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Engineering Principles in Cell Biology
Patent Project Part II

Patent Exploration of Synchrotron Biosciences: Small-Angle X-Ray Scattering and Circular Dichroism Technologies

Background:

It is desired that both synchrotron radiation circular dichroism (SRCD) and small-angle x-ray scattering (SAXS) be applied to a high throughput screening (HTS) assay in search of pharmacologically active ligands. These ligands will target proteins including, but are not limited to viral envelope proteins, human receptors, enzymes for transcription, proteolytic modification and bacterial proteins essential for pathogenesis. This will require special consideration of how the biological samples could be transported to and targeted by a synchrotron beam. X-ray transparent microplates, UV transparent cells, cell changers and microfluidic capillary systems coupled to synchrotron beamlines are generally unavailable and unpatented tools which are in demand. The fabrication of these tools will be used to 1) transport materials quickly to a synchrotron beam and 2) allow data collection from the biological material while inside this apparatus or transported to a new apparatus especially for data collection. The said apparatus may be one of the aforementioned plates, cells of capillaries.

Small-angle x-ray scattering (SAXS) is a physical diffraction process which occurs at the atomic level. Intermolecular spacing in non-periodic materials can range from the tens to thousands of angstroms (Å). Opposed to many x-ray diffraction experiments on crystals, biological samples can be examined by SAXS in an amorphous or mesomorphic state. Amorphous materials exhibit no regular order, opposed to the repeating lattice formation of crystals. Mesomorphic states of matter are an intermediate between crystalline solids and isotropic liquids which diffract light.

This technique is useful for the biologist who seeks study the structure of a macromolecule in solution, thereby negating the difficulties of growing crystals from a bioactive material. The sample may be a protein, nucleic acid, or a complex of these macromolecules. Results from a solution SAXS experiment can indicate a conformational change upon ligand binding or assist in model building of complexes at low resolution in accordance with crystal structures. SAXS measurements can produce the molecular weight, size, and shape of the sample. The radius of gyration (R_g), length distribution function ($P(r)$), and maximum dimension (D_{max}) are a quantitative analysis of global conformation and can be produced for proteins in solution with various small molecule inhibitors. Currently at Brookhaven National Laboratory (BNL), SAXS is available at X21 of the National Synchrotron Light Source (NSLS). Equipped with a wiggler, the beam operates at 8KeV producing a 0.5 mm x 0.5 mm beam which can be shown on glass capillaries.

Synchrotron radiation circular dichroism (SRCD) is an emerging technique available at six beamlines worldwide, two of which are located in the U.S., both at the NSLS. Conventional CD is found in many labs; however SRCD provides more flux

which allows the user to collect data at lower wavelengths unavailable on bench-top equipment. Compiling CD measurements over a broader spectrum results in a more complete waveform which describes the structure more completely. The low wavelength region of the vacuum ultraviolet (VUV) contains additional electronic transitions adding to the information content of a waveform. SRCD data will be truncated at 168 nm due to the absorption of water. Little is known about the specific structural contributions in the VUV (under 200 nm), as data is scarce. The availability of this data is due to the high signal-to-noise ratio obtained by nitrogen purging and UV transparent calcium fluoride cells at the beamline.

The strength of CD relies on the fundamental chiroptical properties of proteins in which backbone fluctuations result in convolutions of spectra. Polypeptide chromophores exhibit electronic transitions within a molecular orbital as a function of secondary structure which generate peaks at particular wavelengths. Essentially, a particular CD spectrum will be representative of a protein or nucleic acid's backbone conformation.

Patent Search for “High Throughput Screening”:

This sector contained the most patents related to the invention proposed by this author. A patent search for “high throughput screening” in the abstract of the U.S. Patent Database produced 1164 results. More specific searches of the patent database for the terms “high throughput protein screening assay” produced results between one and two hundred results. Many of these inventions are applied to specific targets such as G-protein coupled receptors, lipoproteins and their cholesterol receptors, monoclonal antibodies, cancer markers, glycosylated proteins, growth factors, DNA libraries, inhalation anesthetics, and other pharmaceuticals. Therefore, the theoretical patent in preparation will deal with only specific pathological proteins from viruses or bacteria.

Due to the volume of patents in this field, the search for inventions applicable only to the assays in development will be described. This does not limit the assay technique, but will instead focus on the methods for handling large arrays of samples, the movement of fluids between chambers and general assay design. Several of these screens are designed to specifically test small fluid volumes for favorable crystallization conditions. However, we are interested in a high throughput test which can demonstrate a conformation change upon binding of a ligand.

