BUILDING NEW VACCINES FOR THE 21ST CENTURY

Tiny nanolipoprotein

particles deliver

the antigens and

adjuvants needed

to create vaccines

against infectious

diseases.

SINCE the invention of vaccines, humankind has had a potent weapon against debilitating diseases such as smallpox, polio, diphtheria, tetanus, pneumonia, and influenza. Vaccination may also provide an effective approach to protect warfighters against biological threat agents and thus is a major research focus supported by the Department of Defense.

Traditional vaccines have drawbacks along with the benefits. Those made from attenuated, or weakened, microorganisms often pose safety concerns that limit their use in special populations, especially in people with compromised immune systems. New preparations can take years to bring to market, and research and development costs can soar into millions of dollars. In addition, most formulations require cold storage and have reduced lifetimes outside the cold chain. Some vaccines, such as the one for anthrax, require repeated injections over several months to elicit the protective immune response, plus an annual booster to maintain that effectiveness. As a result, vaccines have limited utility in protecting naive, or unexposed, populations.

Livermore researchers have developed a technology that reduces or eliminates these drawbacks in vaccine development and could one day lead to treatments that save thousands of lives. The new approach with such huge potential uses the tiny

Nanolipoprotein particles (NLPs) are naturally occurring molecules that serve as the structural components of cell membranes. This reconstructed image reveals the structural details of an NLP from different angles. (Courtesy of Baylor College of Medicine.)

Top

Top, rotated

Top, rotated

Side

10 nanometers
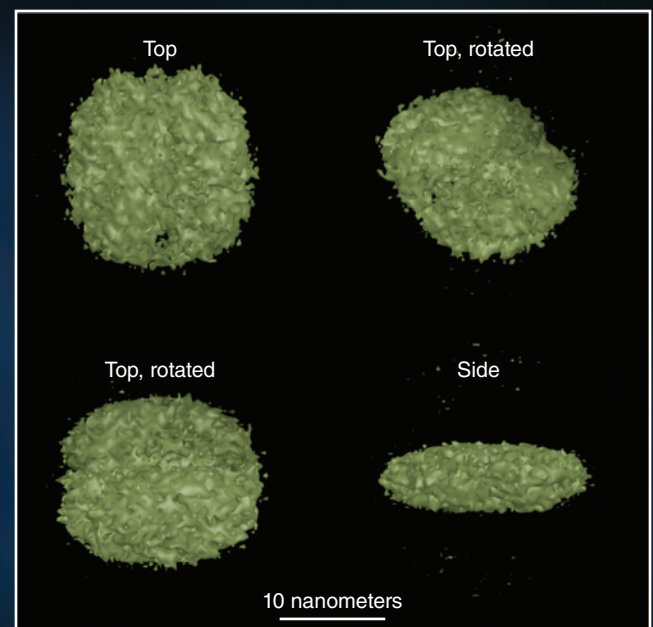
nanolipoprotein particle (NLP) as a platform that can be conjugated, or attached, to protein antigens to quickly produce potent, custom-targeted vaccines.

Many Uses for an Intriguing Particle

NLPs are an amalgam of naturally occurring apolipoproteins and lipids. Discoidal in shape and nanometer in size, they are similar to "good" and "bad" cholesterol—those high- and low-density lipoprotein particles that move fats and lipids through our bloodstream. NLPs self-assemble in a relatively simple manner and, much like a set of interlocking building blocks, can provide a structure or platform for connecting other molecules.

Livermore chemist Paul Hoeprich and his colleagues in the Physical and Life Sciences Directorate began working with NLPs in an effort to better understand the structure and function of membrane-associated proteins in support of the Laboratory's ongoing biodefense research. Membrane proteins are involved in an array of cellular processes required for organisms to survive, including energy production, communication between cells, and drug interactions. "They are the first responders for what passes through every cell in the human body," says Hoeprich. "They connect the outside, or extracellular, world to the inside, or intracellular, world."

Membrane proteins are tricky to study, however, because they are hydrophobic and notoriously insoluble. In addition,

when removed from their natural lipid environment, they often become so distorted that they are no longer biologically active.

