Runnin' on Near Empty with Hydrazine as Fuel Source for Catalytic Micromotors

Tiny motors that move antidotes to deadly chemical and biological agents throughout the body need even tinier fuel sources. A team of researchers managed by DTRA CB/JSTO's Dr. Brian Pate, including Wei Gao and Joseph Wang at the University of California, San Diego, recently published results demonstrating that catalytic micromotors can be powered by extremely low concentrations of chemical fuels, including hydrazine.

The near fuel-free requirements of these new catalytic micromotors also show promise for the design and development of chemically powered nanomachines for targeted drug delivery, bioisolation, and environmental remediation in applications for warfighters and first responders.

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JSTO in the news

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"Ships of the Desert" Could Soon Provide Better Protections against Biological Weapons

Warfighters going into extreme conditions, such as desert heat, soon will have more rugged detectors against biological weapons, thanks to a protein derived from an animal accustomed to living in that environment. Recent work managed by DTRA CB/JSTO's Dr. Ilya Elashvili and performed by researchers at the U.S. Naval Research Laboratory (NRL), led by Dr. Ellen Goldman, highlighted two methods to increase the utility of single domain antibodies (sdAbs). These sdAbs are binding domains derived from the heavy chain antibodies found in camelids such as camels and llamas, by further enhancing their melting temperature and solubility.

Although sdAbs are typically more soluble and thermally stable than other antibody-derived binding domains, increasing their ability to survive high heat challenges can improve the performance of field portable detection devices in austere environments.

These studies were reported in a pair of articles: "Enhanced stabilization of a stable single domain antibody for SEB toxin by random mutagenesis and stringent selection" published in *Protein Engineering Design and Selection*, and "Negative tail fusions can improve ruggedness of single domain antibodies" published in *Protein Expression and Purification*.

SdAbs, recombinantly-expressed binding domains, offer alternative binding reagents that provide the affinity and specificity similar to traditional antibodies, but they are much more rugged when exposed to harsh chemicals or high temperatures. Not only do the reagents withstand denaturation better at higher temperatures, but sdAbs also often recover their 3-D structure and binding ability even after denaturation at high temperatures, unlike traditional antibodies which lose their ability to recover. However, not all sdAbs possess this ability to recover, and some sdAb clones are prone to irreversible aggregation after heat denaturation. Therefore, understanding how to manipulate sdAbs to resist aggregation can result in improved reagents with better shelf life and reduced logistical demands, such as shipping and storing without refrigeration.

U.S. Marine Corps photo by Cpl. James Clark

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"Ships of the Desert"... *(continued from page 1)*

The team reported earlier the development of an A3 sdAb, which binds with excellent affinity to the potential biothreat agent *Staphylococcal enterotoxin B (SEB)* and possesses one of the highest melting temperatures (Tm of 83.5°C) yet reported (see the December 2013 *JSTO in the News* article, "Unraveling the Secrets of Protein Stability"). In a demonstration to push its melting temperature up closer to boiling, the A3 sdAb underwent a process termed random mutagenesis and stringent selection. A library of variants was constructed in which amino acid changes were introduced randomly through the protein. The variants were heated and those that retained their ability to bind antigen were selected. Through this process, a derivative of A3 was isolated that had a melting temperature 6.5 °C higher, while maintaining its ability to recognize toxin with high affinity. This demonstrated that even an sdAb with an extraordinarily high melting temperature is able to be improved. This strategy should also be applicable to sdAbs with more typical melting temperatures.

Engineering the ability of sdAbs to resist aggregation is another method to improve these reagents. The scientists found that improvement could be accomplished by appending a negatively charged segment to sdAbs. The sdAbs with the negative tail were much less prone to aggregate when kept for extended periods of time at high concentrations above their melting temperature. In some cases, this did not translate into improved ability to function after heating, but in others, such as with the anti-SEB sdAb A3, the fusion of the negatively charged segment led to both aggregation resistance and improved binding performance after heating. An unexpected result of this work was that sdAb proteins with the negative segment were able to refold after heat denaturation into an active state even when produced under circumstances that typically abolish refolding ability.

This work demonstrates that sdAbs can be engineered to increase their melting temperatures and improve the probability of function after heating above their melting temperature at high concentrations for long periods of time. The methods used to improve the sdAb should be generalizable and could be applied to any sdAb reagent. These are important advancements towards the realization of sdAbs as improved binding reagents for integration into antibody based biosensors.

