IDENTIFYING SMALL MOLECULE AGGREGATORS WITH PHOTONIC CRYSTAL BIOSENSOR MICROPLATES

BY

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THESIS

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Abstract

Small molecules identified through high-throughput screens are essential elements 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 a-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, while the microplate-based sensor format enables compatibility with high-throughput automated liquid handling methods used in pharmaceutical screening.

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Chapter 1: Introduction

1.1 – Pharmaceutical Drug Discovery – Natural and Synthetic Product Isolation

Pharmaceutical drug discovery programs employ a wide variety of high-throughput screening (HTS) methods to identify lead compounds for further development [1-6]. These strategies generally include the isolation and categorization of a broad source of potential drugs or drug-like compounds prior to the screening and evaluation of these compounds over an extended research and development cycle (Fig. 1)[7]. Two primary isolation strategies include extraction from natural products and combinatorial chemistry approaches designed around a specific compound. These approaches are often used in tandem with one another – once a specific natural product is found, combinatorial chemistry can often be used to enhance its functionality and therefore optimizing its utility as a pharmaceutical solution. Compounds created from hybridization of these two strategies are known as semi-synthetic compounds or derivatives.

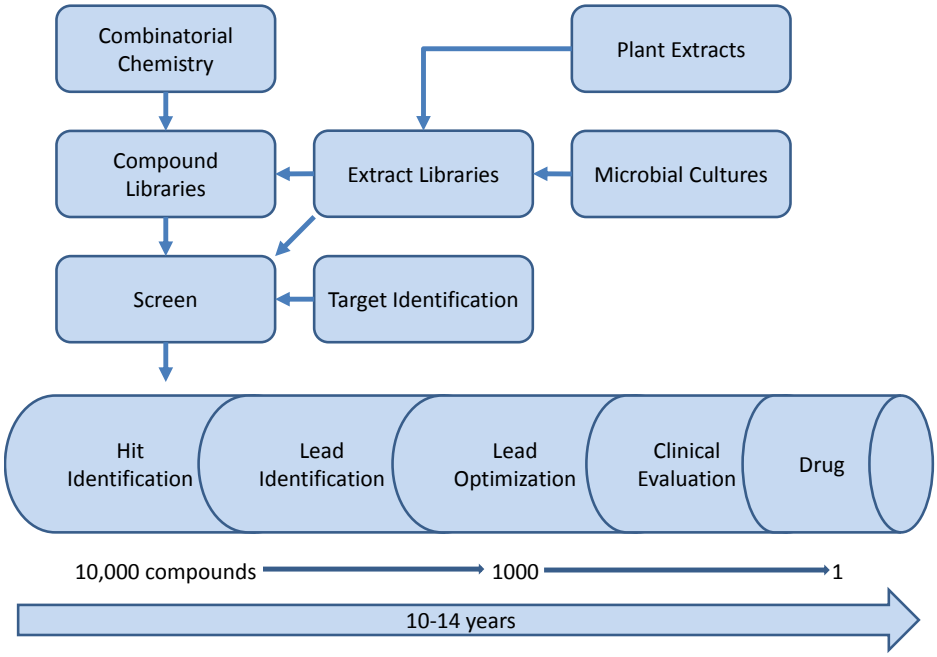


Figure 1: Process schematic for drug discovery. High-throughput screening of naturally-occurring organic compounds remains the most productive method through which to seek novel therapeutics. Despite recent advances in compound synthesis and isolation, however, it remains a complex process, often requiring over a decade for a particular compound to progress from an initial screen to a clinical use. Shortening the time invested in any of the steps highlighted above would result in a significant reduction in time-to-market for a particular drug (Adopted from [7]).

These semi-synthetic compounds possess a storied history in the pursuit of therapeutic compounds, and continue to be a valuable source for new studies. Supporting this assertion, over 60% of currently approved drugs are naturally occurring substances [8, 9]. There are three primary sources of new compounds in the high-throughput pharmaceutical screening arena: natural products, semi-synthetic derivatives of natural products, and synthetic compounds produced using combinatorial chemistry techniques on natural product models [8, 9, 10]. Basing the search for novel inhibitors on novel compounds is not only a choice of convenience, however. Interesting relationships between naturally occurring compounds and their cognate receptors can be related to the evolution of processes deeply ingrained in cell biology – processes that have since been modified to present functionalities that remain incredibly disparate between the organism producing the natural compound and the organism expressing a target suitable for therapeutic intervention.

While naturally occurring compounds have made great contributions to the state of modern pharmacology, recent advances in combinatorial chemistry have allowed the synthesis of derivative compounds and original compounds with unprecedented efficiency. The resulting compounds present a unique challenge, however, in that they lack the heterogeneity typical of natural products with respect to several qualities desirable for effective drug design. Molecular characteristics including chirality, asymmetry, and charge distribution are critical to the functionality of many currently used drugs [9, 10]. The rapid availability of derivatives increased by combinatorial chemistry has been faulted for this lack of heterogeneity, as these qualities in particular are able to modify efficacy and specificity by orders of magnitude [9] In short, pharmaceutical screening campaigns remain indebted to continued extraction, isolation, and characterization of natural products from novel sources – a pursuit widely acknowledged as lengthy, expensive, and difficult [11, 12, 13].

Using these techniques, it is possible to compose a diverse pharmaceutical screening library. Typical libraries involve the use of many thousands of compounds, possessing a significant degree of heterogeneity with regard to the characteristics mentioned above. The compounds documented in this manuscript originated from the High-Throughput Screening Facility at the University of Illinois at Urbana-Champaign, which maintains a library containing over 180,000 compounds chosen using over sixty computational methods at Chembridge (San Diego, CA) to ensure structural heterogeneity and optimal similarity to effective drugs (Figure 2).

After compiling a diverse library of pharmaceutical screening candidates, the traditional approach calls for an application-specific screening experiment, of which there are three main classes. One type of screen relies on the construction of a colorimetric readout for synthesis of a desired reaction product – examples of such reactions are discussed in the *Current Detection Methods* section. Some screening experiments are designed around modifying cell viability – as such, these experiments are typically analyzed by culturing small quantities of the cells of interest before conducting a high-throughput reading step in context with potential pharmaceuticals at physiologically relevant concentrations. High-throughput reading assays for these experiments include flow cytometry (fluorescence-activated cell sorting, FACS), apoptosis detection assays (fluorescence-based and colorimetric), and live-dead cell sorting assays. This collection of assays provides excellent information with regard to the library compounds' effectiveness toward a single application, but little in terms of information that may be applied broadly and across multiple screening campaigns.

Such broadly applicable information has traditionally been limited to such physical constants as hydrophobicity, molecular weight, density, solubility, and charge, but these qualities have yielded little in the way of predicting a molecule's success or failure for a particular pharmaceutical application. Recent studies have revealed some traits that are broadly undesirable among drug candidate, and the possibility of computational methods to predict problematic candidates is currently under investigation. The pharmaceutical industry, however, continues to rely on evidence discovered from *in vitro* experimentation with compound libraries in order to decide which compounds should be discounted from consideration and which compounds merit further study and optimization.

While the current strategies have proven successful for certain problems, drug discovery remains a challenging and costly process. Screening campaigns often take over a decade and hundreds of millions of dollars to complete – as compounds progress through the screening process, the assays used to test their validity increase in length, complexity, and cost [12, 13]. For these reasons, optimizing the efficiency of drug discovery remains challenging, yet worthwhile objective. In this vein, the development of a broadly applicable screen to remove undesirable drug candidates at the earliest opportunity would affect the pharmaceutical industry favorably.

1.2 – Small Molecule Aggregators and Promiscuous Inhibition

Throughout a screening campaign, candidates are removed for a number of faults causing incompatibility or reducing compounds' suitability for biological use. One issue prominent among such screening efforts is the formation of multimeric aggregates by candidate compounds. Such aggregates are known to result in nonspecific interactions with many proteins [1, 15-19], leading to unreliable outputs from several types of screening assays [7, 13]. Compounds that can form large aggregates and inhibit the interactions with the target protein are often referred to as "promiscuous inhibitors" due to their ability to alter the function of many different proteins in a nonspecific manner [1, 19, 20]. 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 (Figure 2); recent studies have also shown that in some cases aggregation can lead to non-specific enzymatic activation [21].

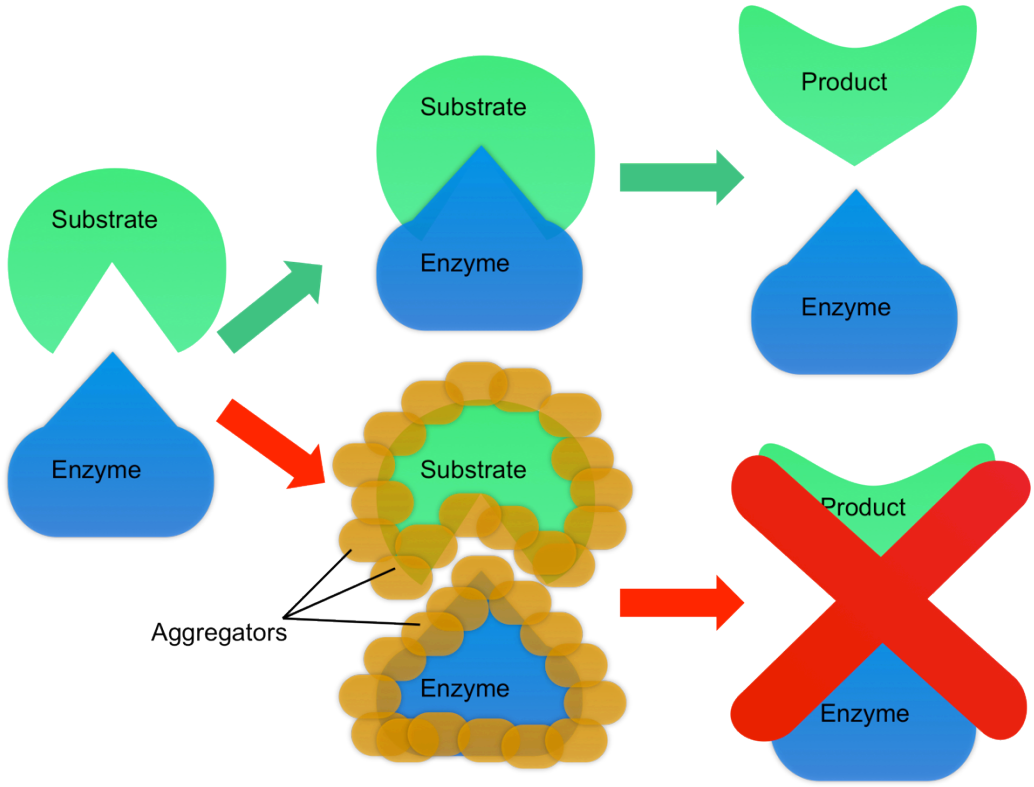


Figure 2: Small molecule aggregation as a mechanism for promiscuous inhibition. Aggregation of prospective drug candidate molecules can result in sequestration of the enzyme and/or substrate, inhibiting product formation and preventing enzyme molecules from performing their desired function. Because this mechanism of inhibition remains nonspecific, broad classes of enzymes remain affected, thereby reducing the candidate molecule's utility as a specific, well-controlled inhibitor of a particular target reaction.

