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Non-optical screening platforms: the next wave in label-free screening?

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The use of optical biosensors for compound screening was first demonstrated in the mid-1990s, but there has been limited uptake in the market owing to issues of limited throughput and a lack of applications for key receptor classes. Recently, several start-up and established tools companies have exploited non-optical detection modalities that seek to address the shortcomings of more established optical approaches. Platforms based on acoustic resonance, electrical impedance, microcantilevers, nanowires and differential calorimetry are beginning to appear with commercially available products targeted at post-high-throughput screening hit confirmation and mode-of-action studies. This article highlights key advances in commercial label-free analysis platforms, which complement more traditional optical system and which also allow novel assay formats for the analysis of previously intractable targets.

Introduction

To analyse a molecular interaction without the use of reporter labels, it is necessary to couple a molecular recognition element (e.g. an antigen or target receptor) to a transducer that converts a chemical or biological interaction into an electrical signal. A biosensor is thus defined as a unique combination of a receptor for molecular recognition and a transducer for transmitting the interaction information into an electrical signal. In turn, a transducer is more specifically defined as a device for converting energy from one form to another for the purpose of measurement of a physical quantity or for information transfer. In theory, there are several different physical phenomena that can be exploited in biosensing: acoustic waves, thermal capacity and heat transfer, photons, neutrons, ions, radioactive particles, electrons, electric fields and magnetic fields. In practice, most analytical platforms in routine use today use optics, with few of the more novel transducer modalities having transitioned from academic instrument prototypes to robust commercial platforms. Nevertheless, non-optical approaches have the potential to complement or even displace many of the currently available optical technologies that have dominated the label-free screening market since the 1990s.

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The previous article [1] reviewed the key optical approaches to biosensing and the benefits and limitations associated with these technologies. Here, I highlight platforms based on acoustic resonance, electrical impedance, microcantilevers, field effect nanowires and differential calorimetry. Products based on these technologies are now beginning to appear in commercially available products targeted at post-high-throughput screening hit confirmation, compound triaging and mode-of-action studies. Selected companies are detailed in Table 1, and the accompanying products are briefly reviewed in the following sections.

Impedance assay systems for cell-based screening

Cell-based assay technologies that provide high-content information (high-content screening) have recently generated significant interest in the hit-to-lead discovery process. Most of the cell-based systems on the market are based on fluorescence detection; however, three groups have recently developed innovative cellular assay technologies, based on frequency spectrometry and bioimpedance measurements that might offer a complementary approach to traditional high-content screening. Impedance measurements have long been exploited in commercial biosensors [2], and the basic principles for application to cell analysis were first reported by Giaever and Keese, then at the General Electric Corporate Research and Development Centre (http://www.ge.com) [3]. In contrast to other methods of monitoring cellular signal

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TABLE 1

Selected non-optical label-free platform developers

Provider	Technology	Product	Website
Acea Biosciences	Cell electronic sensing	RT-CES™	http://www.aceabio.com
Akubio	Resonant acoustic profiling	RAP◆*id*-4™	http://www.akubio.com
Applied BioPhysics	Electric cell-substrate impedance sensing	ECIS™	http://www.biophysics.com
Bioforce Nanosciences	Microcantilever	ViriChip™ NanoArrayer System™	http://www.bioforcenano.com
Calorimetry Sciences	Differential scanning calorimetry Isothermal titration calorimetry	N-ITC III, N-DSC III MC-DSC, IMC, INC	http://www.calscorp.com
Cantion/NanoNord	Microcantilever	CantiLabPro	http://www.cantion.com http://www.nanonord.dk
MDS Sciex	Cellular dielectric spectroscopy	CellKey™ System	http://www.mdssciex.com
MicroCal	Differential scanning calorimetry	VP-ITC, VP-DSC	http://www.microcalorimetry.com
	Isothermal titration calorimetry	VP-Capillary DSC	
Q-Sense	Quartz crystal microbalance	E4, D300	http://www.q-sense.com
Protiveris	Microcantilever	VeriScan™ 3000	http://www.protiveris.com
Thermometric	Isothermal titration calorimetry	–	http://www.thermometric.com
Vivactis	Microplate differential calorimetry	MiDiCal™	http://www.vivactis.com
Xerical	Nanocalorimetry	–	http://www.xerical.com

transduction, impedance measurement of cellular responses can provide high information content in a simplified, label-free and non-invasive fashion.

