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Using Living Spores for Real-Time Biosensing

BCR Technology Detects & IDs Single Bacterial Cells

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Generally defined, biosensors are analytical devices integrating biological elements and signal transducers. The biological element interacts specifically with an analyte, producing a signal that the transducer recognizes and converts into a measurable parameter.

Over the last four decades, a large variety of biological species have been used for biosensors including antibodies, enzymes, proteins, nucleic acids, cell organelles, and microorganisms.[1] For bacterial detection, however, available biosensors are not rapid, sensitive, or cost-effective enough to substitute for time-consuming culture-based testing currently approved for diagnosis of infectious diseases.[2]

One emerging technology developed by **BCR Corp.** (Jamestown, RI) uses microbial spores as nanodetectors, which emit fluorescent light signals in response to specific bacteria in the surroundings. Spores are rugged organisms, ranging from 100–500 nm in diameter, with virtually no metabolic activity but still retaining considerable molecular functions.

Spores as Nanodetectors

Spores are suitable for use as detectors because they have the ability to sense environmental changes and to respond using explosive molecular mechanisms that transform dormant spores into rapidly growing cells.[3]

In addition, spores can survive prolonged harsh conditions including high temperature, extreme pH levels, and the presence of organic solvents. The spore's level of functionality, therefore, may surpass that of other cells currently used for biosensing, and perhaps, that of future molecular-scale machines based on silicon technology.[4]

The spore-based biosensor recently developed by BCR Corp. exploits the fact that dormant spores of various *Bacillus* species carry out no detectable macromolecular synthesis or oxidative metabolism, but acquire, within minutes, normal cell functions in response to specific "germinants" in the environment.[5] *(Table 1)*

The biosensor operating system, LEXSAS (Label-free Exponential Signal-Amplification System), uses living spores of either *Bacillus subtilis* or *Bacillus cereus* as detectors. *Figure 1* shows the LEXSAS operation, using as an example a test for bacteria with alanine aminopeptidase, an enzyme traditionally used for bacterial classification.[6]

The LEXSAS uses spores suspended in buffer containing diacetyl fluorescein (DAF) and L-alanoyl L-alanine (Ala-

Figure 1. Biosensor Operation

Test sample containing bacteria is mixed with spores (nanodetectors)
↓
Mixture is filtered through Biosensor Array
↓
Biosensor is rinsed with buffer containing Ala-Ala and DAF
↓
Biosensor is incubated at 37°C for about 5 min
↓
Bacteria trigger a chain reaction among the spores
↓
Chain reaction generates fluorescent products from DAF
↓
Fluorescence images of biosensor array are captured at time intervals
↓
Digital images are processed to yield 4-D biochemical data
↓

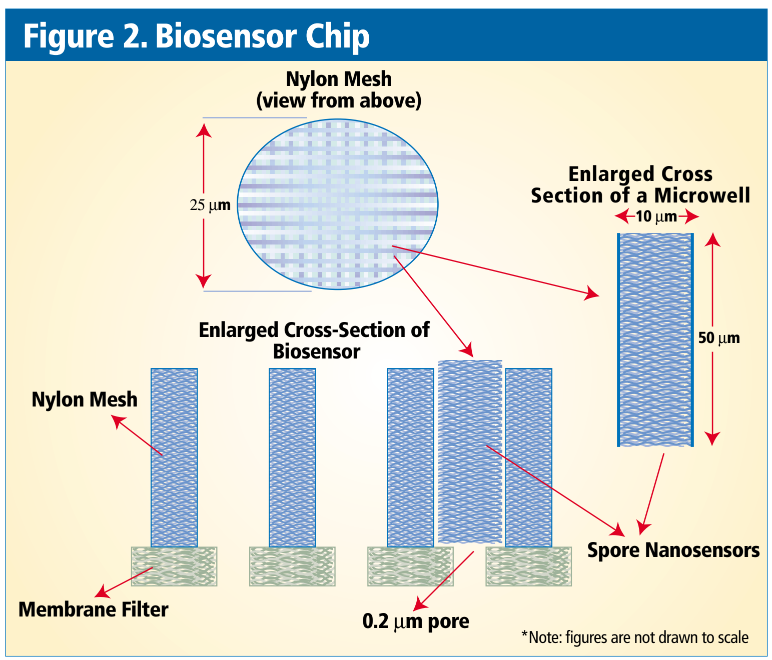

Figure 2. Biosensor Chip

Ala), a "germinogenic" dipeptide hydrolyzed by alanine aminopeptidase. (The term "germinogenic" has been coined to indicate any enzyme substrate that generates a germinant as a result of an enzymatic reaction.) Examples of germinogenic enzymes and corresponding substrates suitable for the LEXSAS are listed in *Table 2.*

Biosensor Mode of Functioning

The biosensor response to bacterial analytes is a chain reaction caused by reiteration of a series of particular events. In the first step, the bacterial analyte hydrolyzes Ala-Ala to produce L-alanine, which is a specific germinant for the spores. A critical point is that spores do not recognize Ala-Ala as a germinant.

Second, the newly formed L-alanine triggers the spores near the analyte to generate de novo alanine aminopeptidase and de novo acetyl esterase activity. Third, the de novo alanine aminopeptidase activity causes a chain reaction, generating more L-alanine (from Ala-Ala), which in turn produces more de novo aminopeptidase activity. The de novo acetyl esterase activity is used as a reporter. Fourth, the chain reaction is monitored at time intervals using the fluorescent output due to DAF hydrolysis by de novo acetyl esterase.