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 HTS assays [3]. Therefore, HTS methods can be improved if aggregating compounds in a given library can be identified, characterized, and eliminated before screening is performed [1-3, 15, 18]. 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 [1-3]. 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.

1.3 – Current Detection Methods for Small Molecule Aggregation

Dynamic Light Scattering (DLS) Spectroscopy

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 molecules aggregates by measuring the time-dependent fluctuation of scattering intensity of a coherent light source illuminating particles suspended in solution [1, 3, 18]. DLS relies on two fundamental assumptions in the computation of particle size – the first is that the particles of interest are subject to Brownian motion. The second assumption is that the particles are uniform in size, distribution, and composition [22]. Briefly, coherent light scattered from two or more particles constructively or destructively interferes at the detector. By calculating the autocorrelation function of the light intensity, it is possible to determine the time scale along which particular motion occurs within the sample. This information, in combination with the assumption of uniform particle size and distribution, is sufficient to estimate the particle's hydrodynamic diameter.

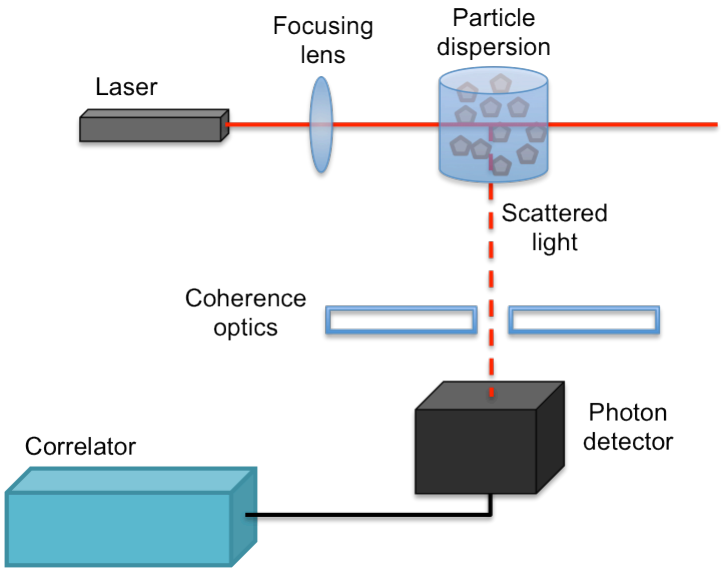


Figure 3: Dynamic Light Scattering instrumentation. Dynamic light scattering uses a focused, coherent light source to illuminate the sample of interest. Scattered light is captured by the detector, and the intensity is recorded as a function of time to compute the autocorrelation function for each sample. This information can then be used to calculate the particles' perceived hydrodynamic diameter. The DLS particle sizing model relies on Mie theory scattering, which assumes both a uniform particle distribution as well as uniformly spherical particles.

While DLS serves as an effective tool when these assertions remain true, small molecule aggregates can form in many shapes, including irregular non-uniform clumps, thin sheets, and fibrous tendrils [1-3, 19, 23-25]. As a result, the fidelity of DLS can be compromised when used for characterizing such compounds, resulting in increased error of the particle-sizing algorithm. Additionally, due to the complexity of the instrumentation necessary to perform DLS spectroscopy as well as the time needed to perform the assay, multiplexing of this assay is compromised. Finally, samples must often be manipulated to approximate a uniform distribution – thorough mixing immediately before reading is necessary for reliable results, resulting in a further increase in the time necessary to perform such a screen, as well as an additional source of error. Though DLS can be effective on small scales if experimental conditions are managed well, small molecule aggregators have been shown to present several complications for which this technique remains poorly suited, especially in the context of high-throughput screening.

Enzyme Inhibition Assays

Other common methods for identification of aggregating compounds include enzyme-based inhibition assays. These assays typically rely on the enzyme-catalyzed formation of a colored product, producing a detectable colorimetric change in solution. To increase throughput,

it is possible to multiplex the assay, using a plate reader to measure the absorbance of reaction product over a range of concentrations in context with several drug candidates simultaneously. Enzymes typically used for these reactions include AmpC β-lactamase, α-chymotrypsin, or dihydrofolate reductase [1, 19, 26]. Bacterial expression vectors are used to provide a plentiful source of the protein before it is purified via affinity chromatography. The chosen enzyme is then mixed with a specially altered substrate that produces a colored reaction product upon completion of the reaction. α-chymotrypsin, for example, proteolytically cleaves the carboxy-terminal bond of glycine, tyrosine, phenylalanine, leucine, and methionine. When this reaction is conducted with N-acetyl-*L*-phenylalanine *p*-nitrophenyl amide, the enzyme-substrate complex forms the yellow intermediate, *p*-nitrophenolate. The concentration of this intermediate can be determined by measuring the absorbance of the reaction mixture at 410 nm, and comparing the absorbance to a range of absorbance readings for a positive control dilution series. Comparing reaction progress over a range of concentrations allows calculation of the reaction rate. When aggregation and non-specific enzyme inhibition occur, the reaction rate is altered and a change in kinetics can be observed. To conclusively establish nonspecific inhibition, it is common to use one or more of these assays - molecules found to be promiscuous inhibitors can then be removed from further consideration as drug candidates.

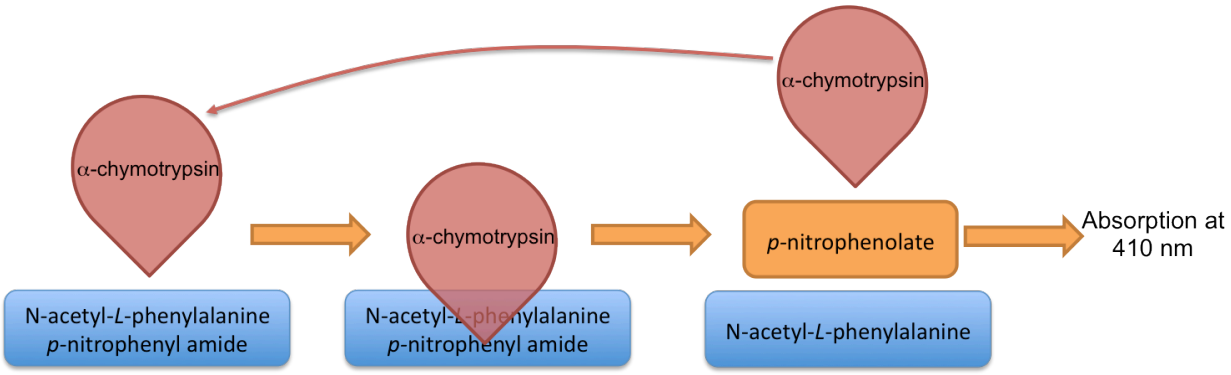


Figure 4: Enzyme inhibition assays. The α-chymotrypsin enzymatic inhibition assay depends on the use of a substrate specially designed for colorimetric detection as the reaction progresses. When N-acetyl-*L*-phenylalanine *p*-nitrophenyl amide is cleaved by α-chymotrypsin, forming *p*-nitrophenolate and a substituted phenylalanine. The product *p*-nitrophenolate absorbs light at 410nm, and so can be detected using a spectrometer. This assay can be performed in context with drug candidates to assess their predilection toward nonspecific inhibition via mechanisms including small molecule aggregation.

Unfortunately, small molecules with significant absorbance in the range of the enzyme-substrate intermediate can affect the absorbance output signal without respect for their potential for aggregation. Because the readout is not a direct product of small molecule aggregation, but rather an indirect measure of the compounds' effects on apparently unrelated enzymatic reactions, It is possible to avoid conflicts using several such reactions to study each compound of interest, but additional screens increase the time and cost of effectively ruling out promiscuous inhibitors. For these reasons, a screen directly relating the properties of the compounds of interest would be a welcome addition to screening methods designed to detect aggregating small molecules.

Surface Plasmon Resonance

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 [15]. SPR is a more reliable method than either DLS or enzymatic inhibition as it directly quantifies the affinities of the molecules observed. Briefly, SPR optical sensors are made up of an optical system, a transducing medium relating the optical domain to the chemical/sensing region, and an electronic system to support the sensor and to allow data processing, collection, and analysis. The transducing medium transforms changes that take place in the sensing region into changes in refractive index. The optical part of the sensor contains a source of illumination and an optical structure in which surface plasmon waves are excited and interrogated. During interrogation, the sensor generates an electronic signal to be processed by the electronic system. To interrogate the sensor, light is directed via a waveguide into a metal layer built into the sensor and interacting directly with the analyte. Changes in the analyte alter the refractive index within the sensing region, causing a change in the sensor response.

Commonly used approaches for detecting SPR signal include measurement of the optical wave intensity near the resonant condition and measurement of the resonant condition using either angular or wavelength-based interrogation techniques [27-30] (Figure 5). For aggregate screening, SPR instrumentation can be constructed using a flow cell through which drug candidates are pumped across a sensor functionalized with protein. In the event that a small molecule is prone to nonspecific aggregation on the sensor, the signals for a range of concentration can be used to characterize its nature and kinetics. SPR has been used to

categorize such aggregation as nonspecific, promiscuous, super-stoichiometric, or detergent-sensitive, among other qualities [31].

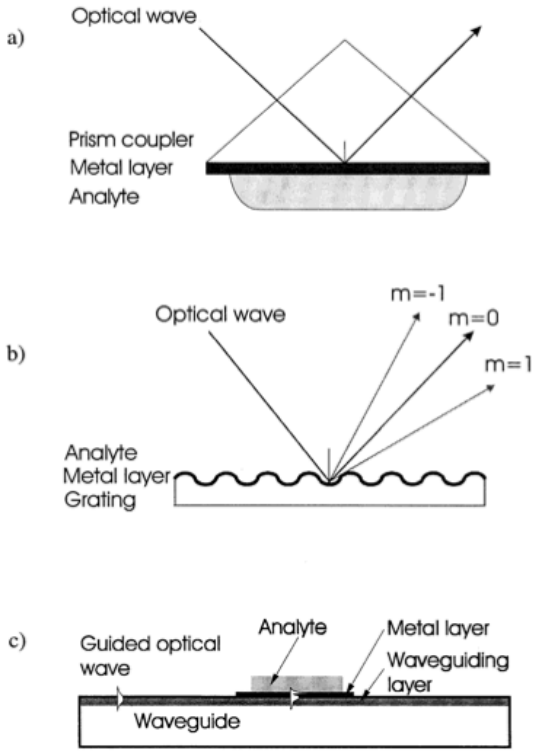


Figure 5: Surface Plasmon Resonance instrumentation. SPR instrumentation has been successfully implemented in several varieties; three popular variations are depicted here. Prism-coupled SPR uses a prism-coupled instrumentation analogous to that used in Total Internal Reflection Fluorescence (TIRF) microscopy (a), while adequate sensitivity has been demonstrated using grating-coupled instruments as well (b). Finally, waveguide-coupled systems have also been used for biomolecular detection (c)[30].