Thirty years ago, a pioneering study led by Steven Sligar at the University of Illinois at Urbana-Champaign used NLPs to solubilize membrane proteins. Building on this effort, the Livermore researchers decided to adapt the tiny particles for their membrane protein study. NLPs mimic the membrane protein's natural cellular environment, but they are smaller and more stable in aqueous environments than the cell membranes themselves. With funding from the Laboratory Directed Research and Development (LDRD) Program, Hoeprich led a research effort to develop and integrate novel technologies for producing and characterizing membrane proteins. (See *S&TR*, July/August 2008, pp. 20–22.)

Exploring the "Universal Platform"

Once the researchers demonstrated they could create NLPs that capture and stabilize membrane proteins, they focused on developing the particles as platforms for other types of biomolecules. "We saw that NLPs might offer intriguing applications in biosecurity, energy security, and health care," says Hoeprich.
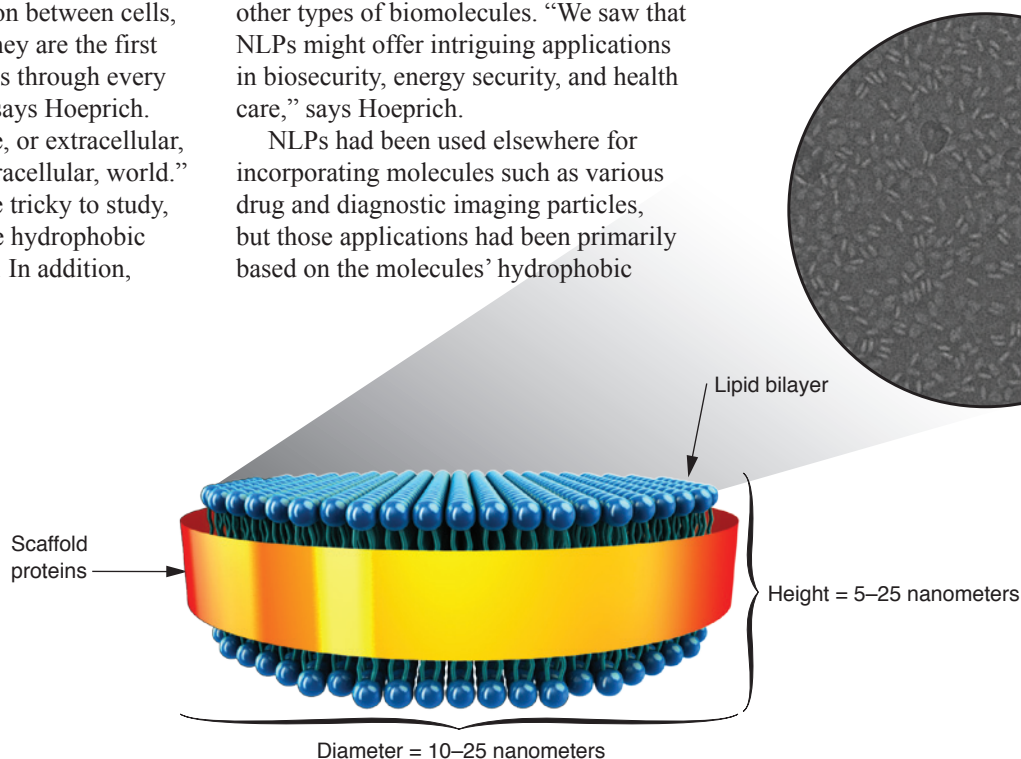
NLPs had been used elsewhere for incorporating molecules such as various drug and diagnostic imaging particles, but those applications had been primarily based on the molecules' hydrophobic
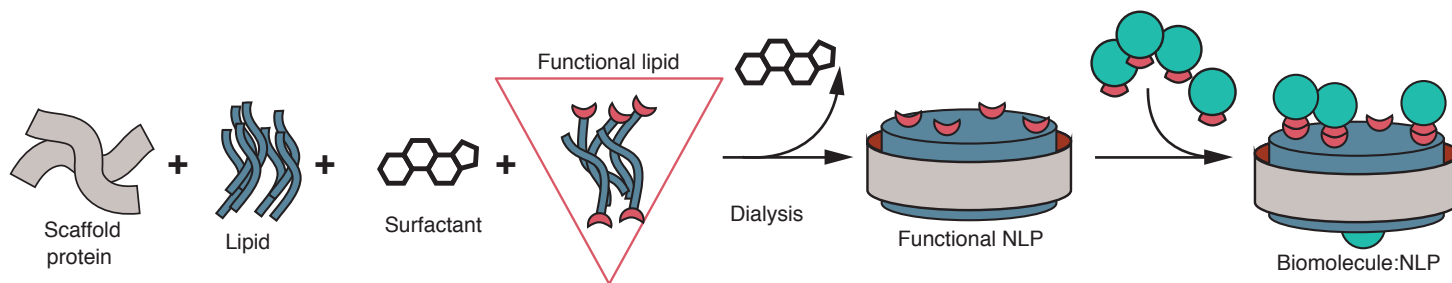
nature. "Restricting the interactions to simply hydrophobic molecules limited the range of potential applications," says Hoeprich. "Because NLPs are multifunctional, we realized that, by changing the lipid involved in the process, we could target particles for specific tasks, such as being a platform for vaccines."