Figure 3. Micrograph of biosensor illustrates (in pseudo color) green fluorescence read-out due to spore activation in the presence of a target bacterium. Microwells are 20 x 20 nm.

An important property of the LEXSAS is that detection sensitivity can be increased simply by decreasing the reaction volume. For example, reducing the reaction volume to about 5-picoliter (5×10^{-12} L) allows for real-time detection of a single bacterium.

The inverse relationship between assay sensitivity and reaction volume is well known in enzymology and is exemplified by fluorogenic assays of ß-galactosidase that have been extended to single enzyme molecules simply by measuring the enzymatic activity in 4-pL microdroplets.[7]

Application

To analyze clinical specimens at single-bacterium sensitivity, BCR has designed a biochip filter consisting of an array of tens of thousands of microsieves of 5-pL volume each.[5]

Figure 2 provides an over-view of the construction of a first-generation biochip-filter constructed from commercially available materials. The biochip's filtration cap-acity is essential because it serves to pack the spores and the bacterial analyte, and to eliminate interfering soluble materials or small particles often present in clinical specimen.

Also important is that the biochip permits tens of thousands of parallel assays to be conducted using a charge-coupled device (CCD) imaging system for high-throughput data acquisition and processing.

Under blue-light epi- (echoplanar imaging-)illumination, individual microsieves within which a chain reaction has occurred show detectable fluorescence *(Figure 3)* and, therefore, the total number of fluorescent microwells equals the number of bacteria in the sample. It should be noted that sample-volume is not critical, because the biosensor is designed as a filtration device. In fact, larger sample volumes increase detection sensitivity.

Other notable benefits of the BCR biosensor include low cost, the ability to test samples with little or no preparation, portability, applicability to automated high-throughput testing, and linear dynamic range, extending from 1 to ~50,000 bacterial cells per sample.

Table 1. Spore-Forming Bacteria & Corresponding Germinants

Bacterium	Germinant
Bacillus cereus	Adenosine, or Inosine and L-alanine
Bacillus licheniformis	Glucose or Inosine
Bacillus megaterium	Glucose or L-proline
Bacillus stearothermophilus	L-leucine or L-valine
Bacillus subtilis	L-alanine

Table 2. Germinogenic Enzymes & Corresponding Substrates

Germinogenic Enzyme	Germinogenic Substrate
Alanine aminopeptidase	L-alanoyl L-alanine
Pyroglutamyl aminopeptidase	L-pyroglutamyl-L-alanine
Proteases	Benzoyl-L-arginyl-L-alanine
Coagulase	N-tosyl-glycyl-1-prolyl-L-arginyl-L-alanine
Esterases	L-alanyl-ethanol
Phosphatases	Adenosine 3′-monophosphate
β-D-Galactosidase	Adenosine-β-D-galactopyranoside
β-D-Glucuronidase	Adenosine-β-D-glucuronide
β-Lactamase II	L-alanine-cephalosporin or Adenosine-cephalosporin

Bacterial Identification

Since the germinogenic enzymes used in the LEXSAS coincide with those traditionally used for bacterial culture classification *(Table 2)*, the BCR biosensor can be used for establishing an innovative bacterial identification system based on differences in enzymatic activity rate measured quantitatively at the single-cell level.

To obtain the appropriate data, different germinogenic substrates are used in the biosensor, and fluorescence images are captured and pro cessed at time intervals. As in conventional methodology for pathogen identification, the enzymatic-rate profiles should be characteristic.

The basic difference between profiling using enzymatic rates and conventional profiling using cultures is illustrated using *Escherichia coli* and *Klebsiella pneumoniae*. Although 97–98% of cultures of these bacteria give positive reactions in conventional tests for β-galactosidase, *E. coli* cells in fact have significantly more β-galactosidase per cell than do *K. pneumoniae* cells. Therefore, in contrast to the culture method, the cellular difference may be easily measured in the biosensor using adenosine-ß-galactoside as the germinogenic substrate.

Applicability of Technology

A real-time bacteriologic biosensor would have a critical impact in public health and other major industries now using culture-based testing that usually requires 16–48 hours for completion.

Bioprocess activities that could derive substantial benefits from routine use of a low cost, real-time bacteriologic biosensor are quality control of fermentation products (e.g., antibiotics, beer, and wine), food and beverage monitoring, and sterility assurance. Other potential applications include clinical diagnostics, detecting biological warfare agents, environmental monitoring, testing blood products intended for transfusion, and veterinary diagnostics.

In the clinic, a real-time biosensor could prove especially useful at this time, given that infectious disease is reemerging as a major cause of death in many countries, including the U.S. Early diagnosis of microbial infection could radically improve medical intervention by allowing for monitoring of patients who present symptoms of serious life-threatening infectious diseases such as primary sepsis and bacterial meningitis. Sepsis affects 500,000 patients each year in the U.S. and has a mortality rate estimated at 20–50%.[8]

Furthermore, real-time monitoring of bacterial load in individual patients during antibiotic therapy could be used to determine antibiotic effectiveness in situ. This approach is similar to that recently used to dramatically improve management of HIV infected patients. In this particular case, efficacy of anti-viral therapy is determined in individual patients using PCR to quantify HIV in the blood.[9] GEN

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