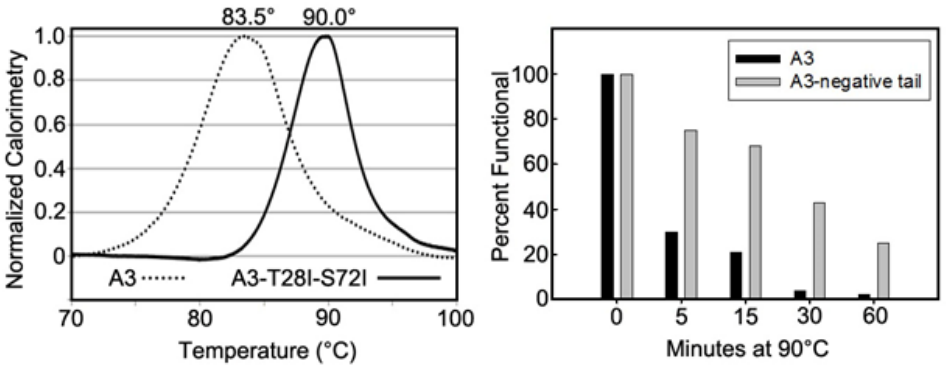

Figure legend: Left panel shows melting data (differential scanning calorimetry) for the anti-staphylococcal enterotoxin B sdAb A3, and a mutant (A3-T28I-S72I) that was generated through random mutagenesis and stringent selection. This work showcased the ability to use mutagenesis techniques to increase the thermal stability of sdAb even when they already possess quite high melting temperatures. The right panel graphs the ability of sdAb A3, and a variant with a negatively charged tail, to function after heating at high concentration to 90 °C. The addition of the negative tail slows aggregation, enabling better retention of function than the unmodified sdAb. (Courtesy: Dr. Ellen Goldman, U.S. Naval Research Laboratory)

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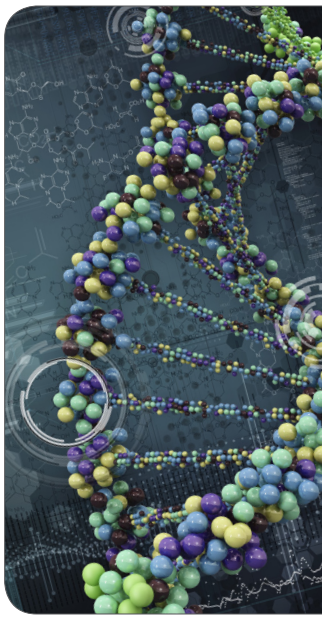
Mining the Literature to Improve Our Ability to Counter Biothreats

The amount of genome data in the published literature has increased exponentially as the price of genomic sequencing has continued to fall.



With that, a key question arises: How can all this data be used to enhance biomedical research and enhance warfighter capabilities? Researchers from Los Alamos National Laboratory (LANL) in New Mexico are finding new ways to mine that data, which could provide clues that will enable more effective countermeasures to biothreats. DTRA CB/JSTO-funded researchers managed by Dr. Dan Wolfe are leading the way by developing methods to mine underutilized data published as text. New methods to search for data in published text and supplemental information will have the potential to facilitate studies of microbial genomes, which will provide insight into how they cause disease and how we might protect warfighters and first responders against those diseases.

The problem is, as the scientific literature grows, medical professionals are having increasing difficulty locating important information to respond rapidly to biothreats and emergent infectious diseases. In

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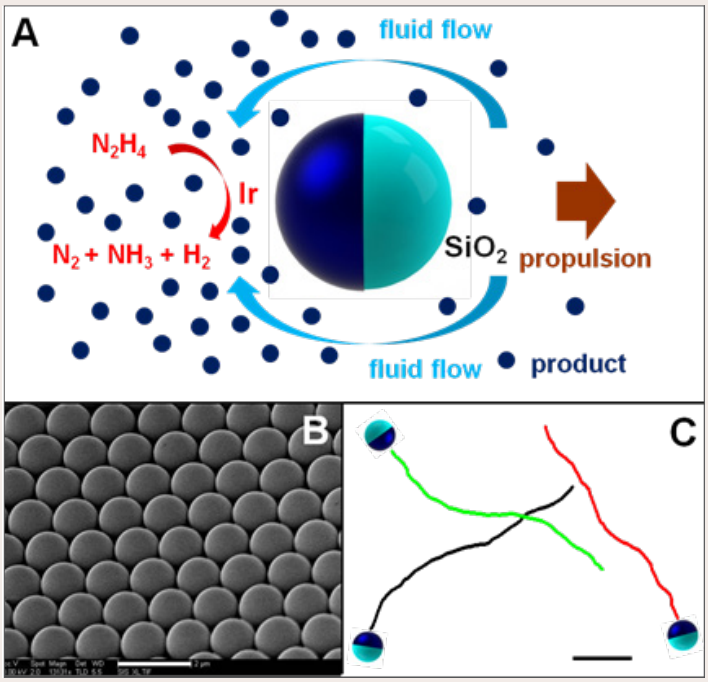
Runnin' on Near Empty with Hydrazine... *(continued from page 1)*