While SPR remains useful for such categorization of drug candidates, its utility is compromised by its reliance on a serial flow cell-implementation. 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 [15, 32-34]. The increased cost and decreased throughput of SPR for detection of small molecule aggregation therefore present challenges that may be addressed with alternative optical biosensor instrumentation.

1.4 – Photonic Crystal Biosensors

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^2 and a large dynamic range [35-36]. As described in previous publications, the PC biosensor is comprised of a subwavelength periodic surface structure that resonantly reflects a narrow band of wavelengths when illuminated with a broadband collimated light source [20-22]. The wavelength reflected from the PC surface is modulated by changes in the refractive index of material within an evanescent field region that extends approximately 300-500 nm from the PC surface into the adjacent liquid media.

Briefly, the sensors are composed of a low refractive index UV-curable polymer (UVCP) molded into a diffraction grating with a 550 nm period and a 200 nm grating depth. This diffraction grating is constructed by pouring the liquid UVCP into a Si master wafer patterned with the desired diffraction grating, and covering the UVCP with a clear PET substrate. After curing the UVCP and removing the Si master, the result is a UVCP diffraction grating attached directly to PET. The grating can then be coated with highly reflective TiO_2, which results in the rejection of light at the resonant wavelength of the sensor. The dimensions specified above, combined with an aqueous medium, allow for a resonant wavelength in the near-IR range. This allows for decreased interference from visible light, and can be monitored with a near-IR spectrometer. Because the sensor and PET substrate are flexible and easily manufactured, they can be fitted to multiwell plates to ease multiplexing, and can be used in conjunction with high-throughput liquid handling systems to further improve throughput. This instrumentation is commercially available in the form of the BIND™ Reader detection instrument (SRU Biosystems, Woburn MA, USA), which was used for the work described here.

The detection instrument illuminates the PC microplate from below with a broadband light source, and uses a spectrometer to measure the peak wavelength value (PWV) of the resonantly reflected light. As biomolecular binding occurs within an evanescent field region, the effective refractive index of the corresponding sensor region is increased. Measuring the PWV before and after addition of the biomolecules allows the calculation of a PWV shift as a result of biomolecule adsorption. The BIND Reader is configured for high-throughput screening, as it is able to gather kinetic data for entire multiwall plates, including 96, 384, and 1536-well plate formats. To do so, the 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 approximately 20 s. The scanning may be repeated to gather kinetic binding information for further characterization of biomolecular binding[22, 23].

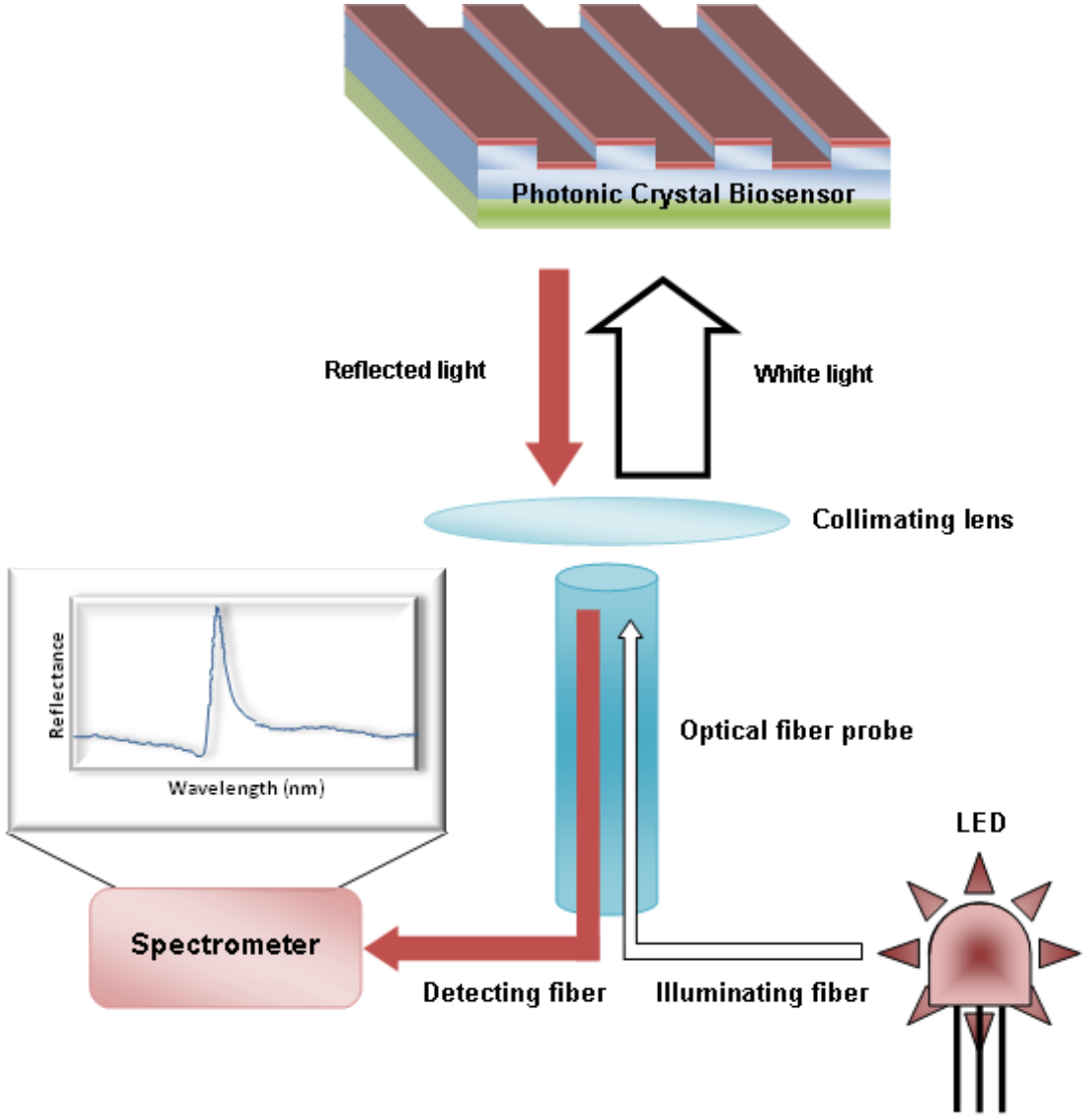


Figure 6: Photonic crystal biosensor instrumentation. The photonic crystal biosensor is illuminated with collimated, broadband white light from an LED source via a fiberoptic probe. The resonant reflected wavelength for each point on the sensor is then relayed through a fiberoptic probe and to a spectrometer that records the peak wavelength value (PWV). When readings are taken before and after addition of biomolecules, it is possible to equate shifts in the PWV for a particular plate well with addition of a particular biomolecule. If the added molecules are drug candidates in the absence of a binding partner, increased PWV shifts are indicative of aggregation, or promiscuous inhibition, therefore negating choice of the compounds for further consideration for pharmaceutical screening [26].

While the BIND system fills the need for high-throughput screening, an alternative imaging method can be used to verify the uniformity of PWV shift across the sensing region. Two types of detection instruments for PC biosensors are used in this work. A second instrument, the BIND Scanner, SRU Biosystems 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 ~22.3x22.3 μm^2/pixel [37-38].

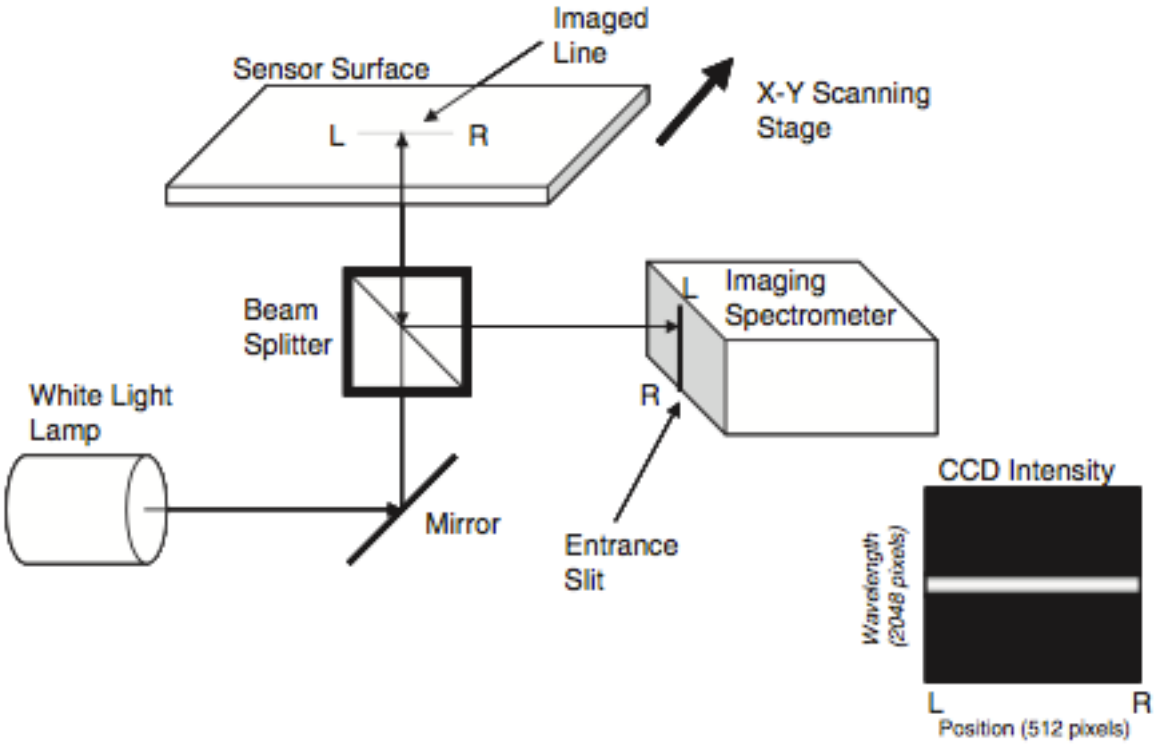


Figure 7: BIND imaging instrument. To verify that PWV shift occurs across the entire PC biosensor surface and is not merely constricted to the center of each well, the BIND imaging instrument can be used. Replacing the fiberoptic detection probes with a beam-splitter and an imaging spectrometer, it is possible to calculate PWV for discrete areas of the sensor, at a pixel resolution of 22.3x22.3μm^2 [36].

