Protein engineering: security implications

The increasing ability to manipulate protein toxins for hostile purposes has prompted calls for regulation

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The rapid pace of research in the life sciences is generating a vast amount of new knowledge that has beneficial applications in industry, agriculture and medicine but could, in some cases, be misused for warfare and terrorism. This dilemma has not escaped the attention of security experts and politicians, who worry that infectious microorganisms could be genetically modified so as to increase their pathogenicity, or that lethal viruses could be synthesized in the laboratory from their DNA sequence data. Another often overlooked area of molecular biology with a potential for misuse is protein engineering, or the design and synthesis of tailor-made proteins for industrial or therapeutic applications (see sidebar). Of particular concern is the possibility that protein engineering could enhance the lethality and stability of known protein toxins or create entirely new 'designer' toxins. This article assesses the security risks that are associated with protein engineering and explores some ways of minimizing them.

Protein toxins are able to incapacitate or kill a human at remarkably low doses

Living organisms produce a wide range of protein toxins, which vary in potency and in the availability of treatments or antidotes (Gorka & Sullivan, 2002). Examples of natural poisons are snake, insect and spider venoms, plant toxins (most notably ricin from castor beans), and bacterial toxins that are produced by the causative agents of anthrax, botulism, cholera, diphtheria, staphylococcal food poisoning and tetanus. Unlike toxic chemicals, protein toxins are non-volatile solids that cannot penetrate the skin and must be injected, ingested or inhaled.

They are also neither infectious nor contagious, although the initial signs and symptoms of toxin exposure can mimic certain disease-induced conditions (Madsen, 2001).

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Protein toxins are able to incapacitate or kill a human at remarkably low doses. Botulinum toxin, for example, is about 100,000 times more toxic than the chemical nerve agent sarin (United States Army Medical Research Institute of Infectious Diseases, 2001). The reason for this extraordinary potency is that protein toxins have evolved to interfere specifically with cellular metabolism. Some classes of toxins have an enzymatic component that inhibits protein synthesis or blocks the release of chemical messengers, whereas others are 'superantigens' that disrupt the intercellular signals that regulate the immune system. Superantigens that are released by staphylococcal and streptococcal bacteria are responsible for most cases of toxic and septic shock, which can kill within 24 to 48 hours (Kotzin *et al*, 1993).

Despite the ability of protein toxins to cause harm, they also have beneficial applications because of the high affinity and specificity with which they recognize and bind to their molecular targets. Botulinum toxin, for example, is widely used to treat spastic conditions, such as strabismus and blepharospasm, and has become popular as a beauty aid because it smoothes wrinkles when injected into facial muscles. Some protein toxins also serve as highly precise research tools for dissecting the molecular

mechanisms of living cells. Researchers continue to isolate new toxins from natural sources, both to study their physiological effects and to screen them for possible medicinal applications.

Until recently, major factors limiting the therapeutic use of plant and animal toxins were the difficulty and high cost of bulk extraction or large-scale chemical synthesis. Now, instead of the labour-intensive process of purifying toxins directly from their original source, an increasing number can be produced in large quantities through the cloning and expression of toxin genes in bacteria, yeast or cultured cells (Ducancel *et al*, 1989; Shulga-Morskoy & Rich, 2005). To facilitate their manufacture, protein toxins can also be truncated to the minimum size that is required for functional activity.

One area of protein engineering that is of particular concern to security analysts is the generation of hybrid or fusion toxins for use in advanced therapeutics. Many protein toxins consist of two or more functionally distinct components known as 'domains'. In such multi-unit toxins, one component (known as the binding domain) attaches specifically to a receptor or antigen on the target cell, while a different part of the complex (known as the catalytic domain) migrates across the cell membrane to disrupt key metabolic processes, such as protein synthesis (vanderSpek & Murphy, 2000). Scientists have created therapeutic fusion toxins by splicing the truncated catalytic domains of ricin, diphtheria toxin or *Pseudomonas* exotoxin onto antibodies or growth factors that bind selectively to cell-surface proteins associated with various cancers and autoimmune diseases (Kreitman, 2001). Fusion toxins incorporating

antibodies against tumour antigens can recognize and kill diseased cells while sparing healthy ones, and are being evaluated for the immunotherapy of cancer. One such immunotoxin has been marketed commercially for the treatment of cutaneous T-cell lymphoma (Foss, 2001). Another application of fusion toxins is in vaccines that deliver bacterial antigens to white blood cells (Barth *et al*, 2002; Shaw & Starnbach, 2003).

