Drug Discovery & Development

Label-Free Methods Are Not Problem Free

Label-free, optical, acoustic, and calorimetric analytical methods do not alter the protein of interest, but they have complications of their own *Diagram of typical biosensor mechanisms.*

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Labeling methods that use fluorescence and radioactivity remain the cornerstone of most biological research protocols, and for good reason. They allow ultra high-throughput evaluation of molecular interactions, the approaches are well established, and for most researchers, to do without them would be akin to throwing out a shabby, yet comfortable, pair of shoes. However, label-free methods, which rely on optical, acoustic, and other types of biosensors, may improve on the standard methods, although achieving high-throughput and sensitivity are still important challenges.

“Fluorescence and ultra high-throughput microtiter plates have dominated detection technology,” says Michael Thompson, PhD, biosensor researcher and professor of analytical chemistry at the University of Toronto, Canada. “Several companies still use large automated drug screening machines that use fluorescence. The technology has a lot of advantages,” he says. However, disadvantages include lack of sensitivity, interference, and cross-reactivity. For measuring proteins, the problems associated with labeling are compounded. The efficiency of labeling varies from protein to protein, making comparisons a challenge. In addition, attaching fluorophores may influence the way in which proteins bind to other molecules and cause background signals, Thompson says.

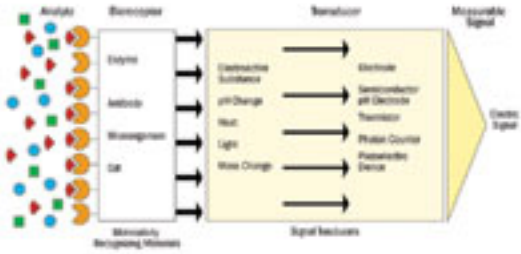

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Diagram of typical biosensor mechanisms.

Label-free detection methods, which use biosensors, have emerged as a potential way to overcome some of these flaws. The most well developed and commercialized approaches are optical methods, which measure properties of evanescent waves. Notably, surface plasmon resonance (SPR) has been around since 1990. Several companies, including Biacore International SA, Neuchâtel, Switzerland, and Applied Biosystems, Foster City, Calif., market SPR-based detection systems that are able to analyze various interactions including small-molecule protein binding and protein-protein binding.

However, SPR is generally not considered to be a high-throughput method. For example, the Biacore S51 model can measure interactions between very small molecules, but has only three channels, one of which is a control, leaving two for samples. The system consists of a chip with a gold surface, and binding events are detected by measuring the change in angle of reflected light. “The S51 model has a

Types of Label-Free Detection	
Category	Description
Amperometric	Detect changes in current as constant

capacity of more than 200 samples, but everything is done in series. So, it is not generally used for anything more than tertiary screening or complementary screening; it's too slow," says Matthew Cooper, PhD, with the Cambridge Center for Molecular Recognition, and founder of Akubio Ltd., Cambridge, UK.

Conductive	Detect changes in conductivity between two electrodes
Capacitive	Used when the biorecognition reaction causes a change in the dielectric constant of medium
Optical	Correlates changes in concentration, mass, or number of molecules to direct changes in the characteristics of ligth
Piezoelectric	Detect changes in potential at constant current (usually zero)
Thermal	Measure changes in temperature

Throwing some hustle into SPR

Various companies have tried to make SPR more high-throughput, however. For example, a system from Applied Biosystems/HTS Biosystems Inc. is a diffraction grating-coupled SPR imager, which images an entire surface rather than just imaging one flow cell. The microarray-based analysis system for proteomics, called the Flex Chip, can perform kinetic analysis of up to 400 individual binding events in a single experiment.

How Biosensors Work

Biosensors are analytical devices that couple a biological material, such as tissue, microorganisms, organelles, cell receptors, enzymes, and antibodies, to a physiochemical transducer. The transducer is typically optical, calorimetric, acoustic, electrochemical, or magnetic. Specific interaction between the target analyte and the biological material layer produces a physicochemical change that is detected by the transducer. The transducer then yields a digital electronic signal proportional to the concentration of a specific analyte or group of analytes. Although the signal may be continuous, discrete measurements can also be made.