It is also important to note the challenges in HT design. Many of the problems with carrying out proposed assays lie in the ability to move small volumes of fluids to specific locations where they can be imaged by the beam of light. Therefore, patents in HTS are often coupled to microfluidics plate design as well.

United States Patent 7,130,747: High throughput screening of ligand binding to macromolecules using high resolution powder diffraction. Von Dreele; Robert B. and D'Amico; Kevin. Los Alamos National Security, LLC. October 31, 2006

Powder diffraction is an established technique in x-ray biosciences. It has been demonstrated that some proteins can be grown into crystal powders, even if single crystals are unobtainable. Polycrystalline materials can be obtained from a much broader range of conditions than single crystals. Other proteins can be grown into both powder

and single crystals. The patent has only one claim which encompasses the x-ray powder diffraction of proteins in a 'slurry' of polycrystalline material and a ligand or solvent 'slurry'. There is no specificity in the claims as to what ligand could be. It is most preferable to obtain a single crystal, however this requires the production of high-quality crystals which survive formation of protein-ligand complexes (i.e. after soaking the crystal in a ligand solution). Only 10% of the proteins deposited in the PDB contain ligands. Because protein-ligand crystals are infrequently grown, the powder diffraction method offers a robust alternative which can detect possible interactions between a target protein and a "suite of possible small molecule ligands".

The technique patented requires powder diffraction data to be recorded in the absence of any ligands and taken again and compared to mixtures of the protein with compounds from a drug library. Anomalies in diffraction data may indicate the formation of a complex between the two materials. The figures detail powder diffraction data from lysozyme and a complex with a known inhibitor, N-acetylglucosamine. In structural biology, powder diffraction is useful because of its sensitivity to conformational changes illustrated by changes in peak position and intensity.

[United States Patent 7,061,605: Apparatus and method for high-throughput preparation and spectroscopic classification and characterization of compositions. Lemmo , et al.. June 13, 2006.](#)

This is an extensive patent from Transform Pharmaceuticals which embodies several methods for conducting a HTS experiment. The advantage of the HTS experiment is that it requires only microgram quantities of material and can be performed in parallel reducing operative time costs. Specific preparation of compounds and methods for determining if similarities exist between samples in an array are specified. The first claim states that the invention consists of a method for screening an array of samples which are consist of a spectrum and are analyzed by a computer. Specifically, the methods in this claim are supported by following claims which declare that the data collected can come from an "infrared spectrometer, Raman spectrometer, NMR spectrometer, X-ray diffractometer, neutron diffractometer, light microscope, electron microscope, second harmonic generator, circular dichroism spectrometer, linear dichroism spectrometer, differential scanning calorimeter, thermal gravimetric analyzer, or melting point analyzer". The spectra is transformed into binary code and transferred to a computer to analyze or compare spectra. The sample can be in solid, crystalline or amorphous form.

The authors elaborate on the need for HTS which can lead to the discovery of novel "physical forms of a compounds". For example, the authors state that in order to generate solid forms of a compounds many thousands of experiments much be completed which test different solvents, additives, pH, thermal cycles etc. Known systems which contain blocks with hundreds of wells for combinatorial chemistry include the TITAN.TM reactor clamp and TITAN.TM.PTFE microplates (Radleys, Essex, UK). Many of these instruments can be used for scanning setting up crystallization conditions.

The strength of this patent does not lie in its methods for setting up assay conditions like many of the other patents researched, but instead resides in the integral sector of data collection. Techniques such as x-ray diffraction, circular dichroism, UV spectroscopy and NMR are all cited by the patent. The technique is required to be

coupled to a data analysis program designed for handling hundreds to thousands of samples. The patent states that these techniques are not easily amenable to high throughput techniques due to the physical handling and data collection required. In the invention summary, the proposed HTS array embodies a method for collecting spectroscopic data and a method for processing and interpreting this data. However, I found this patent extremely broad as it does not specify how to accomplish this task in any detail, as there are several methods in development. The authors do not elaborate on how one spectroscopic method may differ from another in terms of sample preparation and data collection. A patent which uses explicitly uses synchrotron light to collect, specifically, small-angle x-ray scattering and synchrotron radiation circular dichroism would not be covered by this patent. Also, a program designed for synchrotron CD data collection already exists, CDtool, which could be updated for HT techniques.

Even though this patent has several strengths, its ambiguity is its downfall. The forward looking nature of its claims are very strong but do not cover any particular method in enough detail that would allow it to hold up to a specific patent in that technology. Importantly, methods for transporting samples to and from these spectroscopic apparatuses are ignored.

[United States Patent 6,825,032](#): High capacity assay platforms. Sigma-Aldrich Co. Dapron , et al.. November 30, 2004.

