DARPA Integrated Chemical and Biological Detection System

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The threat of attack on military and civilian targets with chemical and biological weapons is a growing national concern. The Defense Advanced Research Projects Agency (DARPA) is developing technologies for detecting these materials in the natural environment. While several technologies show promise as broadband detectors, there is no "silver bullet" that detects all chemical and biological materials at the requisite levels of sensitivity and specificity. DARPA is developing a systems approach whereby several different advanced detection schemes (based on different physical phenomena) are being integrated into a chemical and biological detection suite. (Keywords: Biochemical detectors, Chemical detectors, Lateral flow devices, Miniaturized mass spectrometer, RNA chip technology.)

INTRODUCTION

The number of toxic or infectious agents that could be used in a military or civilian environment is quite large. These materials range from simple inorganic or organic chemicals to complex bioengineered microorganisms (Fig. 1). At one end of the spectrum are vapor-phase nerve agents that will kill nearly instantly. In the middle are biochemicals such as neuroactive peptides that have low vapor pressure but have extremely disruptive effects on human life. At the other end are infectious bacterial agents that will kill in a dose of only one organism but with a longer onset time. Developing a detection system that combines the speed, sensitivity, and specificity necessary for environmental detection of these highly diverse classes of materials, in a package that is field portable, is a daunting task.

DETECTION OF THE THREAT

The approach taken by the Defense Advanced Research Projects Agency (DARPA) is to view the entire spectrum of the threat as a collection of chemicals that increase in size and complexity on going from left to right in Fig. 1. When seen from this view, the threat spectrum can be logically divided into a part that is made up of a single molecular compound (chemical agents, emerging chemical agents, bioregulators, and toxins) and a part that is a complex and ever-evolving mixture of biochemicals (microbes and engineered microbes). Historically, the volatile chemical agents have been detected using vapor-phase detection equipment such as ion mobility spectrometers, surface acoustic wave devices, or gas-sampling mass spectrometers. Similarly, the microbes have been detected using

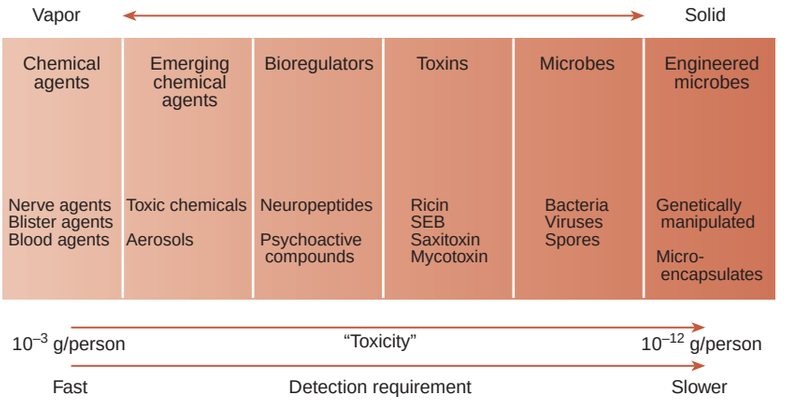

Figure 1. Chemical and biological threats (SEB = staphylococcal enterotoxin B).

antibody-based technology with optical reporters. Inspection of the threat spectrum reveals significant deficiencies in these technologies. The vapor-sensing equipment is completely ineffective for the larger, nonvolatile mid-spectrum and microbial threats. The current antibody-based technologies also do not effectively cover the mid-spectrum threat and suffer from speed and sensitivity problems. DARPA has developed and fostered a systems engineering approach to cover this gap in field detection capability.

DARPA's approach is to invest in the best chemical and biochemical broadband detection capability that is being developed and to augment this capability with the latest highly specific and sensitive identification tools for microbial threats. In particular, DARPA has funded the development of a miniaturized time-of-flight (TOF) mass spectrometer system at APL as a broadband chemical and biochemical detection technology, the multiplexed upconverting phosphor flow cytometer and lateral flow devices at SRI International, and the RNA chip technology at Argonne National Laboratory for identification of specific microbes. These systems have been chosen to match up with the temporal response and sensitivity requirements of the threat spectrum. For instance, it is imperative that many of the chemical agents be detected in near–real time because of the speed with which they act on humans. On the other hand, infective biological agents may take many hours before the onset of debilitation or death. However, early recognition of a potential microbial attack (even without specific identification) is crucial because it enables simple prophylactic measures (such as filtering masks) to be taken to dramatically reduce the casualty rate.