Though photonic crystal biosensors have not previously been used for characterization of small molecule aggregation, their utility has been successfully demonstrated in the pharmaceutical screening arena. Because the sensors can be manufactured with relative ease and at a cost amenable to large-scale screening, PC biosensor-based detection of aggregation is a viable solution for several problems currently restricting the efficiency of pharmaceutical screening campaigns. As the PC detection mechanism relies directly on the properties of the

analytes and not on the detection of an intermediate or reaction product, it results in the direct detection of aggregation. This further reduces costs by restricting the quantities and types of reagents necessary for involved assays including enzyme kinetic assays. The work documented here is aimed to provide detailed characterization of the use and suitability of PC biosensor-based detection of small molecule aggregation, and therefore, small molecule aggregation.

In the course of applying recently developed PC biosensor technology for the 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 [39]. 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. These compounds were purposefully selected to have a wide range of molecular weights while possessing core structural similarities to demonstrate the variety with which aggregation can occur, and the robust nature of the PC detection system. 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.

Chapter 2: Materials and Methods

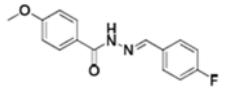
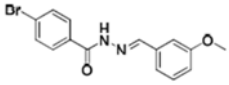
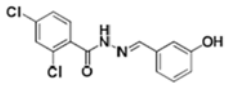
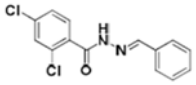
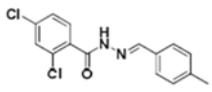
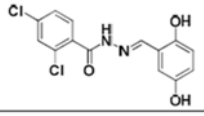
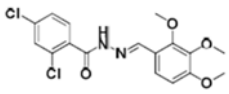
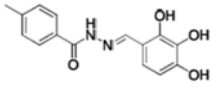
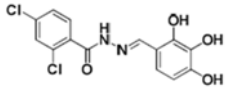
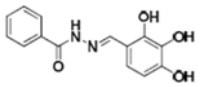
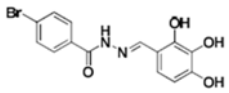
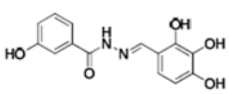
2.1 – Small Molecule Library and Compound Selection

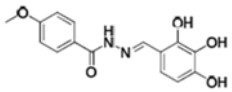
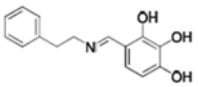
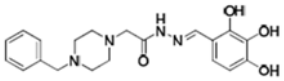
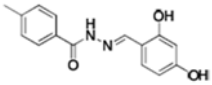
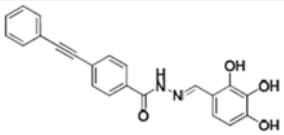
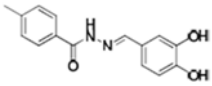
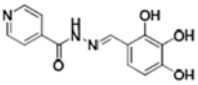
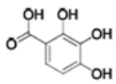
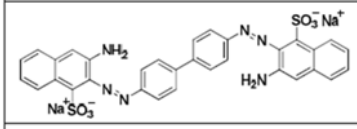
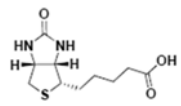
A set of 20 small molecules was selected from an in-house library kept at the University of Illinois at Urbana-Champaign High-Throughput Screening Facility [39-41]. Molecules were maintained as 10mM stock solutions in DMSO. The control molecules biotin and congo red (CR) were also maintained in 10mM stock solutions in DMSO to maintain uniformity between control molecules and library compounds. Congo Red (CR) was purchased from the Agfa-Gevaert Group (AGFA, Mortsel, BEL). Biotin, dimethylsulfoxide (DMSO), phosphate-buffered saline (PBS) solution, and Tween-20 were purchased from Sigma-Aldrich Co. (St. Louis, MO USA) Triton X-100 was purchased from Union Carbide Corporation (Houston, TX USA). All compounds were maintained at -20°C for long-term storage before being transferred to 4°C for the course of experimentation over a period of several weeks. All compounds were allowed to progress to room temperature to ensure proper thawing of DMSO prior to experimentation.

Molecules were chosen to possess a variety of molecular weights and functional groups while demonstrating several similarities in core structure. The compounds have similar core characteristics in the presence of one or more aromatic rings, as well as the presence of several electron-withdrawing groups (EWGs). These characteristics are common among commercially used drugs, as the modification or substitution of particular functional groups can profoundly affect the binding characteristics and inhibitory profiles of the pharmaceuticals, with potentially beneficial or disadvantageous consequences. The selected compounds have a broad range of molecular weights, from 170.1 g/mol to 696.7 g/mol, demonstrating a range typical of commercially available drugs.

Finally, hydrophobicity is an important consideration in the selection of drug candidate compounds, as solubility and permeability vary as a product of the factors listed above. To ensure assay robustness against this variable, the compounds selected were designed to cover a range of hydrophobicity, ensuring that the proposed screening methodology is not biased toward a particular class of small molecules. For a consistent estimate of hydrophobicity, ChemDraw software was used to calculate relative hydrophobicity in the form of the ClogP calculation, where CLogP = log([compound in octanol]/[compound in water]) in a 1:1 mixture of water and octanol. CLogP values for the library compounds range from -3.05 to 4.81. CLogP values above 5.0 indicate exceedingly low hydrophilicity, and therefore low adsorbtion and permeability, and therefore decreased suitability for pharmaceutical use.

Table 1. Small molecule structures, names, and molecular weights.

Chemical Structure	Name & MW 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

Chemical Structure	Name & MW 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
	**CR** 696.7 g/mol -3.05
	**Biotin** 242.3 g/mol -1.33

2.2 – Dynamic Light Scattering (DLS)

Each of the small molecules (Table 1) was allowed to reach room temperature and diluted to 50 mM in deionized (DI) water (0.5% DMSO v/v) to a total volume of 800 μL in a 1 mL glass cuvette. The cuvettes were then placed into the DLS instrument and read for intervals ranging from 10 sec to 2 minutes. DLS spectroscopic 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, and the integration time chosen to minimize fit error for each sample. The default autocorrelation function was used to calculate hydrodynamic diameter and to estimate particle size.

2.3 – α-Chymotrypsin Enzyme Inhibition Assay

A SpectraMax Plus (Molecular Devices, Sunnyvale, CA USA) spectrophotometer (96- or 384-well microplate reader) was first calibrated with a concentration series of α-chymotrypsin (Sigma-Aldrich, St. Louis, MO USA) from 10 ng/mL to 100 μg/mL to determine a fixed enzyme concentration. The 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 while maintaining a dynamic response to changes in reaction rate. The substrate succinyl-AAPF-PNA (Sigma-Aldrich) concentration was fixed at 200 μM in assay buffer (100 mM Tris, 20 mM $CaCl_2$, pH 7.8). After the calibration, each small molecule was diluted to 1, 2.5, 5, 10, 25, 50, 100, and 250 μM 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 μL) and the kinetic OD output was recorded for 30 min at a wavelength of 405 nm.

To compute the reaction rate, the slope was computed for the linear portion (10-15 min as stated above) for the reaction as incubated with each concentration of each inhibitor. A range of compound concentrations was employed to verify that inhibition was dose-dependent, and not merely an inconsistency for a given compound. No incidence of inconsistencies was observed with regard to the dose-dependence of inhibition, though the ability to resolve differences in reaction rate consistently declined beyond 10 μM concentrations and lower. To simplify display of the results, only data for the highest concentration of inhibitor (250 μM) are displayed in Results.

2.4 – PC Biosensor Detection of Aggregation

TiO_2-coated, 384-well BIND™ sensor microplates and streptavidin (SA2)-coated 384-well BIND™ sensor plates were purchased from SRU Biosystems, Inc (Woburn, MA, USA). After washing three times with 80 μL distilled ionized water (DI), the TiO_2 and streptavidin-coated 384-well sensor plates were stabilized with a well volume of 15 μL with four 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 Congo Red. The minimum concentration of CR detected as demonstrating aggregation above background levels was 2.5 μM, and saturation was observed at 50 μM. The assay appeared to demonstrate decreased sensitivity to aggregation at concentrations lower than 50 μM, with a marked decrease in PWV shift at concentrations less than 12.5 μM. In deference to this information, the small molecules within our library (including controls biotin and CR) were allowed to come to room temperature before being prepared at 50 μM with the buffers listed above and 15 μL of each compound was added to the previously equilibrated plate. The PWV shift for each sample was then recorded as a function of time for approximately 5-6 h. Following this read step, the PC biosensor plate was washed with the corresponding buffer for each well, and the PWV shift was assessed before and after the wash step. Endpoint readings refer to data taken after the wash step.

2.5 – Imaging Small Molecule Aggregation on PC Biosensors

To verify that PWV shifts observed in the PC Biosensor procedure described above, PWV shifts of the small molecules at a concentration of 50 mM were monitored using an uncoated PC biosensor microplate with an exposed TiO_2 surface and DI as a solvent. After washing the uncoated sensor three times with 80 μL DI water, the plate was stabilized with 30 μL water for approximately 30 min, using the BIND Reader to establish the point of equilibration. Images were then constructed using a BIND Scanner (SRU Biosystems, Woburn MA USA) at a pixel resolution of 22.3 x 22.3 μm^2/pixel after stabilization with DI water (baseline image) and again after a 5-6 h aggregation period conducted at RT (aggregation image). In order to record the PWV shift due to aggregation, the registered baseline image was subtracted from the aggregation image on a pixel-by-pixel basis, resulting in a final PWV shift image. To increase contrast over the relevant range, the PWV shift depicted in the image was set from -1.40 to 2.10 nm with a user-selected color map as displayed in the color bar.

2.6 – Scanning Electron Microscopy (SEM)

For further verification that observed results on the BIND sensor correlated with the physical aggregation of library compound molecules on the surface of the device, scanning electron microscopy was performed on devices incubated with aggregating and nonaggregating compounds. Uncoated PC biosensor microplate wells with an exposed TiO_2 surface were incubated with 3 μL of each small molecule (50 μM 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 ~50 Å 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 mm under focus at varying magnifications.

2.7 – Measuring the Effects of Detergent Using PC Biosensors

To assess the detergent-mediated inhibition of aggregation, streptavidin-coated 384-well sensor plates were temperature-stabilized in 15 μL PBS or PBS + 0.05% Tween-20. During stabilization, small molecules were permitted to come to room temperature before being diluted to a concentration of 50 μM in PBS or PBS with 0.05% (v/v) Tween-20. Following dilution, 15 μL of each sample was added to the stabilized biosensor and incubated for approximately 5-6 h while being and scanned with the BIND Reader. After concluding the incubation period, wells were rinsed with 30 μL of the corresponding buffer (PBS or PBS with 0.05% (v/v) Tween-20).

For establishment of dose-dependence of the observed detergent-mediated inhibition of aggregation, PBS was prepared with varying percentages (v/v) of Tween-20: 0.00, 0.01, 0.05, 0.10, 0.5, 1.00, and 5.00%. CR was prepared with each buffer to in a dilution series ranging from 0.4-50 mM and 15 μL 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 refer to the PWV values obtained after the wash step.