This invention serves as a direct competitor to the technologies proposed by the author of this manuscript. US Patent 6,825,032 enacted by Sigma-Aldrich is a well designed, properly claimed invention consisting of a high capacity assay between a polymer matrix and its substrate. The matrix is covalently cross linked to other polymers which may be biologically active materials or consist of ligands. The assay platform functions by binding proteins to a polymer matrix substrate which has an affinity ligand consisting of metal chelators, anion/cation exchangers, hydrophobic binding ligands, antibodies, streptavidin, biotin and other proteins. The binding density will be on the nanomolar range/cm². The patent claims several different densities of ligands which could be used on multiwell glass or plastic plates of different dimensions. It goes on to detail how the ligands are crosslinked to the polymer matrix and claim different plastic compositions of the plate and what specific ligands could be used.

These multiwell plates can be used for HTS and include a very dense array of ligands. Previous plates were at much lower densities and unable to capture sub-microgram quantities of protein. A major roadblock in development of this tool is the actual structure or an assay plate itself. "Solid supports made of polystyrene, polypropylene and glass, such as multiwell plates, glass slides, solid chromatography beads, sheets and tubes, are not suitable for the binding and isolation of multi-microgram amounts of high molecular weight target molecules per square centimeter". No previous three dimensional structure was capable of binding high capacity of proteins even with a high density of liganded material. Attempts to covalently link materials to solid plates were unsuccessful.

This patent is a direct competitor to the proposed invention because it has the capability of detecting ligands for multiple proteins. However, these ligands are limited by what is covalently linked to the plate and the sale of individual plates from Sigma, as

well as their detection methods which are different. These plates are designed primarily to be analyzed by MALDI mass spectroscopy. The advantages of this will reveal if a protein is bound to the ligand-polymer substrate be a large increase in MW, however it does not specify if the ligand reduces the activity of the protein, where it binds or if the protein changes conformation. Important examples of this invention include a Nickel-chelating plate which binds to His-tagged proteins. Therefore, cells expressing the protein of interest can be lysed, and their contents spilled on the plate. MALDI detection would be used to quantify protein binding and expression.

Honorable Mentions:

[United States Patent 7,041,509: High throughput screening assay systems in microscale fluidic devices. May 9, 2006](#)

[United States Patent 7,052,545: High throughput screening of crystallization of materials. May 30, 2006.](#)

Patent Search for “Small angle x-ray scattering”:

There are currently 293 patents with the term “small angle x-ray scattering” present in their abstract. However, only 24 patents contain “small angle x-ray scattering assay” and 28 contain “small angle x-ray scattering screening” with several overlapping inventions. Of the search results for “SAXS screening”, 6 out of 28 patents were related to screening tumor cells and tissue with non-invasive cancer detection methods. Most of these refer to breast tissue. One includes cardiovascular imaging while the other results are for optical devices, nanowire fabrication, x-ray imaging and lithography. Searching for “small angle x-ray scattering assay” produced 24 patents, of which 14 are chemicals or pharmaceuticals. Two are about tumor cell preparation, and the rest are a photoluminescent sensor, a piezoelectric sensor and an immunoassay. Only two patents were found relating to the proposed invention, but are designed for a particular purpose using different technologies than the proposed invention. I will most fully describe these two patents. These are current, state-of-the-art technologies may have current and future commercial applications in synchrotron end-station design, microfluidics, drug discovery, pharmacology and structural biology.

[United States Patent 6,808,934: High-throughput biomolecular crystallization and biomolecular crystal screening. Mutz , et al. October 26, 2004.](#)

The following system is based on the research of Mitchell Mutz from Palo Alto, CA. The patent and following publications use an acoustic device to eject fluid droplets of picoliter quantities. Sample size cannot exceed 100 μ l. These drops will form an array and be used for crystallization studies. The acoustic device can also measure the volume and concentration of water in 96, 384 and 1536 drug well solutions which is important for quality control as water can hydrolyze the sample. The extremely small volume of the screen help preserve costly reagents such as purified protein, small organic inhibitors or

peptides used in experiments. Once the array is constructed, the presence of crystals can be detected by the acoustic method and biomolecular crystals can be differentiated from salt crystals. During deposition, two fluids are mixed together – one containing the protein solution and the other a nucleation solution which causes crystals to grow. The fluid ejector is movable relative to the reservoir for deposition allowing for a speedy arrangement of drops. Any ionic, non-polymeric, biopolymer, steroid, retinoid, monomer, cluster of oligosaccharides, amino acids or nucleic acids can be considered ligands which may be present in any of the drops. Proteins may be partially or completely denatured. The method of acoustic radiation provides a quick, economic approach to combinatorial screening. The final claim (89 total claims) states that protein crystal quality can be ascertained by x-ray diffraction.