Likewise, technology from SRU Biosystems, Woburn, Mass., called BIND, can be applied to 96-well and 384-well microtiter plates as well as microarray chips allowing it to achieve more high-throughput capability. The technology uses a surface-binding platform with resonant grating. A white light is shined on the grating and a single color is reflected back. When a molecule binds, the light reflected back changes color. The sensor itself is made of plastic, with a coating of epoxy that produces the desired wavelength of light. "HTS Biosystems' technology can measure molecules down to about 8 kDa, which is about the size of a very small protein or a large molecule, so this may limit its use for certain applications," says Cooper. Sensors from SRU Biosystems can detect down to about 1 kDa, so this technology is "on the edge for good drug screening applications," he says.

Pros/cons of optical sensors

Other disadvantage of optical sensors is that they are very sensitive to changes in the refractive index. Drug libraries are usually solubilized in dimethyl sulfoxide (DMSO), and the refractive indexes of the DMSO solution and the buffered solution are very different, which means instruments must be calibrated for the DMSO content in samples. This makes the assay much more complicated and labor intensive than some other label-free detection methods.

Imaging ellipsometry is a non-SPR label-free optical approach. This technique measures the change in the state of polarization of the light reflected. Fast ellipsometry methods, single or multiwavelength, have been adopted for monitoring growth of cells in situ. In addition, imaging ellipsometry has a lateral or spatial resolution of down to 1 micrometer, which potentially expands this technology into bioanalytics and microelectronics. The technology is also well suited for the detection of DNA hybridization and the

observation of adsorption reactions in real time, according to Nanofilm Technologie GmbH, Göttingen, Germany, which manufactures the EP3 ellipsometer.

Microcantilevers are another optical approach. Technology from Protiveris Inc., Rockville, Md., is based on chemo-mechanical bending of silicon microcantilevers and subsequent detection of changes in light deflection. A microcantilever coated on one side with a protein, antibody, antigen or piece of DNA bends when a complementary molecule binds. The VeriScan 3000 monitors the movement of microcantilevers by focusing tiny laser beams on the microcantilevers' free ends. Thus, the technology can measure protein-protein, protein-DNA, and receptor-ligand interactions.

Cantion A/S, Copenhagen, Denmark, also makes a cantilever technology, but with an electrical rather than an optical readout. According to the company, unlike optical detection methods, the electrical detection system is insensitive to changes in the optical density of the sample making it possible to work with opaque samples such as serum or urine. Thus, changing buffers or introducing liquids with varying indexes of refraction will not influence the electrical signal. The company also says that the technology is suitable for detecting both small and large molecules.

Fiber optics may also play role in future label-free detection methods. Luna Analytics, Blacksburg, Va., is developing fiber optic technology to quantify protein and small molecule interactions. The technology uses a device that modulates the light transmitted through the fiber. The device is sensitive to local density changes due to the addition of polymer coatings with selectivity to specific targets achieved through the use of antibodies, antigens or other specific receptors. Binding of the target causes local density increases, resulting in an optical signal modulation.

Revamping calorimetry

Calorimetric methods have typically been viewed as the "gold standard" for characterizing molecular interactions. Until recently, however, applications have been constrained by the relatively large sample amounts (greater than 1 micromol/L) needed and limited throughput. One form of calorimetry, isothermal titration calorimetry (ITC), measures heat generated or absorbed when molecules interact. An advantage of this method is that ITC does not require immobilization of the reactants. Another calorimetric method is differential scanning calorimetry (DSC) which measures conformational changes in macromolecules. As with optical methods, these methods are being scaled down and reaching new limits of detection. Vivactis NV, Leuven, Belgium, has recently developed a differential calorimetric technology in microplate format for high-throughput screening. The technology uses an array with integrated micro temperature sensors at the bottom of each well. Intermolecular activity is determined by measuring the temperature in each well. Likewise, MicroCal LLC, Northampton, Mass., has developed an "AutoITC" machine that can measure samples in a standard 96-well plate format. Other companies developing similar approaches include Thermometric Inc., Charlotte, N.C., and Calorimetry Sciences Corp., Lindon, Utah.