Chapter 3: Results

3.1 – Motivation and Initial PC Biosensor Results

As noted in the Introduction, we discovered several compounds that showed binding capabilities in excess of expected stoichiometric values in a previous large-scale screening experiment [26]. Figure 1 shows data representative of this phenomenon for the dye Congo Red. Congo Red has been demonstrated as prone to aggregation, and has also demonstrated an ability to inhibit enzyme-catalyzed reactions, presumably through the sequestration mechanism summarized above (Introduction). Similar results were obtained for several other documented aggregating compounds, including the compounds Rose Bengal [11] and Indigo [12]. The large binding signals elicited from these compounds were consistently several times the binding signal of a non-aggregating negative control compound (biotin, shown in Figure 1), and would occur even upon surfaces that were blocked against biochemical binding.

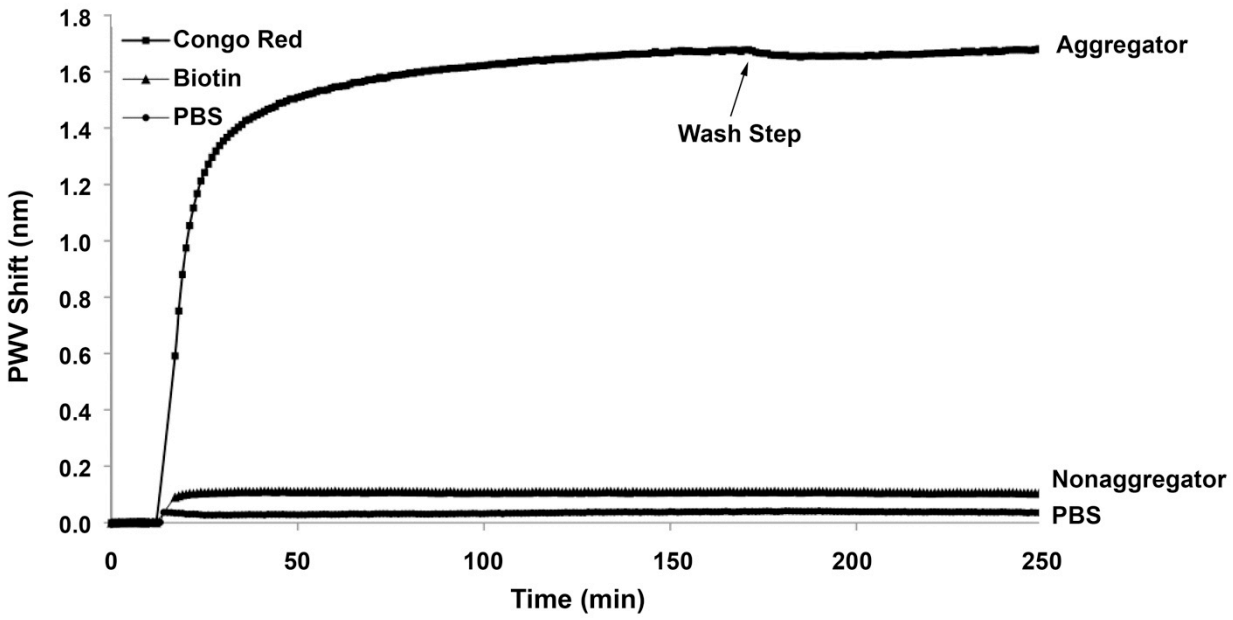


Figure 8. Kinetic plot of PC biosensor PWV shift as a function of time for a typical aggregating compound (Congo Red) and a nonaggregating compound (biotin). Both molecules were introduced in PBS buffer with 0.05% DMSO to separate biosensor microplate wells treated with streptavidin. Aggregation is characterized 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 (3 times with PBS) does not remove the aggregated material from the sensor surface.

Upon closer examination of the binding profiles presented by these aggregators, we noted that the signal failed to reach a stable reading, indicating the continued adsorption of small molecules to the BIND sensor. As discussed earlier, such an increase is likely attributed only to a direct increase in the local refractive index of material within the evanescent field region. Because the only compound added to the buffer was Congo Red, we assert that the increased signal is due to the deposition of additional Congo Red. Supporting this assertion, biotin (an established nonaggregator) results in a Peak Wavelength Value (PWV) shift that is several times lower than that displayed by Congo Red. These observations merit consideration of the PC biosensor system as a detection system for aggregating small molecules. To pursue this study in further detail, we compiled the small molecule library listed above and conducted a number of studies on each molecule using screening methodologies currently employed in the pharmaceutical screening and biotechnology industries.

3.2 – Dynamic Light Scattering (DLS) Spectroscopy Results

The compounds evaluated in this study are listed in Table 1 (Materials and Methods). Library compounds 1-20 were previously uncharacterized as aggregators, while negative controls (DMSO, biotin, buffer) and positive controls (Congo Red and a solution of 100nm beads) were used for comparison. Results of the DLS measurements can be seen 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 hydrodynamic diameter of the particles formed in solution and, therefore, aggregation. The small molecules with high scattering intensity (approximately 300 Hz or greater) include compounds 2, 6, 14, 16, 17, and CR, along with biotin. Low-scattering (approximately 100Hz or fewer) molecules include compounds 1, 3, 4, 5, 7, 8, 9, 10, 11, 15, 19, and the 100 nm bead control. The efficient scattering presented by biotin is interesting in that it is at odds with its low CLogP value and high solubility. This point of contention is evidence of the characteristic unreliability of DLS as a high-throughput screening tool. Furthermore, the information gained through DLS spectroscopy consistently indicates that biotin is capable of forming aggregates that are approximately 300nm in diameter, when this is simply not characteristic of the compound. While useful when the critical assumptions of uniformly sized and spatially distributed particles are valid, DLS spectroscopy remains a tool of limited utility in the drug screening environment due to the significantly variable properties of small molecule candidate libraries.

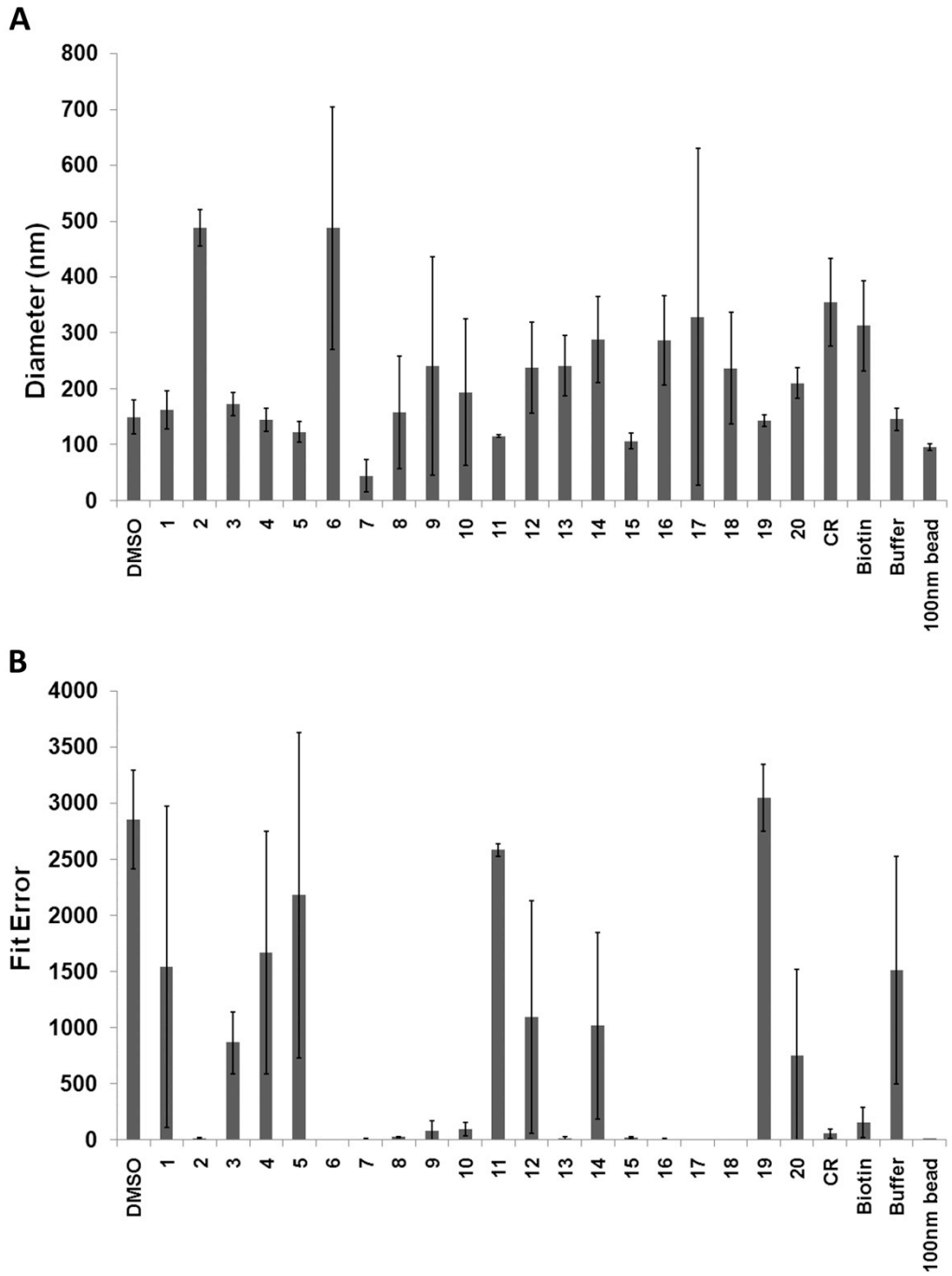


Figure 9: DLS Spectroscopic Analysis for Small Molecule Library. Compounds with greater than 300 Hz scattering intensity signify increased hydrodynamic diameter of the particles. Such compounds include compounds 2, 6, 14, 16, 17, and CR. Compounds with reduced scattering intensity (fewer than 100Hz) include compounds 1, 3, 4, 5, 7, 8, 9, 10, 11, 15, and 19, in addition to the 100nm bead control. (b) DLS fit error for the size measurements obtained in (a). Note that large standard deviations in diameter measurements do not necessarily correlate with large fit errors, indicating that other factors may be responsible for these inconsistencies.

Further examination of the DLS data gives a measure of insight as to the unreliability of DLS spectroscopy for this application. The DLS instrument provided a fit error value for each compound (Figure 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 100nm bead control sample. Additionally, 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. Finally, the fit error for DMSO and for the vehicle control sample are significantly greater than the 100 nm bead control, lending a further decrease in credibility to the DLS assay. These consistent false positive results are indicative of the fact DLS may be limited in the detection of the types and sizes of aggregates formed by drug-like compounds.