Critical for effective medical treatment after an incident is verification of the putative attack and specific identification of the biological agent, albeit with a somewhat relaxed time constraint. The system funded by DARPA uses the rapid detection capability of the mass spectrometer to identify chemical and mid-spectrum threats and to detect and characterize the microbial threat within a few minutes. Using information provided by the mass spectrometer as a trigger, the flow devices and RNA chip technologies are used to provide more specific identification of the threat over a period ranging from several minutes to a few hours. The essential battlefield requirements of protection through early warning of troops and identification of the threat to enhance the outcome of medical countermeasures are incorporated in this system.

MINIATURIZED TIME-OF-FLIGHT MASS SPECTROMETER

The mass spectrometer is the most powerful laboratory analytical tool for analyzing a broad spectrum of chemical and biological materials. Mass spectrometry is a technique for determining the masses of molecules and specific fragmentation products formed following vaporization and ionization. Detailed analysis of the mass distribution of the molecule and its fragments leads to identification of the molecule. These molecular measurements can be done at the attomole (10^{-18} mole) level of material using specialized laboratory-based instruments. The combination of specific molecular identification and extreme sensitivity is what makes mass spectrometry such a powerful tool. While such capability has existed in the laboratory for many years, the development of a small, portable mass spectrometer for potential field detection of chemical and biological substances is described here.

APL is developing a miniaturized TOF mass spectrometer system for automated environmental detection. This system combines a continuously vapor-sampling electron impact ionization method for detecting small chemical substances and a laser desorption/ionization method for detecting higher-weight mid-spectrum and microbial materials. For brevity's sake, we describe only the biological detection scheme here.

For the particulate threat, a trigger will initiate an aerosol sampler to sort, concentrate, and collect material onto a movable tape sample substrate. The sample is moved into the vacuum chamber of the mass spectrometer for analysis. The application of pulsed light from a laser onto the target creates sample ions. The

instrument uses matrix-assisted laser desorption/ionization (MALDI). With this technique, biomolecules as large as 300,000 Da can be ionized and desorbed intact into the gas phase for mass analysis.

The basis for MALDI is the interaction of a pulsed laser beam with a laser-absorbing matrix material into which analyte molecules are dispersed. Pulsed laser energy is absorbed by the matrix and transferred to the analyte, causing it to be ionized and desorbed into the gas phase. In the process, the analyte chemically interacts with fragment ions of the matrix, forming molecular adduct ions. The performance of the mass spectrometer system is outstanding for chemical and biochemical materials, including the mid-spectrum threats described. These biochemicals can be detected when presented individually to the mass spectrometer and can be determined in more complex mixtures.

The ultimate in complex mixtures is exhibited in microbes such as viruses and bacteria. The TOF system is proving useful for initial classification of these materials through the building of databases of spectral information and development of the interpretive software tools for using the database information for detection. The database methods and data are being developed at the University of Maryland, College Park, and the U.S. Army Research Institute of Infectious Diseases using a chemotaxonomic approach.

The chemotaxonomic method recognizes that all materials of biological origin are composed of common building blocks that include inorganic chemicals, small organic molecules, carbohydrates, amino acids, and nucleic acids. These building blocks are linked in precise fashion to form all manner of biological materials. These larger macromolecules are the prime signatures that can be used to identify materials of interest. Chemical markers that are characteristic of broad classes of microorganisms as well as markers that are specific for specific pathogens are being developed. These markers can be quantified extremely rapidly using the techniques of modern mass spectrometry.

The polar lipids present in bacterial cell (and several other) membranes are classically a useful chemotaxonomic biomarker. These biochemicals are also attractive (and useful) as mass spectral biomarkers. The phospholipid biomarker libraries include both molecular ion fingerprints and polar head group analyses derived from fragment ions.

While polar lipids are present in bacterial cells and enveloped viruses, they are absent in most viruses and all proteinaceous toxins. A more universal class of biomarker is based on peptides and proteins. The use of this type of biomarker is complicated by the large number (several thousand) of proteins present in a typical cell. However, proteins are being identified that are characteristic of specific bacteria and viruses.

Finally, the genetic pattern (DNA and RNA) of bacteria and viruses is an absolutely characteristic biomarker. However, current mass spectrometer technology does not allow long-chain DNA and RNA to be measured. DARPA is funding approaches that use chemical and enzymatic production of smaller fragments (oligonucleotides) for direct fingerprinting of viruses. Nucleic acid analysis using mass spectrometry is currently best accomplished by first enzymatically degrading the nucleic acid, using probes immobilized on a surface that capture specific sequences, and then measuring the mass distribution on the surface. With properly designed probes, a mass spectrometer can efficiently generate genetic information.