An alternative to the more classic vaccine production process, which relies on a neutralized or attenuated infectious agent, involves recombinant DNA technology (genetic engineering). This approach produces specific proteins, also called antigens, that will elicit an immune response. "We refer to these newer vaccines as 'subunit' vaccines because they are made with part of a microbe rather than the whole microorganism," says Hoeprich.

Subunit vaccines can be generated in a decades-old genetic engineering method that results in a protein with a polyhistidine tag (His-tag). Traditionally, this tag facilitates purification of recombinant proteins from a cellular reaction mixture. In the Livermore effort, the His-tag enables

NLPs are made of hydrophobic lipids surrounded by a band of scaffold proteins. The cryogenic electron microscopy image (inset) shows the top side of an empty NLP magnified 60,000 times. (Rendering by Kwei-Yu Chu. Micrograph courtesy of H. Change, University of California at Davis.)

Lipid bilayer

Scaffold proteins

Height = 5–25 nanometers

Diameter = 10–25 nanometers

Functional lipid

Scaffold protein

Lipid

+

Surfactant

+

Dialysis

Functional NLP

Biomolecule:NLP

NLPs are produced by mixing scaffold proteins and lipids with a surfactant, such as detergent. Other lipids, such as nickel-chelated lipids, can be added, if desired. The scaffold protein and lipids self-assemble into a disklike structure. When the surfactant is removed via dialysis, the resulting NLP provides a platform on which to attach other molecules at the ends of the functional lipids.

conjugation to the NLP vaccine platform. Thus, coming up with a universal platform to exploit the His-tag on an antigen could help speed vaccine development.

The team explored nickel-chelating NLPs (NiNLPs) as a vehicle to deliver His-tagged protein antigens. "The lipids in these particles have nickel atoms attached to their polar head groups," says Hoeprich. "The nickel binds to His-tagged molecules, turning the particle into the stable platform we needed."

The team's first step was to develop a procedure for producing NiNLPs, attach appropriate His-tagged proteins to them, and examine how well these proteins bound to the particles. Livermore researchers Nicholas Fischer and Craig Blanchette, working with Hoeprich and colleagues from the University of Texas Medical Branch at Galveston, developed a candidate West Nile virus vaccine that used the NiNLP platform. They conjugated a His-tagged protein from the virus to a NiNLP and injected the vaccine into mice. The results were impressive: More than 90 percent of the vaccinated mice survived a viral challenge, compared with 20 percent of the unvaccinated mice. "This project demonstrated that we could indeed use NLPs as a platform to capture and hold antigenic proteins," says Hoeprich.

Creating All-in-One Delivery

In exploring ways to improve the platform technology, the team decided to incorporate adjuvants—molecules that, when paired with antigens, will generate a more rapid and focused immune response. Blanchette led a three-year LDRD project that explored using NiNLPs to bind other pathogenic antigens accompanied by adjuvants. Hoeprich notes that this practice is common in the vaccine industry, although the Food and Drug Administration has yet to approve the use of adjuvant-containing vaccines in the U.S.

"The traditional whole- or killed-cell pathogen approach to vaccine development worked well in the past, but it had many technical difficulties," says Blanchette. "Antigen vaccines were promising, but they lacked the efficacy of more traditional preparations. In particular, there were challenges in efficiently delivering antigens and the required adjuvants in equal measure to the immunogenic cells." Even though the antigen and the adjuvant were "mixed" in solution, they were not bound together. Thus, vaccine developers could not guarantee that a cell would take up both antigen and booster in equal amounts.