Fusion toxins often have characteristics that differ from those of the original toxins. For example, when the catalytic domain of tetanus toxin, which normally targets nerve cells, is coupled with components of anthrax toxin, the resulting hybrid can enter and kill non-neuronal cells, and is therefore more potent than either parent (Arora *et al*, 1994). In addition, protein engineering can enhance the receptor-binding affinity and efficacy of fusion toxins in killing target cells; in one experiment, the use of these techniques increased the cytotoxicity of a fusion toxin approximately 17-fold (Kiyokawa *et al*, 1991).

Although most research on protein toxins has been conducted for peaceful purposes and published in the scientific literature, such work could also be misused for the development of biological weapons. Because many toxins have no antidotes and can take several hours to produce symptoms, they are well suited for covert use (Hamilton, 1998). In several known cases, protein toxins have been acquired for warfare, assassination and other hostile ends. During the 1960s, when the United States had an offensive biowarfare programme, it produced and stockpiled two protein toxins: the lethal agent botulinum toxin and the incapacitating agent staphylococcal enterotoxin B (Moon, 2006). In 1978, the Bulgarian secret service—with technical assistance from the Soviet KGB—used a tiny pellet containing ricin, which was fired from an air gun concealed inside an umbrella, to assassinate the Bulgarian exile Georgi Markov in London (United States Army Medical Research Institute of Infectious Diseases, 2001). Before the 1991 Persian Gulf War, Iraq mass-produced botulinum toxin to fill aerial bombs and missile warheads. And in the early 1990s, the Japanese ‘doomsday’ cult Aum Shinrikyo attempted unsuccessfully to mass-produce botulinum toxin (Leitenberg, 2005).

PROTEIN-ENGINEERING TECHNIQUES

Proteins play many roles in cellular physiology, serving as structural building blocks, catalysing biochemical reactions, performing regulatory functions and, in combination with other proteins, self-assembling into complex molecular machines, such as the contractile fibres that are involved in cell motility or the channels that control ion flow across cell membranes. Protein engineering, which first emerged in the early 1980s, involves the use of chemical or genetic techniques to modify the structure of a protein, thereby increasing its stability or altering its physiological function or activity (Ulmer, 1983). The goal is to enhance the usefulness of proteins for commercial or medical applications, such as heat-stable enzymes for use in laundry detergents and industrial processes, as well as new materials, biosensors, vaccines and therapeutic drugs. Another line of research aims to modify the substrate specificity of enzymes or enhance their catalytic efficiency.

Protein engineering relies on the fact that proteins consist of a linear chain of amino acids that folds up spontaneously into a distinctive globular shape. Nooks and crannies on the surface of the molecule, along with the distribution of positive and negative charges, create highly specific binding domains that operate in a lock-and-key fashion, as well as active sites where enzymatic catalysis occurs. There are two common approaches to protein engineering. In ‘rational design’, the researcher starts with the precise three-dimensional structure of a protein and modifies parts of its amino-acid sequence—either directly by chemical means or indirectly by genetic techniques—to affect its stability and functional characteristics. However, even when the precise structure of a protein is known, it is often difficult to predict how specific amino-acid changes will alter its function. Rational design therefore requires many cycles of modification and testing to produce an engineered protein with the desired properties.

An alternative approach, which is known as ‘directed evolution’ or ‘molecular breeding’, involves generating random mutations in the gene encoding a protein, or shuffling the genes encoding different domains, to create hundreds or thousands of mutant proteins with slightly different structures. A high-throughput screening mechanism is then used to select those rare mutants that possess a desired property. Through repeated rounds of mutation and selection, it is possible to accelerate the evolution of ‘non-natural’ characteristics. An important advantage of molecular breeding is that it permits the directed engineering of a protein without knowing its precise structure or the effects of a given mutation. The main drawback of this approach is that it requires a large library of molecular variants and high-throughput selection techniques, which might not work for all proteins. Some researchers have successfully combined elements of rational design and directed evolution.

