR showed the greatest reduction in nonspecific binding in response to the addition of varying concentrations of detergent. This information was used to plot a PWV shift curve as a function of concentration in respect to Tween-20 percentage (Fig. 7). Decreased PWV shift in response to increased detergent concentration supports the hypothesis that  $\Delta$ PWV as described here is proportional to a given small molecule's propensity for aggregation.

## 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 OD 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.

The ability to not only identify aggregators but to also quantify the degree of aggregation was demonstrated using various concentrations of detergent and compound to modulate the aggregation effect.

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## REFERENCES

1. Ryan, A. J.; Gray, N. M.; Lowe, P. N.; Chung, C. W. Effect of detergent on "promiscuous" inhibitors. *J. Med. Chem.* **2003**, *46*, 3448–3451.
2. Feng, B. Y.; Shelat, A.; Doman, T. N.; Guy, R. K.; Shoichet, B. K. High-throughput assays for promiscuous inhibitors. *Nat. Chem. Biol.* **2005**, *1*, 146–148.
3. Feng, B. Y.; Shoichet, B. K. A detergent-based assay for the detection of promiscuous inhibitors. *Nat. Protoc.* **2006**, *1*, 550–553.
4. Lipinski, C. A.; Lombardo, F.; Dominy, B. W.; Feeney, P. J. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development setting. *Adv. Drug. Discov. Rev.* **1997**, *23*, 3–25.
5. Walters, W. P.; Murcko, M. A. Recognizing molecules with drug-like properties. *Curr. Opin. Chem. Biol.* **1999**, *3*, 384–387.
6. Muegge, I.; Heald, S. L.; Brittelli, D. Simple selection criteria for drug-like chemical matter. *J. Med. Chem.* **2001**, *44*, 1814–1816.
7. Giannetti, A. M.; Koch, B. D.; Browner, M. F. Surface plasmon resonance based assay for the detection and characterization of promiscuous inhibitors. *J. Med. Chem.* **2008**, *51*, 574–580.
8. Rishton, G. M. Reactive compounds and in vitro false positives in HTS. *Drug Discov. Today* **1997**, *2*, 382–384.
9. Walters, W. P.; Namchuk, M. Designing screens: how to make your hits a hit. *Nat. Rev. Drug Discov.* **2003**, *2*, 259–266.
10. Hajduk, P. J.; Huth, J. R.; Fesik, S. W. Druggability indices for protein targets derived from NMR-based screening data. *J. Med. Chem.* **2005**, *48*, 2518–2525.
11. McGovern, S. L.; Caselli, E.; Grigorieff, N.; Shoichet, B. K. A common mechanism underlying promiscuous inhibitors from virtual and high-throughput screening. *J. Med. Chem.* **2002**, *45*, 1712–1722.
12. McGovern, S. L.; Shoichet, B. K. Kinase inhibitors: not just for kinases anymore. *J. Med. Chem.* **2003**, *46*, 1478–1483.
13. Keseru, G. M.; Makara, G. M. Hit discovery and hit-to-lead approaches. *Drug Discov. Today* **2006**, *11*, 741–748.
14. Goode, D. R.; Totten, R. K.; Heeres, J. T.; Hergenrother, P. J. Identification of promiscuous small molecule activators in high-throughput enzyme activation screens. *J. Med. Chem.* **2008**, *51*, 2346–2349.
15. Kaszuba, M.; Connah, M. T.; McNeil-Watson, F. K. Resolving concentrated particle size mixtures using dynamic light scattering. *Part. Part. Syst. Charact.* **2007**, *24*, 159–162.
16. International Organization for Standardization (ISO) 13321: Methods for Determination of Particle Size Distribution Part 8: Photon Correlation Spectroscopy, *Particle Size Analysis* 1996.
17. Myszyka, D. G. Improving biosensor analysis. *J. Mol. Recognit.* **1999**, *12*, 279–284.

18. Papalia, G. A.; Leavitt, S.; Bynum, M. A.; Katsamba, P. S.; Wilton, R.; Qiu, H.; Steukers, M.; Wang, S.; Bindu, L.; Phogat, S.; Giannetti, A. M.; Ryan, T. E.; Pudlak, V. A.; Matusiewicz, K.; Michelson, K. M.; Nowakowski, A.; Pham-Baginski, A.; Brooks, J.; Tieman, B. C.; Bruce, B. D.; Vaughn, M.; Baksh, M.; Cho, Y. H.; Wit, M. D.; Smets, A.; Vandersmissen, J.; Michiels, L.; Myszka, D. G. Comparative analysis of 10 small molecules binding to carbonic anhydrase II by different investigators using Biacore technology. *Anal. Biochem.* **2006**, *359*, 94–105.
19. \*Rich, R. L.; Myszka, D. G. Why you should be using more SPR biosensor technology. *Drug Discov. Today Technol.* **2004**, *1*, 301–308.
20. Lin, B.; Li, P.; Cunningham, B. T. A label-free biosensor-based cell attachment assay for characterization of cell surface molecules. *Actuators B Chem.* **2006**, *114*, 559–564.
21. Cunningham, B. T.; Li, P.; Schulz, S.; Lin, B.; Baird, C.; Gerstenmaier, J.; Genick, C.; Wang, F.; Fine, E.; Laing, L. Label-free assays on the BIND system. *J. Biomol. Screen.* **2004**, *9*, 481–490.
22. Cunningham, B. T.; Laing, L. L. Label-free detection of biomolecular interactions: applications in proteomics and drug discovery. *Expert Rev. Proteomics* **2006**, *3*, 271–281.
23. Lin, B.; Li, P.; Cunningham, B. T. A label-free biosensor-based cell attachment assay for characterization of cell surface molecules. *Sens. Actuators B Chem.* **2006**, *114*, 559–564.
24. Chan, L. L.; Gosangari, S. L.; Watkin, K. L.; Cunningham, B. T. A label-free photonic crystal biosensor imaging method for detection of cancer cell cytotoxicity and proliferation. *Apoptosis* **2007**, *12*, 1061–1068.
25. Chan, L. L.; Pineda, M. F.; Heeres, J.; Hergenrother, P.; Cunningham, B. T. A general method for discovering inhibitors of protein-DNA interactions using SRU photonic crystal biosensors. *Am. Chem. Soc. Chem. Biol.* **2008**, *3*, 437–448.
26. Hergenrother, P. J. Obtaining and screening compound collections: a user's guide and a call to chemists. *Curr. Opin. Chem. Biol.* **2006**, *10*, 213–218.