Artificial Nucleotides Help Identify Multiple Real Biothreats

Warfighters may soon have better sensing and reporting mechanisms to provide a clearer and more timely picture of the chemical and biological threats they face, allowing them to better protect themselves against those threats. This could be accomplished by research performed for DTRA CB/JSTO, led by Dr. Steven A. Benner of the Foundation for Applied Molecular Evolution (FfAME), Gainesville, Fla., who reported on a high-fidelity method to simultaneously detect dozens of dangerous pathogens in a single vessel (multiplexing). Additionally, this multiplexing comes at a cost no higher than it costs today to detect just a single pathogen.

Photo courtesy of DefenseImagery.mil

*(continued on **Page 2**)*

JSTO in the news

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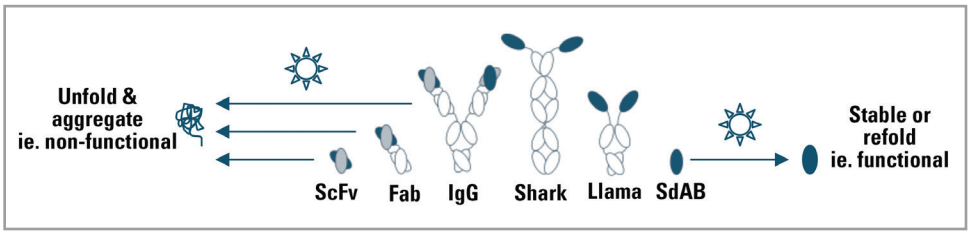
Ricin Detection Sensitivity Improved up to 1000 Times

Warfighters, first-responders and frontline detection personnel will be able to better protect themselves as well as the American public, by improving their ability to detect even miniscule levels of biothreat agent pathogens and their toxins, thanks to work funded by DTRA CB/JSTO. The research yields enhanced abilities to detect and diagnose bio-threat toxins, such as Ricin, at significantly lower concentrations than previously demonstrated -- a 100-1,000 times improvement.

The DTRA Ruggedized Antibody Program project, funded by the Diagnostics, Detection, and Disease Surveillance Division, recently demonstrated greatly enhanced capabilities in detecting Ricin toxin using a combination of recombinant anti-ricin single domain antibodies (sdAbs) developed in DTRA-funded work at the Naval Research Laboratory (NRL) and a novel enabling technology called ultrasensitive single-molecule array (SiMoA) platform, developed by Dr. David Walt at Tufts University.



When conventional antibodies (e.g. from mice and rabbits) and corresponding molecule fragments are exposed to heat their structure unfolds and aggregates causing loss of functionality; hence they require cold-chain transport. However, when heavy chain only antibodies (e.g. from llamas, sharks) and their antibody fragments are exposed to heat- they refold , are stable, and hence remain functional and do not require cold-chain logistics.

Together this new technique is 100 times more sensitive than using the conventional particle-based multiplex immunoassay (Luminex) and 1,000 times more sensitive than the widely used enzyme-linked immunosorbent assays (ELISA).

This technology has demonstrated exquisite analytical and clinical sensitivity, as well as a broad dynamic range. The combination of these two technologies will robustly increase the Department of Defense's diagnostic armamentarium. This could lead to warfighters being able to detect lower levels of the toxin, therefore decreasing false negatives in environmental samples and earlier discovery in the course of clinical intoxication.

SdAbs are recombinant ligand binding antibody fragments derived from the unusual structure of native antibodies found in camels and llamas. These unique heavy chain binding elements offer many desirable properties such as their small size (~15 kDa) and thermal stability, which makes them attractive alternatives to conventional monoclonal antibodies. The fact that they can be rationally-selected, produced in mass quantity by standard recombinant

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Artificial Nucleotides... (*continued from page 1*)