Blanchette proposed using NiNLPs as a single delivery vehicle for both adjuvants and antigens. "We wanted to find ways to attach the two substances to an NLP, all in a single package," he says. Having discrete numbers of antigens and adjuvants bound to each NLP would
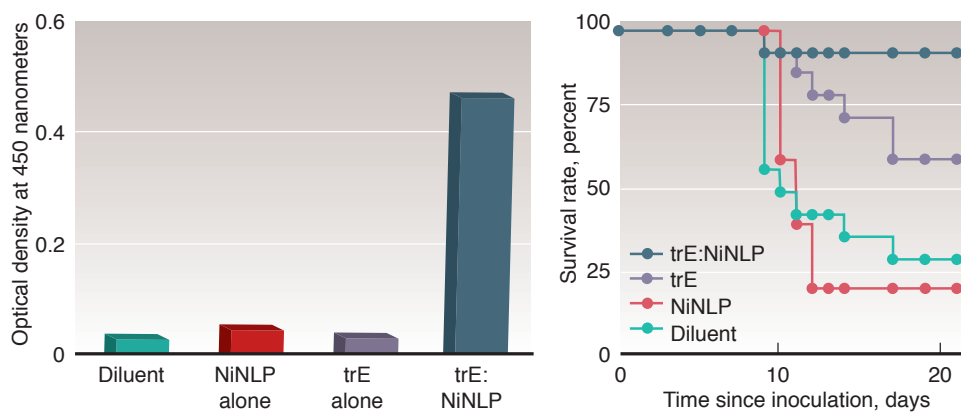
A Livermore collaboration with the University of Texas at Galveston used a nickel-chelating NLP (NiNLP) platform to increase the protective effects of a West Nile virus antigen. In experiments to test the vaccine, mice were inoculated with NiNLP alone, a West Nile antigen only (trE alone), and the West Nile antigen attached to a NiNLP (trE:NiNLP). The diluent acts as the neutral agent or control for the experiments. Graphs show (left) the amount of specific antibodies generated and (right) the survival rate for each inoculating agent. The trE:NiNLP combination had the highest survival rate by far—well over 90 percent more than 20 days after inoculation. Sample size: NiNLP = 5; diluent, trE, and trE:NiNLP = 15 each.

present each cell with defined amounts of both agents, thus making a vaccine more effective.

Blanchette and the team began by identifying lipid-to-scaffold-protein combinations that yielded stable, uniform NiNLPs. Then using analytical size-exclusion chromatography and surface plasmon resonance, they characterized how the His-tagged protein binds with the optimized NiNLP compositions. Next, they conjugated different antigens, including those from bubonic plague and West Nile virus, to the NiNLPs. They also demonstrated that they could control the number of antigen particles attached to an NLP by varying the initial ratios of antigen to NLP during the conjugation step. "We could actually quantify what attached to an NLP, basically engineering a batch of NLPs such that we could reliably control what goes on each particle: antigens and adjuvants," says Blanchette. "It was a huge breakthrough."

The result was a just-in-time production process for fabricating a vaccine candidate from a specific gene and having it ready for use in a matter of hours. "This method could be a viable option for developing vaccines against hundreds of pathogens," says Blanchette.

The next challenge was to address the cold-storage issue. "If a vaccine could be stored at ambient or room temperature and still work, that would be a great advantage, particularly for supplies shipped to distant clinics in hot climates served by poorly developed transport networks," says Blanchette. "It would be a boon for the military and for developing countries."

The researchers discovered that NiNLPs could be dehydrated, or freeze-dried, to form a stable powder. The powder could be stored for months at room temperature and easily rehydrated for field inoculation. Moreover, vaccine administration via dry powder inhalation is a possibility with further formulation and milling.

Interest Ramps Up

In October 2011, Lawrence Livermore and Loyola University in Chicago, Illinois, received a $3.5-million grant from the National Institutes of Health (NIH) to help develop a new anthrax vaccine. It was the first major NIH-funded biodefense grant focused on the promising NLP technology. Livermore scientist Amy Rasley, a coauthor on the proposal, says, "The current anthrax vaccine requires several injections over an 18-month period before an individual

develops a protective immune response. This lengthy inoculation schedule would severely limit the vaccine's ability to protect targeted populations following a deliberate anthrax release."