ACEA Biosciences

ACEA Biosciences (http://www.aceabio.com) has released the real-time cell electronic sensing (RT-CES™) system, based on a micro-electronic cell sensor array integrated into the bottom of standard Society for Biomolecular Sciences (SBS; http://www.sbsonline.org) format microtitre plates. RT-CES works by measuring electrical impedance across the sensors to detect the presence, absence or change in condition of cells. For cell-based assays, cells are grown in the individual, sensor-containing wells of the microtitre plate and placed in a standard incubator. The system can be programmed to collect data as frequently as every minute by sending nominal current through the sensors at the user-defined intervals. The electronic sensors provide information on impedance values, which is then converted to a measure known as the cell index (the impedance of the cells normalized for the impedance of the media alone).

Major application areas include cancer biology, cell adhesion and spreading, receptor–ligand binding and signal transduction analysis, cell proliferation, cytotoxicity, cellular differentiation and environmental toxicology. ACEA foresee key applications in the drug discovery field to be centred on cell proliferation assays, and on the elucidation of compound toxicity (e.g. for the prediction of *in vivo* toxic effects and for lead optimization in secondary screening and the early phases of drug development). Further key applications involve the analysis of receptor–ligand binding, in particular for agonists, partial agonists and antagonists of G protein-coupled receptors (GPCRs).

Applied BioPhysics

Electric cell-substrate impedance sensing (ECIS) from Applied BioPhysics (http://www.biophysics.com) is based on a specialized slide that has eight or 96 individual wells for cell culturing. The base of the device has an array of gold film electrodes that connect the ECIS electronics to each of the wells. Cell densities ranging from a heavy confluent layer to sparse layers can be measured with this approach. The size of the electrodes restricts the maximum number of anchored cells that can be observed (typically from 100 to 1000 cells). However, by using multiple electrodes in parallel, more surface area in a well can be covered to measure up to 4000 cells. The detection electronics are sufficiently sensitive to detect even a single isolated cell response. Key applications exemplified by Applied BioPhysics include the monitoring of cellular behaviour, including cell proliferation, barrier function, attachment and spreading, migration, and invasion under both static and flow conditions. In addition, higher electric fields can be used to carry out automated wound healing assays and, if applied for a shorter duration, to electroporate cells and monitor the subsequent entry of membrane impermeable molecules, such as dsRNA. The technology has also been refined to enable profiling of signal transduction, metastatic potential and *in vitro* toxicity.

MDS Sciex

MDS Sciex (http://www.mdssciex.com) has recently released the CellKey™ system, which uses cellular dielectric spectroscopy (CDS) to measure, quantitatively and kinetically, endogenous cell-surface receptor responses to ligands in live cells. Using this technology, a series of receptor-specific, frequency-dependent impedance patterns known as CDS response profiles, resulting from changes in cellular bioimpedance across a spectrum of frequencies (1 kHz to 10 MHz), are collected every two seconds. The characteristics of the CDS response profiles are used to determine the identity of the signalling pathway being activated by the receptor–ligand interaction, and provide facile access to information on compound selectivity. In addition, these profiles enable quantitative pharmacological analyses such as potency and Schild analyses. Recently published work (Figure 1) demonstrates the