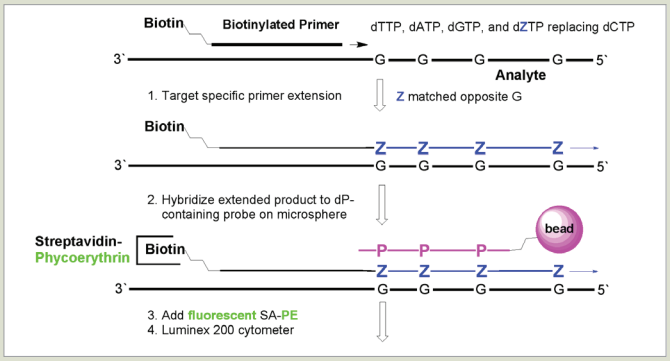
In Department of Defense and homeland defense applications, highly multiplexed and easily updated assays for pathogens are absolutely critical. High-fidelity identification of infectious agents necessitates simultaneous multiple site targeting of a single pathogen, not to mention the possibility of the adversary releasing multiple infectious agents, to allow one-at-a-time detection to be "cost-possible," let alone cost-effective.

In a recently published article in *Analytical Chemistry*, "Conversion Strategy Using an Expanded Genetic Alphabet to Assay Nucleic Acids," the researchers report improving the ability of the popular Luminex instruments to detect the targets in multiplex assays with much higher fidelity than ever before.

This improvement of the technology relies on earlier DTRA-funded work that developed artificial nucleotides and deepened our basic understanding of nucleic acids, including DNA and RNA. This, in turn, allowed the development of a new kind of "augmented" DNA, one where nucleotide letters are expanded beyond the four standard nucleotide letters (G, A, C, and T) found in natural DNA.

Assays that target DNA and RNA are the gold standards for diagnosing infectious disease. This is so, in part, because target DNA and RNA molecules from infectious agents, that often are scarce in biological samples, can be amplified using the polymerase chain reaction (PCR), making DNA and RNA-targeted assays extremely sensitive.

Unfortunately, many of the features that make DNA and RNA molecules good diagnostics targets also create problems. For instance, the amplified DNA molecules from one sample easily become the source of contamination for the next sample. Further, interactions between many DNA probes in assays that try to detect many DNA and RNA molecules simultaneously routinely generate background noise resulting in both false positives (false alarm) and missed false negatives (missed threat).



The ZiP-TAG conversion architecture. Source: Dr. Zunyi Yang, Foundation for Applied Molecular Evolution (FfAME)

In this approach, researchers ingeniously use the nonstandard DNA bases to solve these problems. In order to improve fidelity, the researchers omitted one of the natural nucleotides from the assay mixture forcing the nascent DNA to incorporate a nonstandard nucleotide instead, which can only bind to another nonstandard nucleotide and thereby limit the undesirable interactions.

These research innovations enable biothreat detection that greatly reduces the chance for the false alarm (false positive) and to avoid missing the existing threat (false negative) even in a multi-threat environment. The researchers report that the approach was successfully used to accomplish high-fidelity 12-plex detection of insect-borne RNA viruses, 15-plex detection of respiratory disease panel, and 8-plex detection of HIV targets. This is not possible with multiplexed assays that use only natural DNA as probes and primers.

These findings are of great value to warfighters and homeland defense because it improves speed and accuracy of biothreat sensing and the rapid development of detection and diagnostics elements for emerging threats.

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Seawater-driven Micromotors Clean Up Warfighters' Environments

Looking to find better ways to clean up warfighters' environments affected by toxicants led researchers to study various strategies to explore and expand the potential for implementation of micromotors. These micromotors introduce new paradigms in the assaying, countering and elimination of chemical and biological threats within complex and dynamic environments. Dr. Joesph Wang, University of California, San Diego (UCSD), working under DTRA CB/JSTO funding, published a paper in the journal *Nanoscale* titled, "Seawater-driven magnesium based Janus micromotors for environmental remediation," in which he and his colleagues describe the use of seawater as fuel to propel Janus micromotors. The new micromotors consist of biodegradable and environmentally friendly magnesium microparticles and a nickel–gold bilayer patch for magnetic guidance and surface modification.

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Seawater...