An even bigger issue is that the current vaccine targets a protein expressed in the late stages of the disease. As such, the immune system is not called into action until the disease has taken hold throughout the body. Rasley is working on a team led by Loyola's Adam Driks to develop an alternative that targets anthrax spore coat proteins. By attacking the bacteria at the spore stage, the researchers hope to stop the disease initiation before spores germinate. They also want to develop a vaccine that can be administered intranasally, similar to FluMist, to induce potent immune responses against anthrax at mucosal surfaces.

The team is now exploring which spore coat proteins to include in vaccine formulations. Five such proteins have already been identified. Once all the proteins are characterized, the team will try different combinations, loading NLPs with one, two, or even three proteins at a time. "We will be looking at what combinations of antigens elicit the most robust immune response," says Rasley.
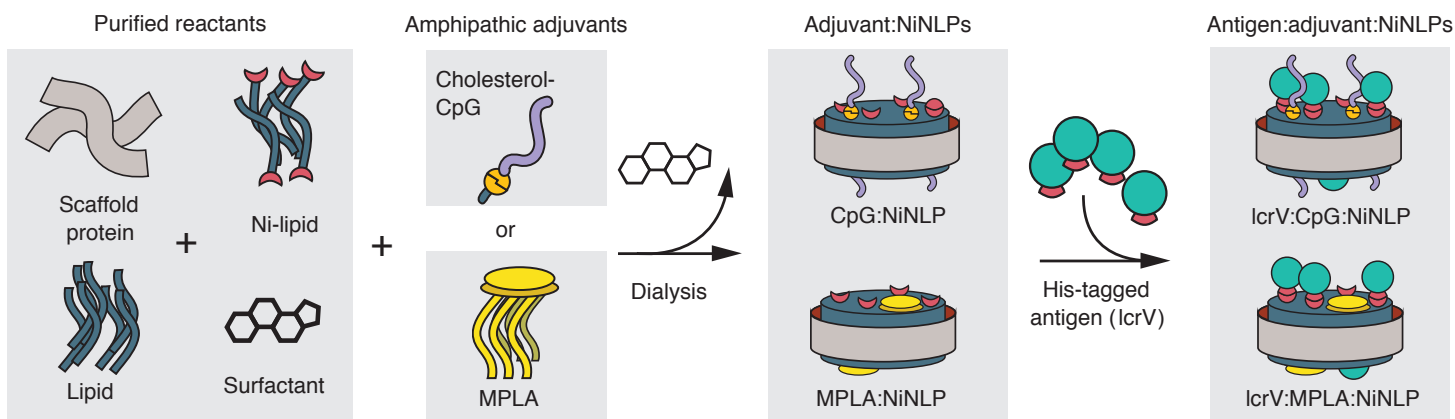
Adjuvants can be included in an NLP by adding components such as cholesterol-modified CpG or monophosphoryl lipid A (MPLA) to the assembly reaction. When the surfactant is removed via dialysis, the adjuvants are anchored to the NiNLP bilayer. Antigens introduced at this stage attach to the nickel atoms at the ends of the lipids, creating an NLP that delivers both substances.