3.3 – α-Chymotrypsin Assay Analysis

Inhibition of α-chymotrypsin was quantified in terms of reduction of the base reaction rate. This reaction rate was determined through the calculation of the slope of the linear portion of data generated from the increase in absorbance at a wavelength of 405 nm over time caused by cleavage of succinyl-AAPF-PNA by α-chymotrypsin. As the reaction continues, absorption at 405 nm increases due to the increased formation of the colorimetric product, paranitrophenolate. Reading entire 384-well multiwell plates allowed multiplexing this assay, although the use of several plates was required to run all combinations of drug candidate concentrations in parallel with uninhibited reactions. The linear portion of the graph (0 - 15 min) was used for slope calculation and comparison to potential inhibition or activation by our compound library. After calculating the reaction rate for each concentration of compound, the resulting rates were normalized to the rate established in the absence of small drug-like molecules (DMSO/vehicle control) to ease comparison. This assay was repeated three times with similar results, displayed in Figure 10. DMSO was run as a vehicle control, while CR and biotin were used as positive and negative controls for inhibition, respectively. To establish dose-dependence for the inhibitory or activating activity displayed by each compound, this experiment was run with a range of concentrations for each of the drug-like compounds in the small molecule library. Concentrations ranged from 1μM to 250μM, and are specifically listed in Materials and Methods. To highlight the reaction-altering properties of the compound library in this assay, the highest concentrations (250 μM) of compounds 1-20, CR, and biotin are depicted in Figure 3. All

percent activity data were normalized to the slope of the line generated from DMSO treated α-chymotrypsin and substrate. Note that several apparent increases in activity occur with compounds previously described as promiscuous inhibitors (CR). This discrepancy may be attributed either to the possibility that the compounds enjoy some spectral overlap with the reaction product, *p*-nitrophenolate, at the wavelength measured by the spectrometer. This highlights a potential weakness of enzyme inhibition assays in general. To rule out the possibility that spectral overlap is responsible for perceived increases in reaction rate, it would be necessary to repeat the assay several times with differing enzymatic reactions and differently colored substrates and reaction products. This would result in substantially increased costs as well as decreased reliability, as sourcing and establishing the efficacy of each batch of enzyme are costly enterprises, in terms of both time and physical resources.

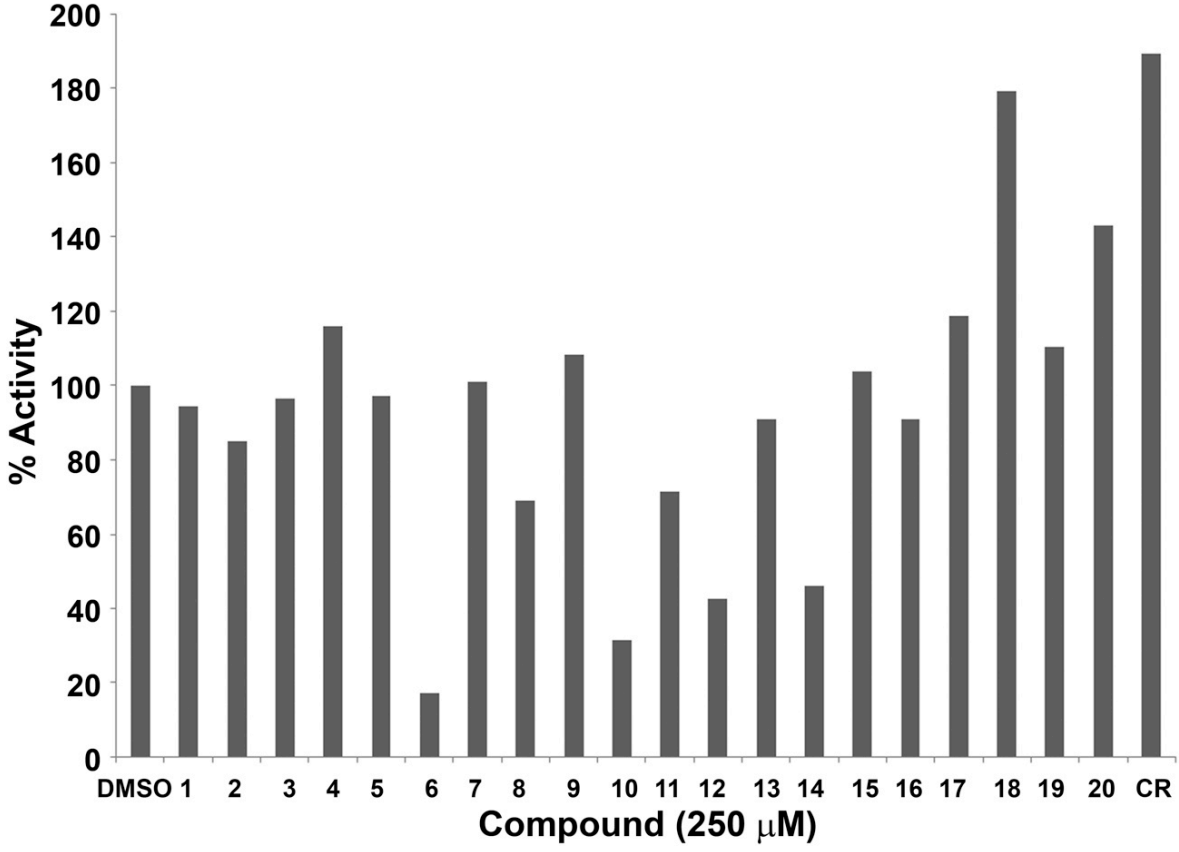


Figure 10: α-Chymotrypsin Inhibition Assay. Each molecule in the screening library was incubated in context with an α-chymotrypsin reaction with the colorimetric substrate succinyl-AAPF-PNA. Inhibitory or activating activity can be observed, as the results were normalized to unaltered progression of the reaction with the vehicle control, DMSO.

3.4 – Aggregation Detection on PC Biosensors

PC biosensor-based assays introduced a reduction in assay complexity as compared to the enzyme inhibition studies documented above. As the PC biosensor results respond directly to the increased mass of an aggregating compound near the sensor surface rather than on an indirect property, meaningful results can be obtained simply from incubating the compounds on the sensors at an optimal concentration. To determine the concentration optimal for establishing the presence or absence of aggregating activity, the PC biosensor assay was run at a series of concentrations from 1 μM to 250 μM with the compound Congo Red, a known promiscuous inhibitor. While detection of a binding profile similar to that shown in Figure 7 was determined for concentrations as low as 12.5 μM, the PC biosensor assay possessed decreased sensitivity at concentrations less than 50 μM. As a result, screening experiments for all molecules took place at a concentration of 50 μM to maximize sensitivity to aggregation while minimizing compound use. The PWV shifts recorded for each compound in the small molecule library are shown in Figure 11. Congo Red was used as a positive control, while biotin, buffer, and the vehicle control (DMSO) serve as negative controls for aggregation. Relative to the negative control samples, the PC biosensor detection instrument recorded an increase in the PWV for several of the compounds, including compounds 6, 8, 9, 10, 11, 12, 13, 15, 17, 19, and 20. Although the sensor surface was washed rigorously with buffer 3 times the wavelength shift remained, indicative of a binding interaction. These results were interpreted as evidence of prolonged nonspecific attachment of material to the sensor surface as a direct result of compound aggregation.

Several compounds incubated on the PC biosensors displayed little to no PWV shift, indicating that there was no bias as to where the molecules settled or bound after prolonged incubation. Though the compounds were added under the same conditions as those highlighted above, compounds 1, 2, 3, 4, 5, 7, 14, 16, and 18 showed little to no binding activity. This indicates that the compounds likely remained in solution, as no evidence of aggregation could be documented via PWV shift. The results for these compounds were similar to those for the three negative control samples. Samples were run in triplicate, and error bars represent one standard deviation above and below the mean. As shown in Figure 11, PC biosensor results were observed to be relatively consistent among replicates.

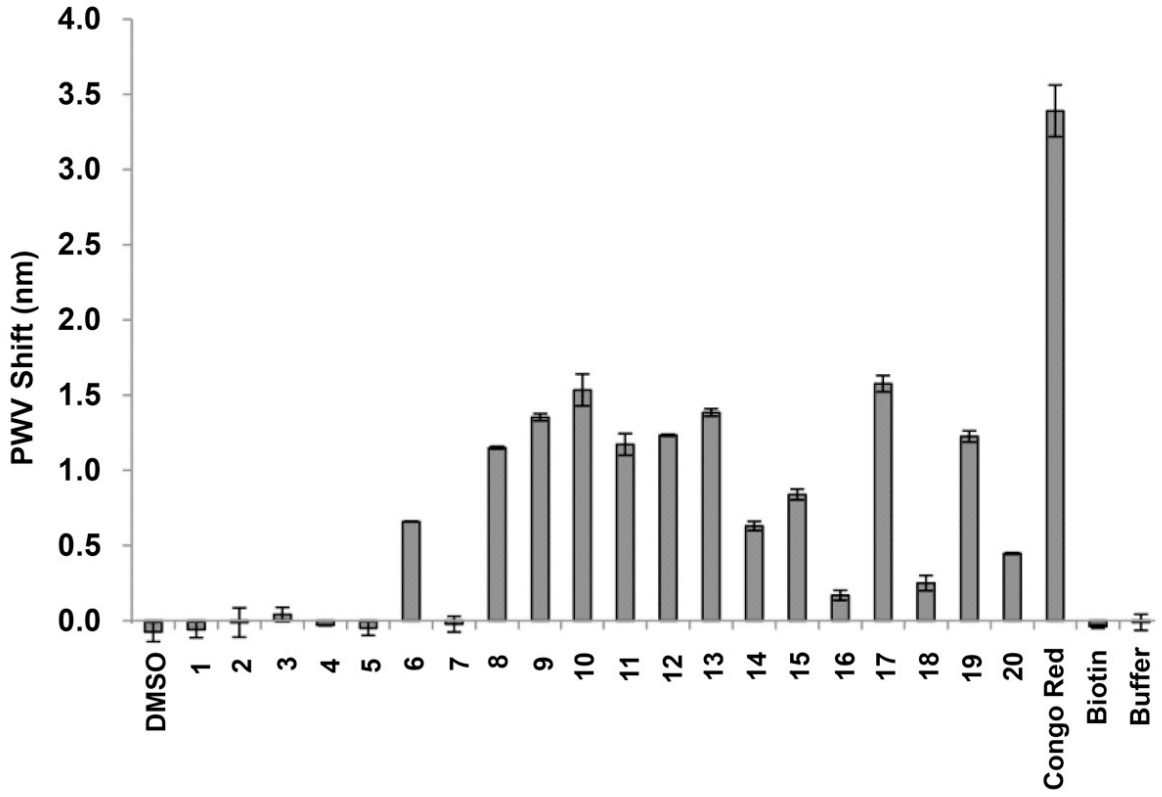


Figure 11: PC Biosensor-Based Detection of Aggregation. Each molecule in the screening library was incubated at a concentration of 50 μM in PBS in a PC Biosensor microplate for a period of 6 h at RT. After the incubation period, all wells were rinsed three times with 80 μL DI water, after which the endpoint read displayed above was taken. A DMSO vehicle control was run to verify that PWV shifts were not a product of bulk refractive index shifts.