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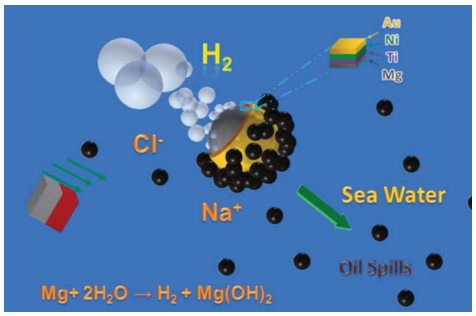

Schematic of the Mg-based seawater-driven Janus micromotors. Source: Dr. Joseph Wang, University of California, San Diego

Micromotor surface modification has been demonstrated to enable selectivity for a wide variety of target molecules and cells. In the application described in this work, alkanethiols were introduced to enable selectivity for oil droplets. Fundamentally, the seawater-driven micromotors, which utilize macrogalvanic corrosion and chloride pitting corrosion processes, eliminate the need for external fuels and offer efficient and prolonged propulsion towards diverse applications in aquatic and other saline-rich environments. Use of magnesium (Mg) is an attractive candidate material for the design of water-driven micromotors as it is a biocompatible 'green' nutrient trace element, vital for many bodily functions and enzymatic processes. In other words, not only does it have the ultimate potential to effectively and efficiently clean up a contaminated site, but it also has the potential to do so in a way that does not cause more harm to the warfighters.

In addition, Mg is a low cost metal, and Mg2+ is present in different natural environments, such as seawater. The new water-driven motion capability should greatly expand the scope of applications for chemically powered nanomachines and environments in which they can be exploited. That compatibility with and use of seawater might allow military members to make use of non-potable water sources, such as the ocean, as part of their remediation efforts.

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Ricin Detection...

(*continued from page 1*)

protein expression manufacturing methods, and have potential to eliminate the cold-chain for these reagents makes them highly attractive binding molecules. In other words, sdAbs provide more uniform, consistent production and function, while making them cheaper and more stable than conventional antibodies and potentially could eliminate the need for refrigeration of these reagents – a big plus for forward-based troops or first-responders who can keep the reagents at ambient temperature.

One limitation of sdAbs to date has been that, while the analytical sensitivityhas been sufficient for most environmental detection, the use of these novel binders for diagnostic targets in clinical sample matrices was difficult due to the inability to consistently produce high affinity binders.

SiMoAs are based on binding single protein molecules to capture antibody-coated magnetic nanoparticle beads to form sandwich antibody complexes. After protein binding and immunocomplex formation, the beads are allowed to settle into femtoliter-sized reaction wells. These small volume wells are sized to allow only a single bead to localize in each well. The wells are sealed with a fluorogenic substrate. Each immunocomplex is labeled with an enzymatic reporter, which generates a high local concentration of fluorescent molecules in the femtoliter wells. The confinement of the fluorescent product provides increased sensitivity over conventional fluorescent ELISA where the fluorescent product generated diffuses into a large volume. Beads carrying a single immunocomplex generate a detectable signal producing an "on" well, and the percentage of "on" wells (percentage of active beads) is directly proportional to the analyte concentration. SiMoA has been established as an ultrasensitive method for detecting single molecules of proteins PSA, botulism toxin and HIVp24 antigen. Coupling sdAbs with SiMoA provides the increased sensitivity needed for diagnostic targets in clinical samples, while still taking advantage of the production, cost and stability benefits provided by sdAbs. In addition, this technology has the potential to be applied to many different agents including bacterial, viral and other toxins as DTRA CB/JSTO expands this work in the future.

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The Defense Threat Reduction Agency's (DTRA) Research and Development (J9) Directorate, Chemical and Biological (CB) Technologies Department, serves as the Joint Science and Technology Office for Chemical and Biological Defense. This newsletter highlights the organization's accomplishments to protect warfighters and citizens through the innovative application of science and technology research.

Reducing Residual Hazards from Contaminated Human Remains

Looking to reduce the fate and residual hazard of chemical warfare agents (CWAs) to warfighters and civilian first responders charged with handling contaminated human remains and personal effects, researchers funded by DTRA CB/JSTO are looking to develop a model to predict the residual hazards after death.

Led by Dr. Daan Noort of TNO Defense, Rijswijk, The Netherlands, the team reported on the results of a quantitative study which provides critical data that advances warfighter understanding of the "agent fate" of CWAs within human remains. Additionaly, the research could be used to develop a prediction model on how long a CWA within human remains is a hazard to the living.