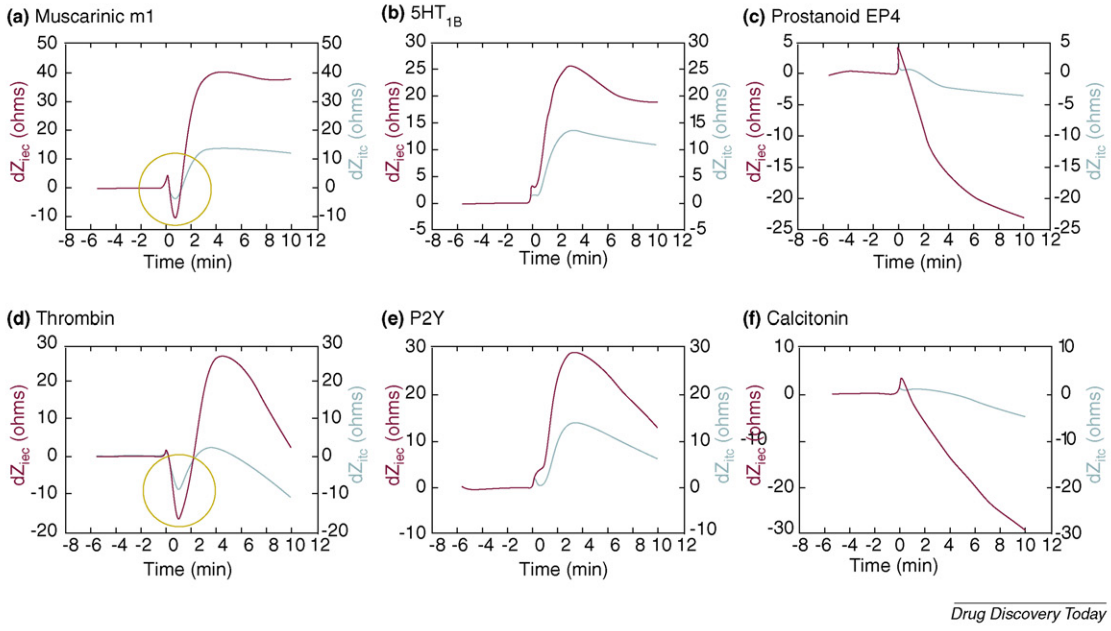

FIGURE 1

Graphs illustrating characteristic GPCR-mediated response profiles in CHO_{m1} cells. Stimulation of different classes of GPCRs leads to unique kinetic response profiles, such as that shown for transfected muscarinic acetylcholine M1 receptor **(a)**, and the endogenous serotonin $5HT_{1B}$ **(b)**, prostanoid EP4 **(c)**, thrombin **(d)**, purinergic P2Y **(e)** and calcitonin **(f)** receptors. The CDS response profiles representing G_q GPCRs typically demonstrate an initial decrease in Z_{iec} and Z_{itc} (circled in yellow), followed by an increase in Z_{iec} and Z_{itc}, whereas over the entire time period Z_{iec} and Z_{itc} decrease in the context of G_s GPCR-mediated responses. By contrast, Z_{iec} and Z_{itc} increase over the entire time period in the context of G_i GPCR-mediated responses. Modified, with permission, from Ref. [4].

effectiveness of the system in profiling many endogenous ligand-induced cellular responses mediated by the three major classes of GPCRs (G_s, G_i and G_q), as well as several protein tyrosine kinase receptors in many different cell types, including primary cells [4].

Feedback from pharmaceutical companies thus far indicates that the value of the technology is in measuring endogenous receptors because it eliminates the need for genetic and chemical manipulation of the cells. In addition, the data gained from the CDS response profiles provide information about the selectivity of compounds for the target of interest. The team at MDS Sciex foresees applications in receptor panning, identification of G protein-coupling mechanisms, deconvolution of signal transduction pathways and the pharmacological analysis of partial, full and inverse agonist or antagonist action.