3.5 – Aggregation Imaging on PC Biosensors

Compounds 1-20 provided a trial set for the ability of PC biosensors to detect aggregators. To verify that these readings were the result of consistent and reproducible shifts in PWV, we confirmed results for a subset of molecules chosen to exemplify aggregating and nonaggregating activity as observed on the PC biosensor surface. Specifically, based on the PC biosensor data in Figure 11, compounds 1 and 2 were selected for further study as non-aggregators, while compounds 8 and 19 were selected as aggregating small molecules. After

washing and temperature stabilization in PBS, PC biosensors were incubated with each of these compounds at the screening concentration of 50 μM in PBS for a period of approximately 6 h. Using the BIND imaging instrument, pre- and post-incubation scans were obtained for comparison and calculation of a PWV shift image, each displaying peak wavelength value as a function of position over the entire PC biosensor microplate. Using image registration, the pre-incubation PWV image was subtracted from the post-incubation PWV image, and the results were recorded as displayed in Figure 12 below. 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 (DMSO) showed no appreciable binding signal. The PWV shifts recorded by the imaging detection instrument are of magnitudes consistent with those measured using the optical fiber probe detection instrument (approximately 1.0 and 1.2 nm for compounds 8 and 19, respectively). The PC biosensor imaging data show 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. This would seem to indicate that aggregation formation occurs at sizes consistent with the order established during the DLS assay, well under 1 μm in diameter, and so sufficiently below the pixel resolution of the BIND imaging instrument (22.3μm x 22.3μm/pixel).

Upon closer inspection of the PC biosensor imaging data, there appears to be a prevalence of PWV shift on the outer boundary of each well. This may be a reflection of increased deposition on the plastic sidewalls of the well plate. An alternative explanation would be preferential deposition at locations containing the UV-curable polymer used to secure the biosensor to the well plate. Because the polymer is pressed between a bottomless well plate and the PC biosensor before being cured, excess polymer can leak slightly into the well during fabrication. Such a surface may provide additional incentive for compounds to adsorb at these locations. The consistent increases in PWV shift at the bottom right corner of each well are due to mechanical degradation of the sensor due to rough handling of the pipette tip used for aspiration. Because the illumination spot of the BIND Reader remains within the center of each well, it is unlikely that effects of either of these observed mechanical defects could be observed in the PC biosensor screening data discussed earlier.

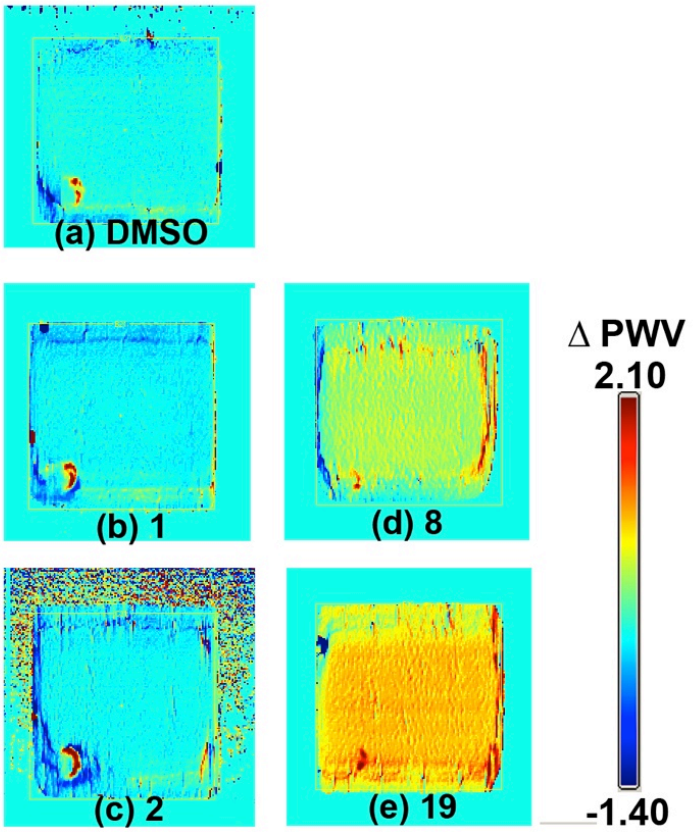


Figure 12. PWV shift images of selected individual 384-well microplate wells, gathered with the PC imaging detection instrument, demonstrating uniformly high levels of aggregation distributed across the biosensor surface for several compounds ((d) 8, (e) 19) and lack of wavelength shift for two non-aggregator compounds ((b) 1, (c) 2) and (a) DMSO reference solution.

3.6 – Confirmation of Small Molecule Aggregation on PC Biosensors via SEM

To provide further verification that the results observed on PC biosensors were indicative of physical aggregation of small molecules, scanning electron microscopy was used to qualitatively demonstrate the presence of compound aggregates as they occur on the biosensors. The small molecules described in Figure 12 were incubated on PC biosensors, using 3 μL of each compound at 50 μM, including DMSO as a negative control. Experiments using sensor surfaces treated with the vehicle (DMSO) and compounds 1 and 2 resulted in a clean grating surface image when examined by SEM, in which no particulates or other deposits could be observed (Figures 13a, 13b, 13c) across the sensor surface.

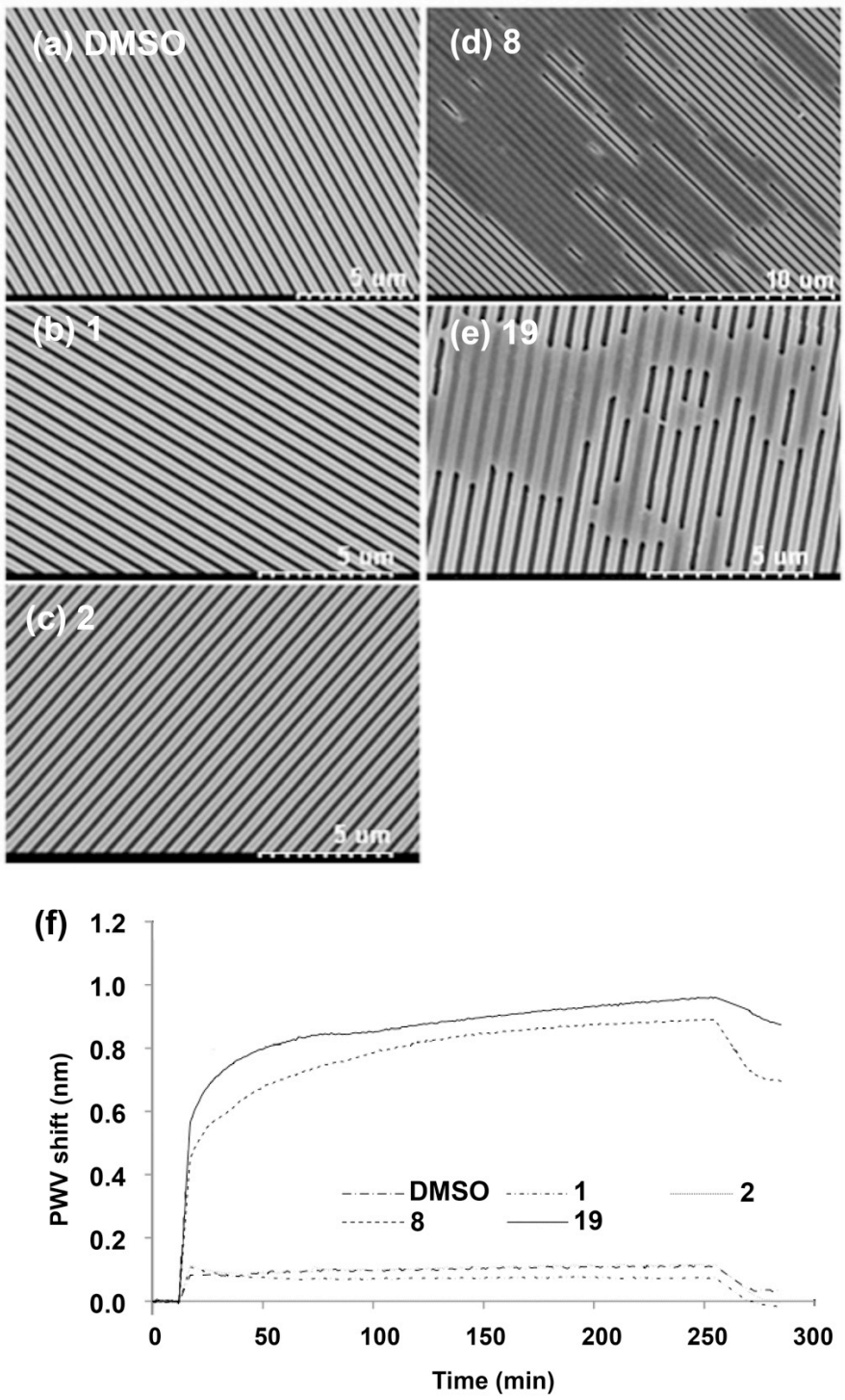


Figure 13. SEM images of (a) DMSO, compounds (b) 1, (c) 2, (d) 8, and (e) 19. The two aggregators (8, 19) showed gel-like substance attached on the sensor surface, while such substance could not be located on the non-aggregator and DMSO surfaces. A kinetic plot containing all five molecules shows that (f) the aggregators slowly increased to a high PWV shift signal even after a rigorous washing step.

In contrast, the sensors incubated with 50 μM concentrations of compounds 8 and 9 (that had registered a positive binding signal in the PC aggregation assay) exhibit a gel-like substance attached to the surface when imaged by SEM (Figures 13d, 13e). 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 entire sensor as suggested by the PC biosensor imaging measurements shown in Figure 12. The material has the appearance of a thick film with a highly variable shape capable of conforming to the grating period and filling gaps in the diffraction grating surface. These films are characteristic of the shapes of small molecule aggregators and promiscuous inhibitors reported by Shoichet et al. [11]. In no case were aggregate formations in the shape of round or spherical objects observed.

For a direct comparison of the long-term aggregation present with compounds 8 and 19 and the non-aggregating compounds 1 and 2, the kinetic data from the PC biosensor screen are displayed in Figure 13f. Aggregating compounds display a kinetic profile similar to that of Congo Red, while the nonaggregating compounds and vehicle control display little in the way of binding activity (as displayed by PWV shift), and reach stable values with relative ease. The aggregating compounds, by contrast, enjoy a steep initial increase in PWV shift, followed by a gradual increase, despite hours of incubation given to achieve stabilization. As with Congo Red, this continued lack of stability likely stems from continued deposition of aggregating small molecules on the sensor surface and within the evanescent field region, indicative that these molecules possess non-specific binding activity.