As pointed out, chemical building blocks making up bacteria and viruses can be measured by mass spectrometry. In addition, the broadband mass spectrometer can be used to measure contaminants (spent media, encapsulation materials, and other cofactors of weaponization) that are present in weaponized biological materials. However, the information produced is quite complex, requires significant interpretation, and relies on the building of an accurate database. Although this technique is improving steadily, it does not yet provide the definitive identification (down to the strain level) often necessary for biological detection and disease treatment. To fill this gap in rapid identification of biological agents, DARPA has invested in a handheld flow-through biodetector, an advanced flow cytometer with upconverting phosphor reporters, and RNA chip technology with both fluorescent and mass spectrometry readouts.

FLOW CYTOMETRY WITH UPCONVERTING PHOSPHORS

DARPA has also invested in the development of a miniaturized flow cytometer and a handheld bioassay incorporating a novel reporter system for multiplexed detection and identification of biological agents. The flow cytometer system is based on the flow of a fluid stream carrying a low density of biological particles past an illuminating laser. As the individual particles enter the interaction area of the laser, light is scattered into several different optical detectors. Information about the size and shape of the particles can be gathered from the spectral and angular distribution of the scattered light. The flow cytometer is a well-established tool in the clinical laboratory for identifying and counting large cells such as white and red blood cells. More recently, the technique has been applied to much smaller bacterial cells in the microbiological research laboratory. The laboratory systems are quite sensitive and provide useful information about the identity of the biological materials, particularly when antibody

probes are used. More recently, the sensitivity and specificity of the flow cytometer have been enhanced through the use of specific dyes and fluorescently labeled antibodies. Furthermore, with the judicious selection of labeled reporters, multiplexing of multiple agents can be obtained.

While this tool has become increasingly useful in the laboratory, it has not transitioned to a field-portable device because of the requirement for a high-power laser system and the weak signals from the reporter systems used. SRI has been developing a unique class of reporter materials based on mixed rare-earth oxides. These man-made oxides have remarkable optical properties unlike anything in nature. Their electronic structure is such that they exhibit an efficient near-infrared, two-photon absorption followed by a visible-light fluorescent emission. Thus, these materials can be used as reporter molecules based on absorption of near-infrared light that is readily obtained using small, light, and inexpensive semiconductor lasers. Furthermore, the visible emission line characteristic of natural materials is not observed, so the emission can be detected without background effects, thus dramatically reducing sample noise and enhancing signal sensitivity. The mixed oxide upconverting phosphor materials have been surface modified with various binding sites and are available with a variable mixture of rare earths so that the emission spectrum of the probe can be chosen for a given analyte. The net effect is a highly efficient multiplexing of the flow cytometer.

The upconverting phosphor materials have paved the way for miniaturization of the flow cytometer and have led to the enhancement of antibody-based handheld assays. Because the reporter probe can be excited with small semiconductor lasers, the entire system can now move out of the laboratory and into the field. Although the technological advances have brought flow cytometry into the arena of fieldable resources, the lack of available specific probes has limited their battlefield use. Focus on the flow-based systems for field use has shifted to the production of highly specific probes for identifying microbes. Antibodies that identify a particular microbe within a genus or species are abundant for some microbes such as *Staphylococcus*. Until very recently, there were no antibody-based probes that could distinguish potentially pathogenic strains of *Bacillus* and *Yersinia* from nonvirulent species. This is a critical point for environmental detectors, which take samples in areas that may be naturally rich in these genus members.

Novel approaches to synthesize small, specific-recognition molecules that could be used to identify these organisms are under way at the University of Alabama, Birmingham (UAB) and Utah State University (USU). UAB and USU have undertaken peptide phage display against pathogenic strains of *Bacillus* and other pathogenic microbes to screen for small, specific molecules. While UAB is using a combinatorial library of random peptides, USU is combining this approach with the analysis of specific bacterial receptors as a means of discovering useful probes for identification. Other researchers at UAB have recently reported the first antibodies that can speciate the *Bacillus* genus.