In their recent *Journal of the American Chemical Society* article, "Catalytic Iridium-Based Janus Micromotors Powered by Ultralow Levels of Chemical Fuels," the scientists showed these iridium-based Janus micromotors required substantially lower fuel levels (up to 10,000 times lower), compared to commonly-used peroxide based and platinum (Pt)-based micromotors. In addition, they display efficient propulsion of more than 20 um/sec. This study represents the first example of hydrazine use as the sole fuel for catalytic micromotors.

Micromotors are beneficial to the warfighter because they are a *non-invasive* targeted drug delivery system that affords a reduction in frequency of dosage, provides a more uniform effect of the drug, and reduces side-effects and fluctuations in circulating drug levels. Micromotors are also capable of imaging certain parts of the body which enable new force structure digital modalities, thus allowing for medical diagnoses/clinical decisions from remote locations in support of operational medical missions, and might significantly reduce the burden on Department of Defense tactical networks relative to the transmission of digital medical imagery.

Gao's DTRA CB/JSTO-funded work developing versatile advanced nanomachines for a wide range of biodefense applications recently earned first place in the 2013 American Institute of Chemical Engineers (AIChE) Bionanotechnology Graduate Student Award competition. In June of this year, he plans to defend his thesis titled "Synthetic micro/nanomachines and their applications: towards fantastic voyage," which includes a heavy component of DTRA CB/JSTO-funded work, and he plans to continue research of potential interest to DTRA CB/JSTO at the University of California, Berkeley by working on "artificial electronic skin," a new class of smart materials that provide a sensor and/or actuation network on a skin-like substrate.

The field of micromotors and countermeasures delivered at the nanoscale level is seen to have significant impacts for warfighters and first responders.



A) Schematic of catalytic Ir/SiO2 Janus micromotors powered by hydrazine. N2, H2, and NH3 molecules are generated at the Ir surface, creating a zone of high product concentrations. Fluid flows from the SiO2 side to the Ir side due to the product gradient. As a result, the motor moves towards the SiO2 side. B) Scanning electron microscope (SEM) image of an array of assembled spherical Ir/SiO2 micromotors. Scale bar, 2 μm. C) Tracking lines illustrating the distances traveled by three micromotors in a 0.001% hydrazine solution over 1 s. Scale bar, 5 μm. (Courtesy: Wei Gao, University of California, San Diego and the American Chemical Society.)

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New Micro-sized Motors Could Yield Big Results in Fight Against Chem-Bio Threats

They might be small, but their results could be mighty in the effort to give warfighters more tools to protect against chemical and biological weapon attacks. A proof-of-concept microscale pump, made possible by the recent discovery of new factors underlying biological catalyst activity and transport, might enable a new paradigm in medical diagnostics and countermeasures.

The basic research project, managed by DTRA CB/JSTO's Dr. Brian Pate, has uncovered the roles of non-reciprocal conformational changes, as well as substrate and cofactor concentration gradients in the activity of DNA polymerase and is generalizable to other biological enzymes.

The identification and understanding of these previously unexplored factors influencing enzymatic activity provides potential new targets for both diagnostics assays and medical countermeasure development, including prophylactics and therapeutics. In part, these might rely upon the ability of enzymes, once immobilized, to act as microscale pumps of their substrates.