(a) Control   NLP-injected   (b)   (c)
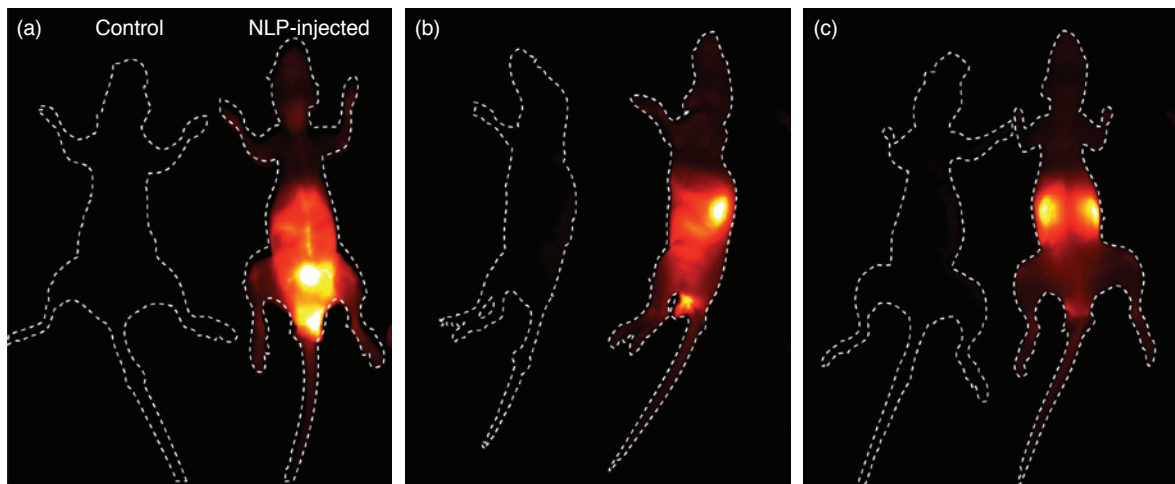
Fluorescent images taken 4 hours after injection reveal the NLP uptake in a mouse viewed from the (a) underside, (b) side, and (c) top. In each panel, a control mouse (left) injected with saline shows no fluorescence. The NLP-fluorophore solution (right mouse) accumulated most prominently in the liver and kidneys.

Focusing on Adjuvants

Vaccines, long considered the gold standard in countermeasures for disease prevention, do not exist for many biothreat pathogens and are not effective in some individuals, such as those with compromised immune systems. To better protect populations from a broad range of traditional and emerging biothreat agents, researchers are exploring innovative approaches that allow for rapid response to a bioterrorist attack or an acute disease outbreak.

Rasley is leading one such effort, also funded by LDRD, that uses NLPs and adjuvants alone, without antigens. This novel treatment targets the innate immune system to provide an immediate, nonspecific protective response to a broad range of pathogens.

A person's innate immune system begins to function immediately when it encounters a pathogen, whether a known, newly emerging, or unknown infection. "It's the body's surveillance system, the first line of defense," Rasley says. "An infection of any kind triggers an immediate cascade of events. This response is very old and very basic—it appears even in fruit flies." The ultimate goal is to develop a therapeutic approach that does not require a pathogen to be identified or its biology to be completely understood. Because the treatment does not target the pathogen directly, it is less

likely to induce antibiotic or antiviral resistance and has the potential for broad-spectrum efficacy.

Adjuvant-laden NLPs could quickly induce a protective immune response. For example, if a microbial threat agent were intentionally released, an inoculated individual, such as a first responder, would most likely not be incapacitated and thus could help facilitate protection of a larger community. "These particles might provide an alternative treatment that could be given alone or in combination with existing therapies," says Rasley. "Plus they might be especially useful for people who cannot tolerate traditional vaccines."

A large part of the study is determining what happens to the NLP–adjuvant structure in vivo, to understand how the particles distribute in the body and how long they remain stable. "We attach a fluorescent tag to the particles, inject them into mice, and track where they go," says Rasley. For the most part, the distribution and half-life of adjuvant-laden NLPs in mice depends on how the particles are administered. "NLPs administered intranasally in mice appear to remain localized in the lung for an extended time compared with those injected into the peritoneal cavity, subcutaneously or intramuscularly, where the particles appear to be cleared from the body much more rapidly," Rasley explains.

When an adjuvant such as CpG is added, the immune response is much more robust and the uptake in the spleen increases dramatically. "We found significant improvement in the immune response to CpG and MPLA [monophosphoryl lipid A] when they are conjugated to NLPs," says Rasley. "We also see enhanced uptake of CpG in the spleen. We're now trying to tease apart why this happens and what mechanism is at work."

DTRA Weighs In on NLPs

A project funded by the Department of Defense's Defense Threat Reduction Agency (DTRA) is focused on optimizing NLP antigen–adjuvant vaccines for two pathogens: *Francisella tularensis* and *Burkholderia*. *F. tularensis*, the bacterium that causes the disease tularemia, is highly virulent and spreads easily by aerosols. *Burkholderia* is probably best known for its species *B. mallei*, a bacterium used in World War I as one of the first biological weapons to infect animals and humans. *B. mallei* and its cousin *B. pseudomallei* are highly infectious and exhibit significant resistance to antibiotics. The Centers for Disease Control and Prevention regard all of these bacteria as viable biological warfare agents.