Acoustic systems

Akubio

Resonant Acoustic Profiling, from Akubio (http://www.akubio.com), is based on piezoelectric quartz crystal technology that measures the build-up of molecules on the surface of an oscillating surface to provide real-time binding information on the molecular interactions [5]. Target molecules are attached to the surface of quartz crystals through a variety of specific coupling chemistries, and then the sample for analysis is applied via a microfluidic flow-based delivery system. Ian Campbell (Director of Business Development, Akubio) comments: 'RAP [resonant acoustic profiling] provides researchers with the ability to perform accurate, sub-nanomolar, real-time characterizations of interactions using drug candidates in buffered solutions, DMSO solutions, and crude matrices such as culture media, periplasmic extracts, hybridoma supernatants, urine and serum'. Launched in September 2006, the first instrument from Akubio Ltd, the RAP◆*id*-4, integrates the RAP detection technology with automated sample handling from 96- and 384-well plates and deep well tubes. Fitted with four individually addressable sensors, the instrumentation can provide specificity, affinity and kinetic characterizations for more than 384 samples per day. This is achieved by analysing samples in parallel, rather than in series, as has been the case with competitor biosensor systems. Ian Campbell elaborates: 'In the RAP◆*id*-4 system, users can parallel process four samples simultaneously, and then use the Workbench analysis tools to analyse the data from more than 384 samples with a few mouse clicks. This represents a significantly increased capability for a label-free platform, which should enable the technology to be adopted much earlier in the drug discovery process'. Primarily targeted at analysis of protein–protein interactions in the development of biopharmaceuticals and antibody therapeutics, the technology can also be used to measure small molecule–protein interactions (Figure 2).

Most optical label-free detection methods ultimately measure changes in the dielectric constant or refractive index of a solution in close proximity to the surface of the sensor substrate. Although they are powerful techniques under extremely well-controlled conditions, the advantages are often minimized when trying to apply these methods in routine analysis procedures. This is because optical methods rely on proximity-based detection, and any analyte that is within an evanescent sensing field (typically 300 nm for most surface plasmon resonance devices) is detected as 'bound'. This is the case whether it physically bound to the

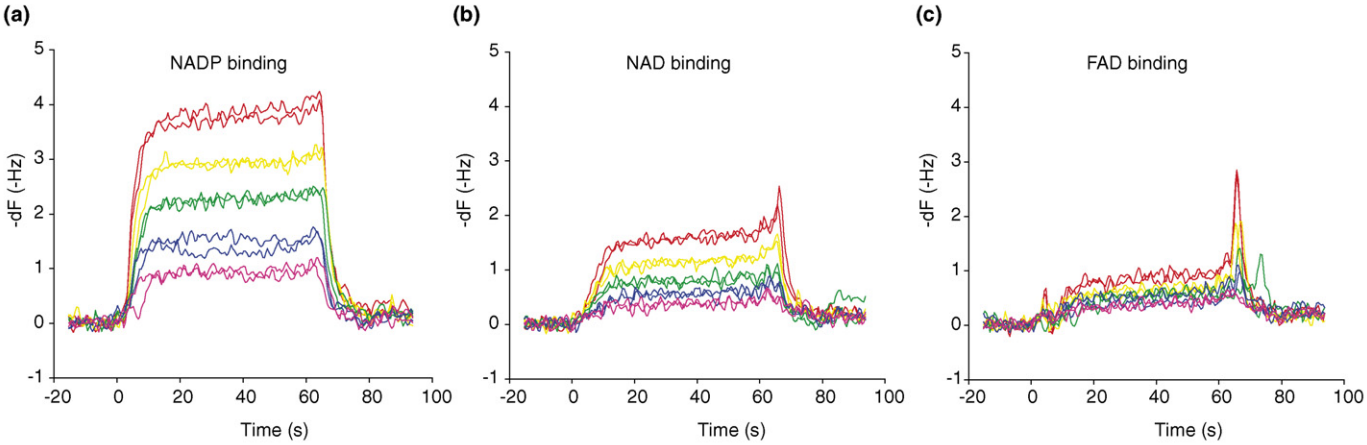

FIGURE 2

Binding of preferred substrate, poor substrate and non-preferred adenine nucleotide cofactors to the metabolic enzyme glucose dehydrogenase. (a) NADP (765 Da, preferred substrate) binding shows a clear equilibrium concentration–response relationship. **(b)** NAD (663 Da, metabolite) shows a lower equilibrium binding level, and compressed concentration–response relationship. **(c)** FAD (830 Da, non-preferred substrate) shows low levels of equilibrium binding. Reproduced, with permission, from Ref. [5].