The sounds of bonds breaking

In addition to optical and calorimetric biosensors, acoustic biosensors also allow the label-free detection of molecules and analysis of binding events. These instruments are generally based on quartz crystal resonators, which can monitor the change in resonant frequency and resistance that occurs when molecules interact. The technology can be used to monitor a wide range of biological interactions between molecules as small as 200 daltons. Similar to other methods, acoustic biosensors allow the real time

monitoring of binding. However, much more detailed information can be obtained about an interaction than with SPR biosensors.

Summary of Biosensor Applications	
General area	Example(s)
In vitro diagnostics	Blood glucose monitoring
In vivo diagnostics	Renal failure monitoring
Environmental monitoring	Biochemical oxygen demand (BOD)
Food and drink industry	Food composition, food sensory analysis, and food pathogen detection
Bioprocess monitoring	Fermentation monitoring and control
Agriculture	Crop diseases, plant nutrients, and pesticides
Research & Development	Biomolecular interactions
Military	Biological and chemical warfare agent detection

“With acoustic wave physics, you can get several different types of signal,” says Thompson, “and this can then lead to chemical information, such as conformation and structure, that you don’t get from some of the optical methods,” he says. Thompson’s group is developing acoustic applications to investigate a number of biological events such as DNA transcriptional chemistry, hybridization events, protein-protein interactions, and protein-small molecule interactions.

Incorporating novel acoustic technology, Akubio’s Rupture Event Scanning (REVS) uses sensitive acoustic technology to detect the sound of bonds breaking as molecules are shaken off a vibrating resonator. REVS can be used to size, separate, and detect particles such as bacteria, viruses, and cells. More strongly attached particles detach at larger amplitudes of oscillation, so that separation and detection are performed in the same experiment.

Furthermore, the magnitude of acoustic energy (i.e., the loudness of the sound) is proportional to the number of ligands on the surface, which allows quantitation of the sample. A related technology is Resonant Acoustic Profiling (RAP) which Akubio recently acquired from GlaxoSmithKline, Brentford, UK. This system couples acoustic detection with microfluidic delivery systems to allow detection of proteins, DNA, small molecules, and other analytes in real time. This system gives information on the specificity, affinity, and kinetics of an interaction in minutes, and being entirely electronic, can be readily multiplexed.

While optical, calorimetric, and acoustic biosensor approaches are the most well-established detection methods, alternative approaches are also under investigation; many of them involve the rapidly emerging field of nanotechnology. For example, silicon nanowires are being developed for the purposes of arrays, says Charles Lieber, PhD, at the Department of Chemistry and Chemical Biology, Harvard University, Cambridge, Mass.

“One of the properties of the semiconductor that makes it so useful is that they can be turned on and off with charge,” says Lieber. “So, any biological species that is polar or charged in solution will change the conductivity of the wire when it binds.”

Nanowires will add to label-free detection primarily by improving sensitivity and being amenable to use in arrays, says Lieber. “Nanoscale structures can result in exquisite sensitivity because they’re so small that when something binds at the surface, it really affects the entire wire,” he says. Lieber also predicts that nanowire arrays can be used to investigate virtually any system. In addition to simple protein detection or nucleic acid detection, the method is being used for small-molecule screening of kinase

inhibitors. “You can get quantitative information on the kinetics of small-molecule inhibition, and this approach could be used for any kinase,” he says.

Similarly, Nanogen Inc., San Diego, has developed microarray technology that exploits the charge present in most biological molecules. Applying an electric current to individual test sites on NanoChip microarrays enables rapid movement and concentration of the molecules. Through electronics, molecular binding is accelerated to a thousand times faster than traditional methods. The technology involves biotinylating DNA samples, hybridizing complementary DNA reporter probes, and applying stringency to remove unbound and nonspecifically bound DNA after hybridization.

Ion Channel Switch (ICS) technology, developed by Ambri Ltd., Chatswood, Australia, involves a self-assembling synthetic biomembrane. The biosensor is a two-molecular-layer self-assembled membrane structured after the ion-channel-forming peptide gramicidin. The binding of the target molecule to the antibody fragment alters the population of conduction ion channels pairs within the tethered membrane, resulting in a change in the membrane conduction.