"No vaccines have been approved to treat these infections, and researchers face

Biosecurity Research Prepares for the Coming Storm

Lawrence Livermore was founded 60 years ago to develop and deliver advanced capabilities for national defense, and many of these technologies have improved the nation's biosecurity. This long history of innovation has also provided the building blocks for future technological advances.

A key area of innovation has been flow cytometry. The Laboratory's remarkable advances began in the 1970s, when researchers developed improved methods to sort cells and used them to process isolated chromosomes. Further breakthroughs in the 1980s, including the idea of attaching fluorescent probes to chromosomes, opened up many new avenues of biological research and ultimately led to the Human Genome Project, an international effort to sequence the 3 billion base pairs that make up human DNA.

In the mid-1990s, Livermore scientists Joe Balch, Anthony Carrano, Allen Northrup, and Ray Mariella developed a miniature flow cytometer that used an immunoassay system to examine proteins and other materials on a cell's surface. They also designed a new approach for polymerase chain reaction (PCR) to dramatically shrink the size of PCR instrumentation and speed up the process for identifying DNA samples.

Both devices were much smaller and more efficient than the laboratory-size instruments then in use for DNA analysis. (See *S&TR*, June 1998, pp. 4–9.) The flow cytometer excelled in international field trials held by the U.S. Army in 1996 and helped launch Livermore's biodefense program. At the Army's field trials two years later, the portable PCR unit proved that DNA could be quickly and accurately identified outside a laboratory setting.

It thus shortened the response time for responding to a natural disease outbreak or an intentional release of a biothreat agent.

"At Livermore, we focus on identifying and understanding emerging and future biological threats," says David Rakestraw, a program manager in the Global Security Principal Directorate. One of the great threats facing the nation involves some of the smallest organisms in the world: viruses and bacteria. Today, when a new disease or pathogen comes to light—such as severe acute respiratory syndrome or methicillin-resistant *Staphylococcus aureus*—it can take researchers 10 to 15 years to develop an effective countermeasure, such as a vaccine or antibiotic. And those countermeasures often come with a multimillion-dollar price tag.

In addition, says Rakestraw, biological sciences are advancing at such a fast pace, the technology needed to manipulate viruses and bacteria has far outstripped the capabilities available for developing an antidote. "Groups from two universities in different countries recently demonstrated the ability to modify a virus to allow its transmission to hosts that previously were not susceptible to infection," he says. "Such capabilities could lead to catastrophic outcomes if misused."

He adds that this technology is becoming more available to a broader range of people. "Because access to these tools is spreading so quickly, we want to speed up our ability to develop a response to a biological threat," says Rakestraw. The Laboratory is applying advanced scientific tools and nanoscience techniques to create biological entities, such as nanolipoprotein particles (NLPs), that may provide an effective path for quickly delivering countermeasures to biothreat agents.

The Laboratory's research to develop medical countermeasures takes a three-pronged approach, starting with a foundational understanding in traditional bioscience and biotechnology. "To that, we add sophisticated measurement tools such as high-resolution microscopy to characterize nanometer-scale particles," says Rakestraw.

The third arm of research is using the Laboratory's high-performance computing resources to model biological processes. Simulations allow researchers to examine molecular structures in detail and predict responses to new or revised treatments. "Livermore researchers can simulate particles with a wide range of sizes and properties and view how they form and respond," says Rakestraw. Measurement techniques are used at this stage as well, to confirm the predicted structures and response.

He notes that biosecurity efforts must take a broad multidisciplinary approach to effectively address the evolving biological threats to the nation. "Our goal is to develop the skills needed to rapidly mitigate existing and emerging biothreats," says Rakestraw. "The nation needs a flexible and agile biodefense strategy. At Livermore, we focus on quickly identifying and characterizing any pathogen, whether known or unknown, and on creating the capabilities needed to rapidly develop and deploy medical countermeasures."