[United States Patent 6,889,727](#): Apparatus and method for the preparation of experiments using proteins contained within gels. Olson, et al. May 10, 2005.

This patent filed by Abbott Laboratories, IL is a method of dispersing protein containing materials such as gels, or lipid mixtures into an assay container. The method specifically refers to both soluble proteins and membrane proteins. The novel apparatus is a gel dispenser coupled to a liquid handling robot for moving the liquid dispensing pipette. The system includes at least one pump to move incompressible fluids which pump out the gel and at least one additional pump to deliver reagents to the pipette probe. The gel dispensing apparatus is comprised of a gel dispensing barrel, a barrel bore, piston, nozzle and pump. While there are many claims regarding the gel dispensing mechanism (17 claims), there are none regarding the target surface for deposition. The amount of gel dispensed will not exceed 1 μ l. The patent's focus is on membrane proteins, which are notoriously difficult to express, purify and crystallize. The authors suggest solubilizing membrane proteins in buffered detergent solutions and mixing them so that a cured lipid bilayers will be fixed. The cubic lipid phase depends on water:lipid ratios and can be studied by x-ray diffraction or scattering. The majority of the background focuses on the problems of dispensing protein/lipid solutions using syringes, an important area in high throughput screening of membrane proteins. The authors site another paper which described a method for transferring homogeneously hydrated lipids to capillaries for x-ray diffraction.

Patent Search for “Circular Dichroism”:

A patent search for circular dichroism” in the abstract text revealed 62 entries. Many of these inventions are regarding data analysis software which are included with the purchase of any bench-top machine. Jasco (JP) is the top producer of such equipment. Many patents are regarding optical and electrical components of CD spectrometers, and only several were applicable to the invention at hand. I decided to include an interesting upgrade to a bench-top CD machine which is coupled to an HPLC device. The second patent is a method for separating optical isomers, which is a common problem in the purification of pharmaceuticals. This is because during the synthesis of many drugs, stereoisomers (enantiomers) are produced. These chiral products differ by a single plane of

symmetry and have many of the same electric and physical properties making them difficult to separate. However, in vivo, one enantiomer may be toxic and the other will be efficacious.

[United States Patent 6,118,536](#): Circular dichroism detector for HPLC. Sakamoto; Mitsunori. Jasco Corporation (JP). September 12, 2000.

This patent is regarding a circular dichroism spectrometer with emission in the UV region emanating from a Hg or HgXe lamp. A photoelastic modulator transforms the light into circularly polarized light which is passed through a flow cell. The unique device was designed to analyze the flow through products from high pressure liquid chromatography (HPLC), the dominant method for purifying proteins. Discussion of prior art includes a CD machine which was too large for typical installations, only can read CD at 2 nm intervals and not was designed with analyzing HPLC products in mind. To alleviate these problems, the inventors at Jasco eliminated the effects of stray light to observe higher CD signals when coupled to HPLC. The new invention is embodied by a light source with strong emission in the UV range, diffraction grating for wavelength dispersal, modulator for perturbing linearly polarized light into circularly polarized light, a flow cell along the optical path and a light detector opposite the beam flanking the flow cell. This system provides much higher sensitivity than previous systems and is successful at separating optical isomers from each other when purified on a gel filtration column.

[United States Patent 5,780,242](#): Bioassay for the screening of ION channel active molecules. Nickel; Alfred A. July 14, 1998.

This is an assay for Sodium channels of the cell membrane whose interactions with other molecules can be measured by circular dichroism. This technique can also be used to diagnose the disease state of potassium, calcium and preferably sodium channels by studying a dysfunctional protein's structure. The first claim states that abnormalities in the sodium channel thermal protein can be detected by conformational changes which appear on CD. Particular experiments are directly patented such as 1) testing molecules which bind directly to the channel to inhibit its function and 2) thermal denaturation of Na channel proteins and their CD spectra in application to disease diagnosis. Several neurotoxins from arthropods and pesticides are known to alter the function and structure of this channel. The protein can be obtained directly from diseased tissues of the CNS, cardiovascular system, peripheral NS or tumors. The temperature of the system will be between 30 and 35 degrees Celsius and scanned by CD at 222 nm wavelength. This technique may have applications to diseases such as cardiac arrhythmias, angina, cystic fibrosis, and epilepsies. Drugs which target this channel include local anesthetics, anticonvulsants, and some psychoactive drugs.

Works Cited