The portion of the study that examined CWAs on personal effects provides similar understanding of the agent fate on

Photo by Staff Sgt. Luke Graziani, www.dvidshub.net

those materials. Combined, this will allow the warfighter to develop protocols for the safe handling of contaminated human remains and personal effects. Additionally, this data transition package provides the Joint Project Manager for Protection (JPM P) with the data necessary to support the continued development of the Contaminated Human Remains Pouch (CHRP), as well as the Human Remains Decontamination System (HRDS), Contaminated Human Remains Transfer-Case (CHRT), and Remains Decontamination System (RDS) programs of record, and it provides the needed capability to the warfighter.

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Protein Switches for Biosensing

Researchers at Johns Hopkins University report several findings that could lead to better protein-based biosensors for both Department of Defense (DoD) and civilian applications. Working for the DTRA/CB-funded basic research program, Dr. Marc Ostermeier and his team published two articles on the design, construction and characterization of protein switches with the ultimate aim of developing design rules for a universal biosensing platform for improved diagnostics and environmental hazards monitoring for warfighters. Protein switches are engineered fusion proteins consisting of an input domain and an output domain. The input domain recognizes and responds to a target molecule by activating an output domain. The output domain's function to generate recognizable signals when activated is regulated by the state of the input domain.

Ostermeier's lab has pioneered a directed evolution strategy for building novel protein switches by fusing

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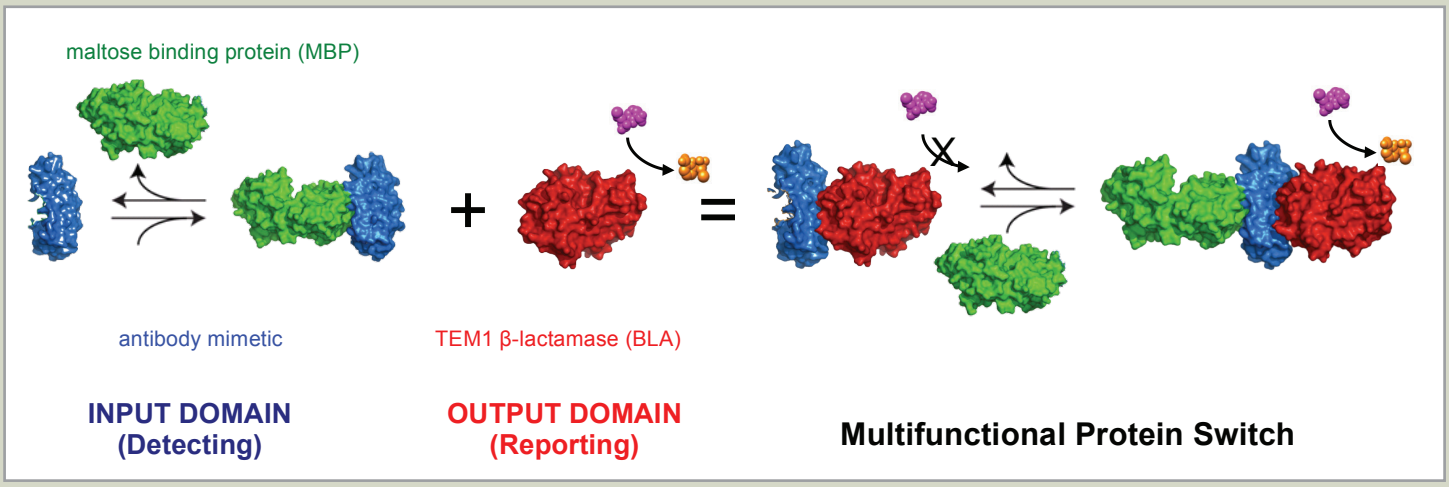

Modular protein switches that sense and report on specific biomolecules have been created by fusion of input (sensing) and output (reporting) protein domains. Source: Dr. Marc Ostermeier, Johns Hopkins University.