The American Chemical Society journal *Nano* article, "DNA Polymerase as a Molecular Motor and Pump," shows how researchers at the Pennsylvania State University and the University of Maine, including first author Samudra Sengupta and corresponding authors Peter Butler, R. Dean Astumian, Stephen Benkovic, and Ayusman Sen, used

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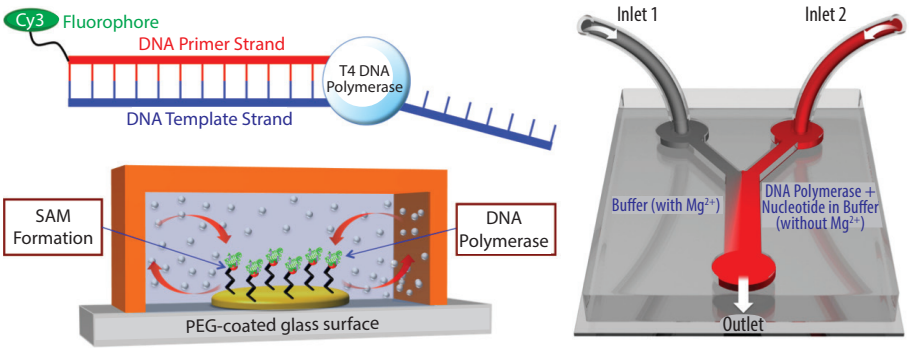
New Micro-sized Motors... *(continued from page 3)*



Depiction of the new methodology for studying DNA polymerase, including exposure of the immobilized enzyme to varied concentrations of fluorophore-tagged substrate and exposure of the enzyme/substrate complex to inorganic cofactor within a microfluidic mixing platform. (Courtesy Dr. Ayusman Sen, The Pennsylvania State University and the American Chemical Society)

fluorescence correlation spectroscopy to demonstrate that the diffusive movement of a molecular complex of DNA template and DNA polymerase is enhanced during nucleotide incorporation into the template, showing a strong dependence on the inorganic cofactor Mg^{2+}. When exposed to gradients of either nucleotide or cofactor concentrations, an ensemble of DNA polymerase complex molecules exhibits collective movement toward regions of higher concentrations. Furthermore, when immobilized to a patterned surface, the molecular complex acts as a pump by transporting fluid and tracer particulars in a directional manner with speeds increasing in the presence of a cofactor.

This principle enables the envisioning of a variety of designs for miniature fluid pumps using enzymes as engines, for example, to enable revolutionary new paradigms in diagnostics and medical countermeasures for the warfighter.

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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 publication highlights the organization's accomplishments to protect warfighters and citizens through the innovative application of science and technology research.

Mining the Literature to Improve Our Ability...

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fact, the scientific literature now identifies millions of microbial genes and associated data. This text data rarely is loaded into public sequence databases that can be rapidly searched. LANL researchers Chris Stubben, Jean Challacombe, and colleagues recently published a new way to take advantage of Open Access publications available on PubMed Central, an archive of over 3 million biomedical and life science articles. They developed a computer program called pmcXML, a gene annotation database that automatically mines and extracts gene locus tags from full text, tables and supplements.

The group demonstrated their data-mining program by identifying gene locus tags of *Burkholderia pseudomallei* as a model organism. Their results show that the locus tags found in unindexed supplementary tables and within ranges like genomic islands contain the majority of locus tags. Significantly, their software provided access to data resources that are not accessed by conventional PubMed searches. The LANL group published their methods in *BMC Bioinformatics* journal article "Mining locus tags in PubMed Central to improve microbial gene annotation."

Researchers hope to convert a full text database into a functional gene annotation database that would be a valuable reference for most microbial genomes that don't have recent or updated annotations available in public sequence databases. This innovative approach will make it easier to identify the biothreats to warfighters and first responders and thus rapidly develop successful countermeasures.

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Quantum Mechanics Build Path to Quicker, More Efficient Chem-Bio Countermeasures

Knowledge of quantum mechanics and the practical application of designs at the sub-atomic level could soon provide a new class of rationally designed, synthetic catalysts that would protect warfighters against chemical and biological threats quicker and more efficiently.

A DTRA CB/JSTO-funded research effort, managed by Dr. Ilya Elashvili, DTRA CB, and carried out by Drs. Christian Schafmeister of Temple University and Kendall Houk of the University of California, Los Angeles, has accomplished a series of catalytic reactions using small, non-natural, shape-programmable scaffolds called "spiroligomers." This opens up the way to address a much larger issue: How to develop catalysts for chem-bio defense, such as nerve agent degradation.