receptor or simply in close proximity to the surface of the sensor. By contrast, RAP measures only those materials that are acoustically coupled to the sensor surface – that is, binding-based detection rather than proximity-based detection. The process of measuring refractive index changes with optical methods to infer mass changes imparts several other intrinsic limitations – in particular, the masking of binding events that occurs in sample environments that have variant refractive indices. In cases where the molecules to be tested have been solubilized in organic solvents, or are components of a crude cell lysate, culture medium or a serum sample, optical-based techniques are often incapable of measuring associated binding events without extensive calibration, dilution or other sample preparation procedures. Ian Campbell states: 'One notable advantage of acoustic detection over more established optical label-free detection is the relative insensitivity of acoustics to changes in solvent. When running samples containing DMSO, optical detection systems suffer from large bulk refractive index shifts that arise from the disparate properties of the organic solvent and the running buffer'. This is because the dielectric constant of water is 80, whereas that of DMSO is 40 (a difference of 100%). To normalize for these large bulk effects, a calibration routine using known serial dilutions of DMSO in running buffer is normally run at the beginning, middle and end of a screening panel [6]. By contrast, acoustic systems are not affected by refractive index changes, but are instead sensitive to bulk effects dominated by the viscosity and density of the solvent – more specifically, the square root of the viscosity:density product [7]. For water, this value is 0.99, whereas for DMSO it is 1.10 (a difference of 11%).

Any system that uses a highly sensitive transducer such as piezoelectric quartz to measure molecular interactions must have a variety of integrated technical controls to facilitate the highest level of sensitivity, accuracy and precision. Previous attempts to exploit this detection method in a commercial format have been limited by poor sample delivery mechanics, inadequate thermal controls and the lack of a multisensor analysis platform. The development of proprietary electronics, fitting algorithms and a low-stress mounting system for the sensor has enabled Akubio to integrate microfluidic delivery together with automated liquid handling in the RAP◆*id* system (Figure 3). Ian Campbell explains: 'User interactions with the platform are facilitated by icon-driven software which provides the researcher with complete flexibility over assay design, and easy editing of individual assay parameters'. This view is reinforced by the Chairman of the company, Andrew Carr (formerly CEO of Amersham Biosciences):

> 'There is a clear message coming from the scientific community that better tools are needed to study bio-molecular interactions in both basic research and therapy development. Scientists want to generate kinetic data on molecules interacting in relatively complex samples such as cell growth media and cell lysates without extensive prior purification. This is today a real problem especially if a relatively large number of samples are to be analysed.'

In the past, acoustic detection has been used to characterize interactions with peptides [8,9], proteins and immunoassay markers [9–13], oligonucleotides [14,15], bacteriophages [16,17], viruses [18,19], bacteria [20–22] and cells [23–28]. The technology can thus be applied to an extremely wide range of biological and chemical entities.

Q-Sense

Q-Sense (http://www.q-sense.com) offers acoustic label-free sensors based on a novel technique, dissipative quartz crystal microbalance (QCM-D). By collecting both the dissipation and the resonance frequency of a quartz crystal, QCM-D can be used to study the formation of thin films of proteins, polymers and the attachment of cells to surfaces. By measuring several frequencies (F) and the dissipation (D), it is possible to ascertain whether the adsorbed film is rigid or soft, and the kinetics of structural changes

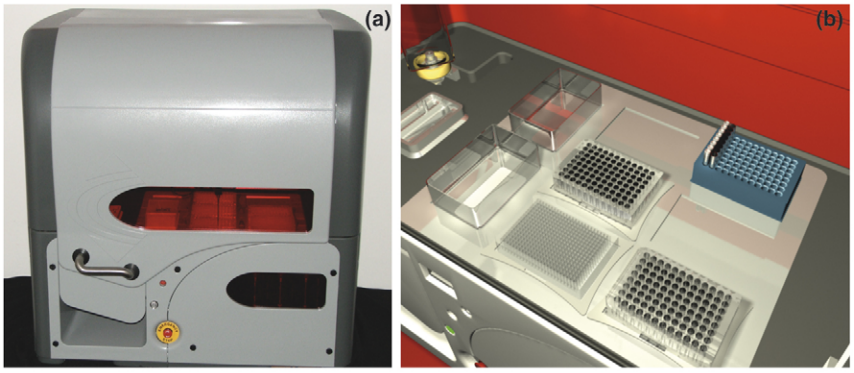

FIGURE 3

The RAP◆*id*-4 system from Akubio (a) with temperature-controlled sample racks and injection ports **(b)**.