Hazards... (con't from page 4)

During the study, hairless guinea pigs were contaminated with various agents (e.g., VX, sulfur mustard) through various routes of exposure. Subsequently, the study measured levels of the intact agent and (potentially) toxic metabolites, both within the animal as well as on the skin, to determine the fate of the agent after the death of the animal. The study also determined the fate of a number of agents on various personal effects, including boots and uniforms. Finally, the study also examined the effectiveness of skin decontamination on hairless guinea pigs contaminated with various chemical and biological agents, including B. anthracis spores.

The study concludes that scenarios can be envisaged in which vapor and/or contact hazards are posed by human remains contaminated by VX or sulfur mustard, require the use of personal protective equipment or other protective systems, like the CHRP and CHRT, during handling and processing. It also demonstrated that decontamination of the remains shortly after death using HRDS and related technologies, such as Reactive Skin Decontamination Lotion (RSDL), would reduce both types of hazards. The study verifies that chemically contaminated personal assets, such as uniforms and boots, also pose a considerable hazard, due to the high persistence of CWAs on and in these materials, in particular the rubber and suede of the boots. Right now, this research reinforces and provides warfighters more information on the importance of properly handling any possibly contaminated human remains and decontaminating any items of clothing or personal effects that might be exposed to chemical warfare agents. In the future, this research could lead to better technologies for handling all contaminated items. For remains contaminated with B. anthracis, a similar level of personal protection is required. Autopsy on such remains is preferably performed in a biosafety environment.

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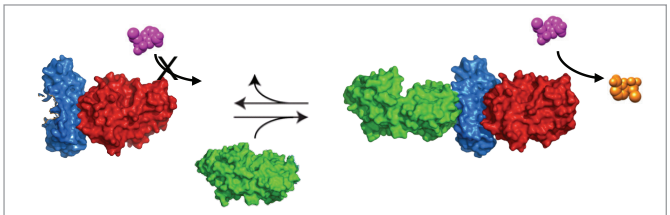
Switch... (con't from page 4)

natural proteins with the prerequisite input and output function of the desired switch. The difficult question is how to fuse the two proteins to generate such a switch.



Multifunctional Protein Switch

Source: Dr. Marc Ostermeier, Johns Hopkins University

The *Methods in Enzymology* article, "Protein Switch Engineering by Domain Insertion," reports a novel inverse PCR technology for building switches. The approach improves on existing technology in which insertions could not be targeted. The method allows the single-pot creation of thousands of different targeted insertions of one protein into the other, thus enriching proteins with the highest potential for switching activity. The Ostermeier lab is currently using this technology to test ideas on which protein domains might best serve as a universal platform for switch development.

The *Protein Science* article, "Non-allosteric enzyme switches possess larger effector-induced changes in thermodynamic stability than their non-switch analogs," describes the characterization of one type of protein switch that functions only in the context of the cell. Such switches represent an important class of engineered proteins for basic science and biotechnological applications in vivo. For these switches, switching is manifested through increased accumulation of the switch protein in the presence of the target molecule the switch detects. The authors uncovered that the linker between the two proteins is the key for the manifestation of this type of switching. They also elucidated some of the biophysical and biochemical phenomena behind the increased cellular accumulation of switch proteins and their switching properties. Namely, the fact that in the absence of the input signal, some of these switch proteins undergo an increased rate of unfolding, decreased conformational stability and increased susceptibility to cellular proteases. Each of these factors alone or in combination serves to decrease switch protein cellular accumulation. Conversely, the input signal increases cellular accumulation of the switch proteins by alleviating one or more of these factors through the stabilizing effect on switch proteins upon their binding to the input signal. This perspective on the mechanism for phenotypic switching is not only helpful to develop design rules for switch construction for applications, but also will be useful to study the regulatory mechanisms of natural cellular proteins.

The switches are designed to contain both the sensing and the reporting components in a single molecular construct whose diameter is less than 1/10,000 of the size of a diameter of a human hair. As such, they have excellent potential for efficacious sensing and reporting not only in sensing platforms for diagnostics applications, but they can also conceivably be imbedded in various protective materials (such as garments to monitor the environmental hazards and/or warfighter's condition) or other gear.

Combined with the potential modularity of switches created by domain fusion, in which different input domains can be coupled to the same output domain, this basic research innovation offers a potential route to a universal biosensing platform as well as selective protein therapeutics approaches for both DoD and civilian applications.

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