In a recent *Journal of American Chemical Society (JACS)* article, "Acceleration of an Aromatic Claisen Rearrangement via a Designed Spiroligozyme Catalyst that Mimics the Ketosteroid Isomerase Catalytic Dyad," the scientists reported the development of a catalyst that accelerates an important carbon-carbon bond forming reaction called the aromatic Claisen rearrangement. Ever since its discovery more than 100 years ago, the aromatic Claisen rearrangement reaction has served as a model reaction towards the understanding of how enzymes work because enzymes accelerate this unimolecular reaction with a relatively simple, single transition state.

The aromatic Claisen rearrangement breaks a carbon-oxygen (C-O) bond while simultaneously forming a carbon-carbon (C-C) bond (Figure 1A). As the C-O bond breaks and the C-C bond forms, a negative charge transiently builds up on the oxygen, labeled ∂-. The isolated negatively charged oxygen is a high-energy species and the primary reason why the reaction is slow.

The catalyst was developed by the Schafmeister/Houk groups to accelerate this reaction. They used the active site of the enzyme Ketosteroid Isomerase (KSI) as a starting point for the design based on its ability to stabilize a similar transient negative charge on an oxygen atom (Figure 1B). From this structure, the Houk group designed an optimal model of the catalyst using quantum mechanical calculations. The Schafmeister group constructed a series of molecules based on their spiroligomer chemistry that mimicked the presentation of the carboxylic acid and the phenol as hydrogen bond donors to stabilize the oxyanion. They then demonstrated that the synthetic catalysts that best mimicked the designed model generated the largest accelerations of the reaction.

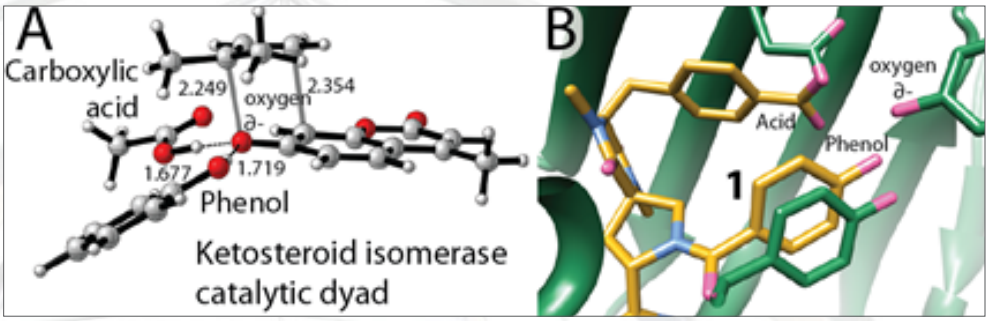
This work demonstrated that the essential catalytic activity of the active site of a large enzyme could be mimicked by the proper presentation of just a few active site residues on a small, pre-organized spiroligomer scaffold that is forty times smaller than the enzyme.

The Claisen catalyst that the Schafmeister/Houk group developed is the first synthetic Claisen catalyst that uses O-H (oxygen-hydrogen) hydrogen bond donors to stabilize oxyanions similar to natural enzymes.

These efforts started five years ago after the Schafmeister group showed a method that synthesizes shape-programmable spiroligomer macromolecules. Since then, the team reported DTRA CB-funded successful work that introduced functional groups at predetermined sites into the scaffold and the ways to create larger and more elaborate structures, enabling the design of two catalysts: a proline-based aldol catalyst (see the *JACS* article, "Hydrophobic Substituent Effects on Proline Catalysis of Aldol Reactions in Water") and a transesterification catalyst (see the *JACS* article, "Spiroligozymes for Transesterifications: Design and Relationship of Structure to Activity").

This latest effort will be the third DTRA CB-funded catalyst for the aromatic Claisen rearrangement.

Going forward, the Schafmeister and Houk groups plan to construct much larger spiroligomer scaffolds within which more complex active sites can be displayed based on the ideas developed in these earlier works.



The transition state model of the aromatic Claisen rearrangement was inspired by the active site of the enzyme Ketosteroid Isomerase (KSI). (A) The model, which was optimized using quantum mechanics calculations to accelerate the Claisen rearrangement reaction, by presenting a carboxylic acid and a phenol group to simultaneously donate two hydrogen bonds and stabilize the negative charge that transiently builds up on the oxygen (labeled ∂-, the oxyanion). (B) The superposition of the most active spiroligomer based catalyst (compound 1, gold) onto the active site of KSI (in green). Similar to the model, KSI presents a carboxylic acid and a phenol to stabilize the negatively charged enolate oxyanion (∂-) that forms during the catalyzed isomerization of "3-oxo-Δ5-steroid." (Courtesy: by Drs. Christian Schafmeister, Temple University and American Chemical Society)