and mass changes can be obtained. Q-Sense offers two products: Q-Sense E4 and Q-Sense D300. The D300 is the original Q-Sense instrument that enables QCM-D studies of a variety of processes taking place on its sensor surface. The company's E4 System allows a wide range of experimental parameters to be varied, including flow rates and temperature (4–40 °C). The multiplexed capability of the E4 System enables simultaneous analysis, where four different samples can be monitored at the same time. In addition to QCM-D, an optional 'add on' flow cell enables simultaneous electrochemistry measurements. Key applications involve the combined measurement of *F* and *D*, often probed at multiple acoustic harmonics, which have been used to identify structural changes in proteins during adsorption to solid surfaces, PNA and DNA immobilization and subsequent hybridization, and the kinetics and changes of morphology of lipid vesicles during adsorption to surfaces.

Calorimetric systems

Calorimetric methods have typically been viewed as the gold standard for characterizing molecular interactions: calorimetry is label free and immobilization free, provides real-time kinetic monitoring, and yields relevant thermodynamic and kinetic parameters in terms of specificity, activity and inhibition through well-understood physics. More detailed reviews of the application of calorimetry in drug discovery have appeared elsewhere [29–32]. However, until recently, applications have been constrained by the need for relatively large (ml) sample volumes, limited throughput and lack of accessibility to the reaction and reference vessel. MicroCal LLC (http://www.microcalorimetry.com) has developed an 'Auto-ITC' machine that can measure samples in a standard 96-well plate format. Other companies developing similar systems include Thermometric (Charlotte, NC, USA) and Calorimetry Sciences (http://www.thermometric.com). In Europe, Vivactis (http://www.vivactis.be) is developing a nanocalorimetric platform that is more compatible with the logistics and processes of drug screening. Vivactis applies its microplate-based differential calorimetry (MiDiCal^{TM}) technology to position nanocalorimetry within the bionutritional and pharmaceutical industries as a (validation) routine screening tool based on energy content changes. This platform enables users to measure the thermodynamics of biological events in a direct and simple way, improving the quantification of metabolic, biocatalytic and binding interactions. Vivactis aims to be the first to offer nanocalorimetry in an open and accessible multiwell format for routine determinations and screening.

MiDiCal^{TM} platforms could offer a solution for the four shortcomings of classical calorimetry: sample amount needed, low throughput, serial single-sample testing and limited accessibility. Sample reduction and increased throughput are achieved on the MiDiCal^{TM} chips by miniaturization (well volume of 25 μl) and parallelization (96-well and 384-well formats). Accessibility to both reaction and reference vessel or 'open' calorimetry is a unique novel feature, making it possible to tune the atmospheric conditions in and around the multiwell plate, and to collect multiple distinct profiles from a single-well experiment by means of sequential additions. Katarina Verhaegen (CSO, Vivactis) adds:

> 'The strength of calorimetry reaches far beyond pure binding events, towards enzyme activity and cellular screens. The technology makes it possible to determine the full kinetics of enzyme-substrate-inhibitor systems (in theory) for all possible systems. Multiple injections of substrate can be done in a single experiment, so K_M and k_{cat} can be determined in a single ITC experiment. For cellular assays calorimetry is a new tool that can complement existing assays by assessing the impact of potentially metabolically active compounds on tissue specific thermogenesis (by monitoring the shape of the thermal signature). One especially important domain is the field of compound toxicity. Different disease domain applications are currently under study at Vivactis: diabetes, obesity, infection, inflammation and cancer (apoptosis). In all of these, metabolic activity is changed in one way or another. The key focus areas for Vivactis are: a) metabolic activity of living cells, yeast strains, fungi and microorganisms; b) enzyme catalyzed reactions; c) and pure binding studies.'