RNA CHIP TECHNOLOGY

The most characteristic molecules for identifying an organism are the genetic materials DNA and RNA. Researchers from the Englehardt Institute in Moscow, working at Argonne National Laboratory, have developed a tool for rapidly identifying microorganisms using a three-dimensional gel pad chip. The target moiety in their investigation is ribosomal RNA (rRNA). Since rRNA is present in many thousands of copies per cell, the selection of this target obviates the need for signal amplification using PCR (polymerase chain reaction)-based methods. Therefore, analysis of natively amplified RNA dramatically speeds the analysis compared to other nucleic acid identification approaches.

In the evolutionary history of life, the RNA molecule and the ribosome structure were among the first objects developed. In particular, the 16S fraction of rRNA is intriguing to study as it appears to be one of the genetic elements that encodes the road map of evolutionary progression and divergence from simple to complex organisms. The 16S rRNA consists of 1500 nucleotides in a characteristic structure. As life has evolved, this molecule has evolved with it. All living creatures, from bacteria to humans, have rRNA materials within their cells. In the RNA structure there are highly conserved regions along with regions that show considerable variability. By judicious selection of RNA complementary probes, regions of the 16S rRNA can be used to detect and phylogenetically distinguish organisms using the RNA chip.

Scientists at Argonne National Laboratory have developed a two-dimensional array of complementary nucleic acid probes immobilized within a 10×60 μm gel pad. The gel pads act as very small test tubes for carrying out the annealing reactions between the complementary probes and the rRNA oligonucleotides. Detection is done by breaking open the cells of interest, cleaving the rRNA, allowing the resulting oligonucleotides to flow over the chip, and measuring the fluorescence of the individual pads using a fluorescent microscope. From a knowledge of the complementary sequence, the sequence of a particular section of RNA can be determined and the organism can be identified. This type of chip can distinguish organisms that differ in only a single base pair at the target site.

The first application of this technology has been the development of a "Bacillus Chip." The genus *Bacillus* is important for biological detection applications because it includes the organism responsible for anthrax. However, the surface structure of spores of the genus *Bacillus* is such that highly specific antibodies for anthrax are not readily available. The Bacillus Chip is a technology that allows anthrax to be specifically differentiated from closely related organisms. One caveat of the rRNA-based chip is that it is of little use in detecting viruses, which do not contain an amplified target such as rRNA, and proteinaceous toxins, which may contain only small variable amounts of contaminating nucleic acids. These caveats may be overcome by amplifying the signal through PCR-based methods or by enhancing the detection of signals that are present in low amounts using other technologies such as the mass spectrometer for readout.

An alternative reporting scheme is also being developed in a collaboration between APL and Argonne National Laboratory. The approach uses a mass spectrometer to read out the bound oligonucleotide. It presents the opportunity for enhanced sensitivity and further multiplexing as a result of the ability of the mass spectrometer to determine multiple bindings to a particular sequence. This hybrid approach embodies the mass spectrometer with the specificity that is missing in the straightforward chemotaxonomy that is currently being pursued.

SYSTEM INTEGRATION

The detection of the entire range of toxic and infective threats is not currently possible with one type of field-portable instrumentation. The best approach to this broadband detection is the TOF mass spectrometer, which can rapidly and sensitively gather information on the entire spectrum of the threat. For chemical and biochemical threats, the spectrometer can both detect and identify threats because of the ability to measure molecular mass and fragmentation patterns. For complex microorganisms, the information that the mass spectrometer currently provides leads to classification of the threat and identification of co-factors of weaponization. The mass spectrometer does not at this point have the database and algorithms for identification of the threat. It is imperative in the short term to have fieldable technologies that address the entire threat spectrum.

The upconverting phosphor flow cytometer and the RNA chip are being integrated into a detection suite that provides a hierarchical approach to detection, classification, and identification. In this scheme, multiple sensors based on different technologies are used. In this way, countermeasures against one technology should prove ineffective against the others.

For the scenario of troop protection in the field, the detection system would work as follows: The system would be stationed in the field, and intelligent particle counters (e.g., the Lincoln Laboratory Biological Aerosol Warning System) would continuously monitor the air for a rise in specific bioparticle counts. When such a rise occurs (or when another trigger is alerted), the aerosol collectors on the system would be activated. Samples would be collected on the mass spectrometer sampling tape, and analysis of the threat would commence. On the basis of the information from the mass spectrometer about the nature of the threat (chemical, biochemical, or microbial), the sample collected for the flow cytometer and RNA chip would be analyzed on those systems. Thus, triggering, detection, classification, and identification would be rapidly and accurately obtained from the integrated DARPA chemical and biological detection system. As other systems for rapid detection and identification are developed, they will be incorporated into an enhanced detection system.

THE AUTHORS

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