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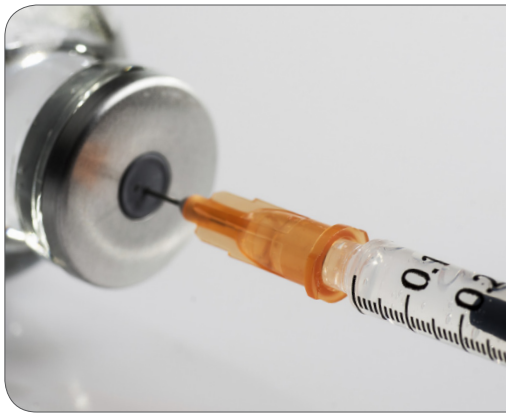
Novel Drug Treatment Against Deadly Marburg Virus Shows Promise Against Other Diseases



Warfighters could soon have better medical countermeasures for a highly lethal virus, as well as other diseases. Scientists at the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID), the lead military medical research laboratory for DTRA CB/JSTO, demonstrated the effectiveness of a small-molecule drug in protecting nonhuman primates from the lethal Marburg virus.

The research, managed by Dr. Erin Reichert, DTRA CB, and performed by Sina Bavari, USAMRIID, along with a host of other Army scientists, was published online in the journal *Nature* and is the result of a continuing collaboration between Army scientists and industry partners. In addition to Marbug virus, the drug known as BCX4430 from BioCryst Pharmaceuticals, Inc., showed promise against a broad range of other RNA viruses, including the emerging viral pathogen Middle East respiratory syndrome coronavirus (MERS-CoV), when tested in cell culture.

In the article "Protection against filovirus diseases by a novel broad-spectrum nucleoside analog BCX4430," the research revealed BCX4430 protected cynomolgous macaques (a nonhuman primate) from Marburg virus infection when administered by injection up to 48 hours post-infection. Also, the drug protected exposed guinea pigs from Marburg virus by the inhalation route. The paper's findings demonstrated how the drug interfered with the internal "machinery" of Marburg virus, preventing it from replicating its genetic material. Additional studies look to see whether that 48-hour therapeutic window can be extended. With funding from the Department of Health and Human Services (HHS) National Institute of Allergy and Infectious Diseases (NIAID), BioCryst plans to file investigational new drug (IND) applications for intravenous and intramuscular BCX4430 for the treatment of Marburg virus disease, as well as conducting Phase 1 human clinical trials.

This work is in line with one of the top biodefense priorities for the U.S., developing filovirus medical countermeasures, highlights cooperation between the Department of Defense and HHS, and demonstrates the importance of government-industry collaboration.

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Fresh Ideas for Bioactive Coatings More than Just Slapping on New Paint

DTRA CB/JSTO-funded work is finding more than just a fresh coat of paint for drug delivery, biosensors and medical devices that could protect warfighters and first responders from chemical and biological threats. Work managed by Dr. Brian Pate looked into bioactive surface functionalization strategies based on chemical vapor deposition (CVD) polymerization, highlighting commonly used surface chemistries.

In a *Journal of Applied Polymer Science* article, "Orthogonal surface functionalization through bioactive vapor-based polymer coatings," researchers Drs. Xiaopei Deng and Joerg Lahann from the University Michigan found that using CVD polymerization results in biofunctional surface coatings can facilitate orthogonal immobilization of more than one type of ligand on a substrate. These results produce coatings with nanoscale thicknesses of wide applicability in biomedical applications, as well as micro- and nanodevices. These nanodevices are of vital importance to many biomedical applications such as drug delivery, biosensors, medical implants, and tissue engineering (for tissue/bone regeneration), thus providing new capabilities for field dressing of wounds. Nanodevices are also of interest for applications including information storage and mechanical actuation for surface acoustic wave (SAW) immunosensors and field ion spectrometery.

Deng, the primary author on this paper, recently received her Ph.D. in Macromolecular Science and Engineering under DTRA CB/JSTO support. Her thesis title is "Biologically Inspired Surface Design Using Chemical Vapor Deposition Polymerization." She received several prestigious awards including the Charles G. Overbeger Award for Excellence in Research in 2012, in part, for her work with DTRA. She has continued on the DTRA project as a postdoctoral research fellow leading the CVD team in the University of Michigan labs.

This overall work on coatings is anticipated to have widespread applications for warfighters and first responders.

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