The NLP work conducted by a broad group of the Laboratory's research staff is an excellent example of this effort. That work not only builds on the Livermore tradition of multidisciplinary science research but also leads the way for future teams to tackle important national challenges.

many challenges in developing effective ones," says Fischer, who leads the DTRA project. "In particular, using inherently safe subunit antigens has failed to provide significant protection against pathogen exposure." The team is working to enhance the potency of candidate subunit antigens by optimizing formulations with multiple adjuvants. "We believe we can improve antigen efficacy, especially if all components are contained within a single particle," he says. "One approach we're working on is a cocktail of adjuvants and antigens on the NLP platform."

The researchers face several hurdles to a successful treatment. First, they must optimize NLPs to handle a diverse range of adjuvants—not just MPLA and CpG, but also flagellin and muramyl dipeptide. Next, they must determine how the immune system responds to NLPs loaded with adjuvants and specific subunit antigens from the bacteria of interest. In the final tests, which will take place at the Battelle Memorial Institute, the team will inoculate mice with the loaded NLPs, expose them to aerosols of the targeted bacteria, and assess the protection afforded by the various combinations.

"We're not in the business of developing protein antigens," emphasizes Fischer. "We're developing enabling technologies that will enhance the end product or vaccine. We believe the NLP technology will do just that."

A Road to the Future

The NLP research is just one example of the Laboratory's efforts in the strategic mission area of biosecurity, which remains a pressing national concern. The success achieved to date offers great hope for developing NLP technology as a medical countermeasure to biological threats. (See the box on p. 12.) These novel particles have moved from acting as surrogates for cell membranes to being possible vehicles for vaccines that can be stored at room
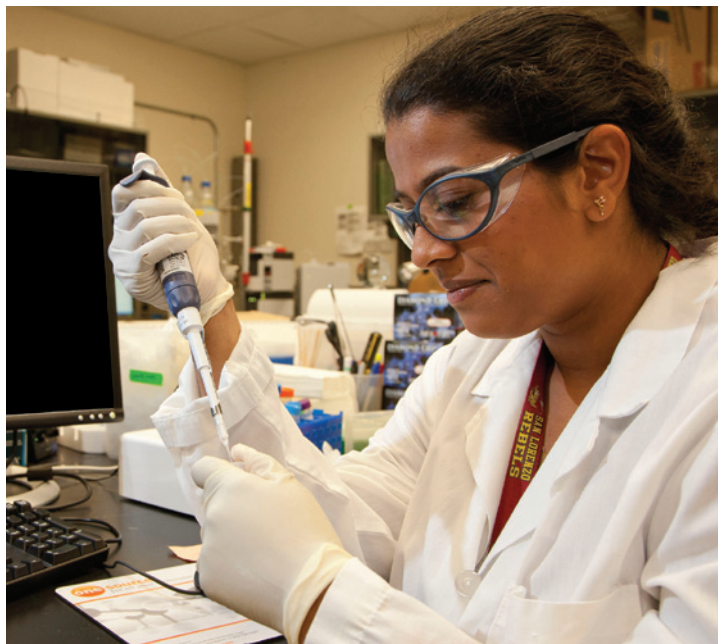
Purna Venkataraman, a former summer student at Livermore, prepares laboratory samples to be used in tests on the NLP platform.

temperature, rehydrated, and injected or perhaps even inhaled directly when needed. Most importantly, NLPs may provide a suitable approach to quickly fabricate and deliver vaccines to protect against some of the most recalcitrant infectious diseases facing humankind.

"Our work is just beginning," says Hoeprich. "NLPs could prove useful in many other applications." In fact, six Livermore programs are examining innovative uses for the particles. For example, when complexed with a hydrophobic drug such as amphotericin B, they provide a treatment for systemic fungal infections in critically ill patients or those with compromised immune systems. Future NLP systems could deposit cancer-fighting drugs directly into the diseased cells, leaving healthy cells untouched. NLP platforms might also carry imaging agents, such as copper-64, so clinicians can more clearly image cancer lesions. Other platforms could be developed to detoxify chemical entities in the blood or promote bone healing and metabolism.

No doubt, as research continues, even more innovations will become apparent. It's a new day, the horizon is open, and the future is bright for these tiny but promising particles.

—*Ann Parker*

Key Words: adjuvant, anthrax, antigen, biodefense, biological threat agent, biosecurity, *Burkholderia*, *Francisella tularensis*, immune system, lipid, membrane protein, nanolipoprotein particle (NLP), nickel-chelated NLP (NiNLP), scaffold protein, vaccine, West Nile virus.

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