Microelectromechanical sensors

These types of sensors based on microelectromechanical systems are submicron-sized mechanical devices normally built onto semiconductor chips. They began to materialize in commercial

products in the mid-1990s as switches; sensors for pressure, temperature and chemicals; and as vibration monitors and accelerometers for airbags. Applications to biological analysis (protein antigens and nucleic acids) appeared from 2000 [33,34]. The most widely used and well-characterized sensors are those based on microcantilevers that deflect or deform owing to changes in binding or surface stress following a specific molecular recognition event. For example, microcantilevers of different geometries have been used to detect two forms of prostate-specific antigen over a wide range of concentrations, from 0.2 ng ml^{-1} to 60 μg ml^{-1}, in a background of human serum albumin and human plasminogen at 1 mg ml^{-1} (mimicking the clinically relevant matrix for prostate cancer diagnosis) [33]. In general, published limits of sensitivity are in the ng ml^{-1} range for proteins or larger analytes when using cantilever deflection or stress measurement. It is also possible to identifying larger (e.g. viral) analytes via affinity capture of pathogen particles onto a planar solid surface. A surface profiler (atomic force microscope) then monitors the changes in the surface features at the affinity capture domains.

More recently, it has been possible to grow nanowire sensors electrochemically with e-beam-patterned electrolyte channels, which potentially enables the controlled fabrication of individually addressable arrays [35]. This creates what is effectively a microscopic field effect transistor that is sensitive to changes in the local electrochemical environment. Applications are centred on gas sensing with palladium nanowires and pH sensing with polypyrrole nanowires. However, biologically functionalized polypyrrole nanowires have been formed by the electropolymerization of pyrrole monomer with entrapped biomolecules (e.g. avidin-, biotin- and streptavidin-conjugated cadmium selenide quantum dots). When challenged with biotinylated DNA, the avidin- and streptavidin–polypyrrole nanowires generated a rapid change in electrical resistance down to a DNA concentration of 1 nM [35]. Field-effect transistors have also been incorporated into microcantilevers to detect biotin binding to an anti-biotin antibody [36]. However, although there is significant early uptake of these approaches in academia, relevant applications to small-molecule screening are difficult to find. There also remain substantial practical and engineering issues regarding the reliable delivery of drug targets to these microscopic features (particularly in a multiplexed format) and the undesirable sensitivity of these devices to environmental vibration.

Conclusion

Biosensor assays are used extensively in many areas of life science research, pharmaceutical discovery, environmental testing and the diagnostic testing of patients. For each of these industries, a wide variety of assay methods have been developed over several decades to answer basic analytical questions: (i) what amount of a material is present within a test sample?; (ii) how strongly do two substances interact with one another?; and (iii) what effect does a substance have on a receptor, cell or patient? The main difficulty in developing tests to answer these basic questions arises from the dramatic variation in the properties of the molecules being studied and the complex, highly heterogeneous environments in which they are found. In addition, medically important biosensor assays have now moved firmly beyond simple measurement of glucose levels (in which blood concentration levels are relatively high) towards more challenging specific detection of low concentrations of analytes, or a panel of analytes. Additional commercial challenges arise from the need to perform millions of such assays routinely in a cost-effective and time-effective manner. The above considerations necessitate continued research and development of novel transduction devices for biosensors that can translate the signal derived from the presence of an analyte into an electrical read-out.

Most platforms on the market use some type of photon detector for signal transduction. These approaches have benefited from the continued substantial development of label-based screening systems that exploit fluorescence for high-throughput screening or high-content screening. The investment in cheaper, faster and more sensitive charge-coupled devices, optical interfaces, solid-state lasers and algorithm (postacquisition processing) development has been substantial, and optical approaches have come a long way since the first commercial biosensor was launched in 1990. Most 'tools' development effort for commercial biosensor products is concentrated on system integration, enhanced content and better user interfaces. Complementing this development focus, smaller, innovative companies are now releasing novel biosensor technologies to the drug discovery and development markets that promise to supply users with novel information about a molecular interaction or an analyte, often at higher throughput and lower cost. The utility of label-free biosensors should become more widely recognized in the market as smaller companies continue to innovate and concomitantly encourage consolidation and more solution-focused product development from the current optical biosensor market leaders.

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