The first commercial application of the technology was as a point-of-care diagnostic system used in hospitals. According to the company, the system has been designed to deliver results in less than five minutes and has the potential to detect and measure drugs, hormones, viruses, and bacteria in whole blood directly from a standard collection tube.

GeneFluidics Corp., Monterey Park, Calif., is commercializing a molecular analysis platform that integrates bionanotechnology and microfluidics. The platform includes a reader and disposable nanobiosensors. It can be used to quantify the presence of target genetic material, proteins, and small molecules in raw samples without the use of target amplification methods such as PCR. According to the company, the low-cost manufacturing methods “will dramatically reduce the cost of molecular analysis and be able to analyze biological molecules at nanometer scale.”

History of Biosensors

1956	Invention of the oxygen electrode (by Leland Clark)
1962	First description of a biosensor: an amperometric enzyme electrode for glucose
1969	Potentiometric biosensor: urease immobilised on an ammonia electrode to detect urea
1970	Invention of the Ion-Selective Field-Effect Transistor (ISFET)
1972/1975	Commercial Biosensor: Yellow Springs Instruments glucose biosensor
1975	Microbe-based biosensor Immunosensor: ovalbumin on a platinum wire Invention of the pO_2 / pCO_2 optode
1986	Bedside artificial pancreas (Miles)
1980	Fiber optic pH sensor for in vivo blood gases
1982	Fiber optic-based biosensor for glucose
1983	Surface plasmon resonance (SPR) immunosensor
1984	Mediated amperometric biosensor: ferrocene used with glucose oxidase for the detection of glucose
1987	MediSense ExacTech blood glucose biosensor
1990	Pharmacia Biacore SPR-based biosensor system released

The scanning Kelvin nanoprobe, an experimental technology, makes use of the principles of Kelvin physics and atomic force microscopy (AFM). The nanoprobe, being developed by Michael Thompson’s team at the University of Toronto, measures the current generated when two materials, one subjected to vibration, are connected. When contact occurs, the equilibration of the Fermi levels of the two substrates leads to a current. The probe uses an AFM-like tip as one of the materials and can detect both topographic

and surface potential maps of a planar surface. Thompson and his colleagues recently demonstrated that surface potential measurements could be made on proteins and nucleic acids attached to substrates such as gold, silicon, and indium-tin oxide. The researchers are currently developing the scanning Kelvin nanoprobe to rapidly scan and analyze spots on DNA microarrays and to examine printed arrays of cells. "It may also be useful for the multiplexed analysis of cell-small molecule interactions and to explore the surface chemical behavior cell populations," Thompson notes.

BioForce Nanosciences Inc., Ames, Iowa, has also used AFM in its NanoArrays. AFM "feels" its way around a sample to create an image, like a nanoscale version of a phonograph needle, says Gary Alianell, PhD, president and chief executive of BioForce Nanosciences. NanoArrays are ultraminiaturized arrays of biomolecules that use approximately 1/10,000th of the surface area occupied by a conventional microarray.
With a specialized reader, sensitivity to the single-particle level can be achieved, the company claims. The technology can be used to measure interactions between proteins, nucleic acids, virus, and small molecules. However, the array's efficacy relies on its ability to test for tens of thousands of interactions at the same time, but tests are statistically accurate only if a few reactions are tested using thousands of tests.

Fast, but unreproducible
According to Electra Gizeli, PhD, group leader of the biosensors group in the Department of Biology, University of Crete, Greece, the most important developments in label-free systems include the emergence of high-throughput technology and systems that can be used to look at individual cells. However, a problem that needs to be overcome is a lack of reproducibility. "Many biosensors are suitable for research purposes but do not yet have the features that are required for commercial development, including things such as batch-to-batch reproducibility," says Gizeli.

"There are no real high-throughput, label-free biosensors out there at the moment," says Cooper, although he estimates that such methods will become available with the next one to two years.
"Biosensors have great potential as a method for investigating chemical and biological samples, not only in drug discovery, but also in the fields of diagnosis and environmental monitoring," he says. "Their impact should continue to grow dramatically over the next decade."