3.7 – Detergent-based inhibition of small molecule aggregation

Previous studies have reported that small molecule aggregation-induced inhibition of aggregation can be ameliorated through the use of detergent compounds such as Tween-20 and Triton X-100. To verify that PC biosensor-based detection sensitivity is capable of detecting detergent-induced restriction of small molecule aggregation, the PC biosensor screening experiment was repeated with and without the use of Tween-20. Each small molecule was allowed to progress to room temperature before diluting to 50 μM in PBS with and without 0.05% (v/v) Tween-20 and adding to a temperature-stabilized PC biosensor. The results are documented in Figure 14a, and show universally decreased response among aggregators as detected using the PC biosensor assay (Figure 11). Peculiarly, samples that do not show

aggregation-like activity in the PC biosensor assay exhibit a slight increase in PWV shift with the addition of detergent. This would seem at odds with the mechanism of the detergent, which relies on micelle formation to sequester individual particles from interacting with one another. One possible explanation for this observation could be that the negative charge imposed by the detergent molecules results in a slightly increased incentive for small molecule binding of the sensor surface. This slight increase, however, is much smaller in magnitude than the decreased PWV shift observed with documented and putative aggregating small molecules.

To verify that these reductions in aggregation were consistently dose-dependent, the most prominent example of aggregation (and aggregation reduction) was chosen. Congo red was shown the experience a 50% decrease in the observed PWV shift value in the presence of only 0.05% Tween-20, so it was established as the compound for studying dose-dependent reduction of aggregation through the use of detergents. As displayed in Figure 14b, 0.00%, 0.01%, 0.05%, 0.10%, 0.50%, 1.00%, and 5.00% (v/v) Tween-20 in PBS were incubated with concentrations of Congo Red ranging from 0.1 μM to 50 μM for a period of 5 h in PC biosensor microplates. The microplates were then rinsed three times with 30 mL DI water before obtaining an endpoint reading for the assay. Results were recorded as displayed in Figure 14b. The logarithmically increasing concentrations of detergent seem to have a roughly linear affect on binding affinity, with negligible reductions in signal occurring at all Congo Red concentrations with Tween-20 concentrations below 0.05% (v/v). Notably, the difference between 0.00% and 0.01% (v/v) is greatly reduced in comparison to the reduction observed for subsequent detergent increases. The reduction in PWV shift resulting from the next concentration (0.05% (v/v) is approximately 60% across the range of concentrations of Congo Red.

These results provide further confirmation that PC biosensors can be used to confirm the presence of aggregating small molecules, and that they can be used to investigate which factors might be responsible for reversing or inhibiting undesired aggregation. Finally, although the binding curves remain relatively consistent, there are some areas of overlap at the lower analyte concentrations. Because sensitivity to analyte binding has been noted as limited at concentrations below 12.5 mM, it is likely that PC biosensor assessment of aggregation below this concentration is currently unreliable. The use of higher precision liquid handling systems may allow further decreases in achievable detection limits in the future, but screening concentrations should remain at 50 μM until such inconsistencies can be resolved.

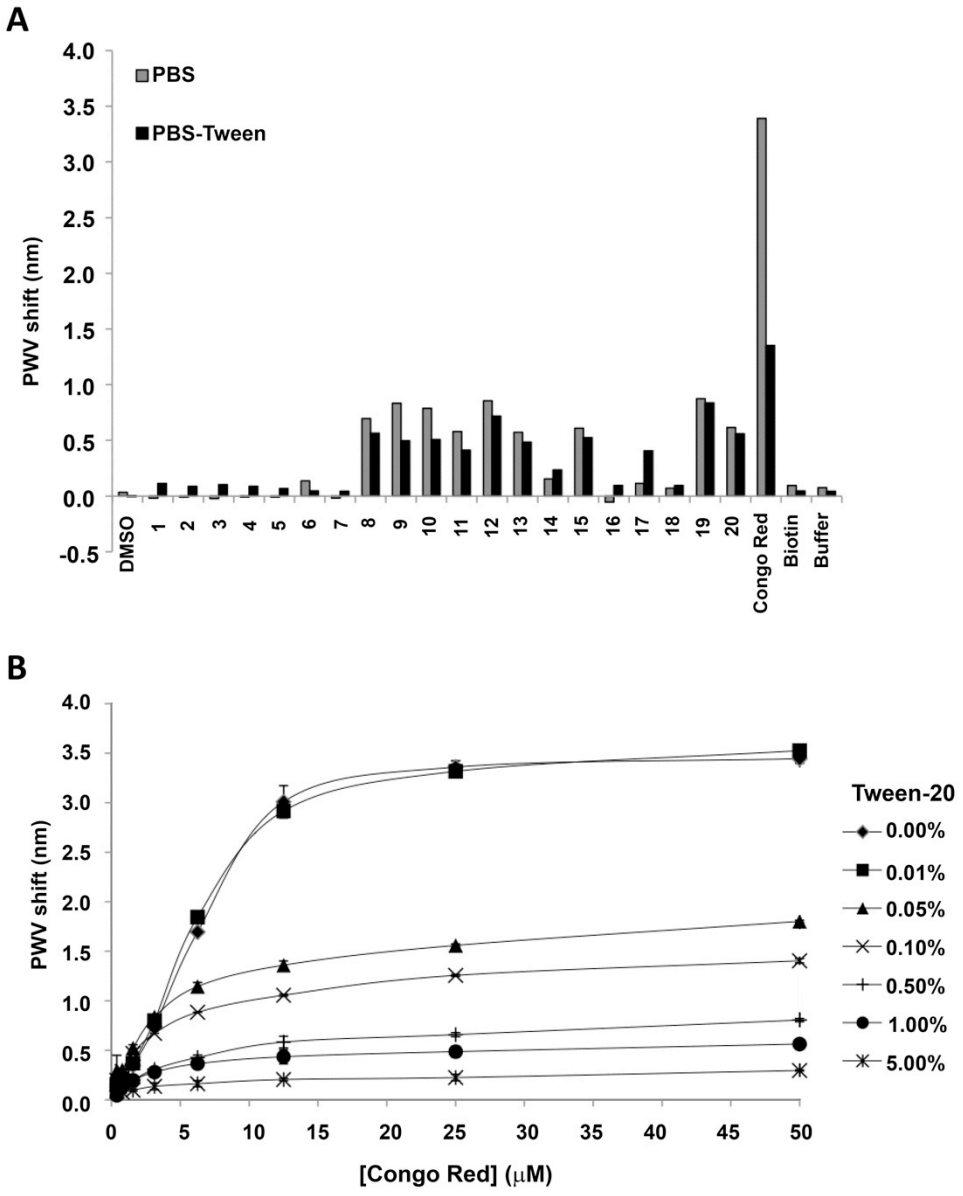


Figure 14: Detergent-based inhibition of small molecule aggregation (a) PC biosensor aggregation measurements showing the effect of 0.05% Tween-20 added to the buffer as a method for reducing compound aggregation. (b) A plot of PC biosensor aggregation signal as a function of aggregator (Congo Red) concentration for Tween-20 percentages of 0-5%, demonstrating that as detergent percentage increases, the aggregation signal decreases, and that aggregation is also dependent upon the concentration of the compound.

Chapter 4: Discussion

The goal of the work presented in this paper was to present PC biosensor assays as a direct means for detecting aggregation of small molecules in the context of pharmaceutical screening assays. In liquid media exposed to the PC biosensors, aggregating compounds appear to result in deposition of material upon the sensor surface; this manifests itself as a substantial increase in the PWV, simplifying the process of these problematic aggregating compounds. To demonstrate the potential utility of this method for pre-screening aggregators from a compound library, the PC biosensor aggregation method was tested for agreement against other commonly used techniques. Additional validation studies included the verification of commonly used strategies for reducing aggregation, and namely whether 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 showed limited evidence of aggregation for several compounds (1, 2, 3, 4, 5, 7, 14, 16, and 18) in addition to three negative controls (DMSO, biotin, and buffer). Several compounds resulted in measured aggregation with the PC assay (6, 8, 9, 10, 11, 12, 13, 15, 17, 19, and 20), in addition to the positive control (CR) (Figure 11). 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 the PC biosensor 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 to 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 observations suggest that the aggregation was not the result of a precipitate and that the measured signals were not caused by such spurious effects as changes in the bulk refractive index of the small molecule buffer. Additionally, the lack of bias toward a particular class of molecules regarding molecular weight, charge, functional group, or hydrophilicity presents the PC biosensor detection assay as an objective method for detection of aggregation. By contrast,

precipitating and colored compounds can cause significant concern for the DLS spectroscopy assay as well as enzyme inhibition assays, respectively.

Although DLS is often used in the pharmaceutical screening industry to measure the size of dispersed particles in solution, dynamic light scattering was not useful for characterizing the aggregations of the compounds in the panel studied in this work. Multiple readings showed disparities among the results for each small molecule, hampering data collection and compromising its validity. Particle diameter measurements of all 22 samples were obtained (including the negative controls), but with typically large standard deviations (Figure 2) 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 an even distribution of uniform spherical particles [14, 15]. However, if the particles do not fit this model, the results can be 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 also 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 sec – 2 min.

Enzyme-based assays remain another commonly used HTS tool 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, however, 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[16]. Therefore, this detection method limits reliability and throughput in achieving the goal of accurately identifying possible aggregators within a small molecule library.

To verify that aggregation observed on the PC biosensor as interpreted from increased PWV shift was indeed the result of physical adsorption of small molecules, physical inspection was performed using SEM to examine the PC biosensor surface. Two aggregators (8, 19), two non-aggregators (1, 2), and one reference sample (DMSO) were examined and compared using SEM. Surprisingly, islands of thick films were found on the surface of the PC sensors exposed to the potential aggregators (Figure 13) and it is probable that these deposits caused the large measured PWV shifts. The observed deposits were absent from sensors exposed to non-aggregators, as well as from the sensors exposed the vehicle control (DMSO). The same samples were scanned using the PC imaging instrument, showing that these deposits are uniformly present over the entire sensor surface area and that the deposits cause a large positive in shift in PWV (Figure 12). These observations are again consistent with large scale deposition of aggregating small molecules on the sensor surface through a mechanism that differs from simple precipitation, and that agrees with previously documentation concerning aggregating small molecules.

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. Additionally, such studies are warranted for borderline compounds in order to learn more about the nature of their aggregation and possible mechanisms or molecular alterations by which such aggregation may be restricted. To this end, we conducted an experiment to confirm that the PC biosensor can be used to observe the effects of detergent on possible aggregators, as well as for related studies in the future. For these experiments, Tween-20 (0.05% v/v) was mixed with the compounds in PBS and a decrease in the aggregation signal occurred for most of the small molecules, including all of the significant aggregating compounds. Congo Red, the most prolific aggregator, showed the greatest reduction (60%) 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 (Figure 14). Decreased PWV shift in response to increased detergent concentration supports the hypothesis that PWV shift as described here is proportional to a given small molecule's propensity for aggregation.

Chapter 5: Conclusion

In this work, the use of PC optical biosensors in a 384-well microplate format is established as a means for identification and quantitative characterization of small molecule aggregation effects. The PC biosensor 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. 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. Importantly, the PC detection assays used in this study showed no bias with regard to molecular weight, composition, hydrophilicity, color, or solubility, indicating that the assay is objective and robust in the face of many variable encountered in high-throughput screening.

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