Another strategy of protein engineering is to expand the set of amino acids beyond the natural repertoire of 20 to include a range of synthetic building blocks. New genetic methods for incorporating such unnatural amino acids into proteins have made it possible to develop peptide-based drugs that can penetrate cell membranes or resist rapid degradation (Benner & Sismour, 2005). The holy grail of protein engineering is the *de novo* design of artificial proteins that do not exist in nature and possess novel properties. Although it is already possible to design simple proteins or close homologues of those with structures that have been determined experimentally, predicting the folding pattern of a large protein from its amino-acid sequence alone remains beyond the state of the art, even with the application of massive computing power.

Still, scientists are making incremental progress towards *de novo* protein design. The folding patterns of protein substructures or motifs, such as α -helices and β -sheets, are well characterized, so these components can be combined to create novel proteins. In addition, certain single-chain protein toxins have a rigid scaffold that can be modified to alter binding specificity and cytotoxicity (Vita *et al*, 1995). Further advances in predictive protein-folding algorithms and automated high-throughput screening, together with the availability of more powerful computers, promise to make protein-engineering techniques more efficient and reliable in the future.

The risk that protein engineering could be misused to develop designer toxins for military purposes has been recognized for some time. More than a decade ago, *The World Armaments and Disarmament Yearbook*, published by the Stockholm International Peace Research Institute, observed that “The ease with which novel engineered bacterial toxins, bacterial–viral toxins and the like can be produced by protein engineering is of military interest...”

(Bartfai *et al*, 1993). One can imagine several possible scenarios involving the offensive use of protein engineering. Improving the binding affinity of toxins or the efficiency of their catalytic domains would increase their lethality and military value. Bioweaponers might also use ‘molecular breeding’ techniques to create new toxins that combine the lethality of botulinum with the stability and persistence of staphylococcal enterotoxin B, allowing them to

withstand environmental stresses and cooking temperatures.

Fusion toxins might also be of interest for warfare or terrorism because their properties and physiological effects differ from those of either parent toxin, making them difficult to detect, diagnose and treat. An anthrax–tetanus fusion toxin, for example, would give rise to unfamiliar signs and symptoms, obscuring the aetiology of the illness and delaying proper medical treatment (Gilsdorf & Zilinskas, 2005). On a more speculative note, protein engineering might be used to convert a normally harmless protein into a toxin by causing it to act on new substrates or bind to different proteins or DNA sequences. Alternatively, it might be possible to design a synthetic toxin that is functionally identical to, but antigenically distinct from, a natural toxin, thereby making it resistant to an existing vaccine (toxoid). Further research might also reveal the characteristics that turn a benign protein into a superantigen that can trigger toxic-shock syndrome.

At present, advanced protein-engineering techniques require a high level of expertise, putting them beyond the capabilities of most developing countries and all known terrorist organizations—with the possible exception of a group that actively recruits former bioweapons experts from a state programme or disgruntled scientists with advanced training and experience. Nevertheless, the technical hurdles are gradually diminishing. Technologies such as bioinformatics, solid-phase protein synthesis and industrial microbiology are

diffusing worldwide, making the development and production of engineered toxins increasingly accessible to countries of concern.

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Because protein toxins, such as botulinum and ricin, have legitimate applications in therapeutics and biomedical research, minimizing their potential for misuse is a challenge (Tucker, 1994). As non-living chemicals produced by living organisms, toxins are covered by both the 1972 Biological and Toxin Weapons Convention (BTWC) and the 1993 Chemical Weapons Convention (CWC). The BTWC prohibits the development and production of toxins for offensive ends “whatever their origin or method of production”, although it permits work with toxins for “prophylactic, protective or other peaceful purposes” (BTWC, 1972). Similarly, the CWC bans the production of toxins “except where intended for purposes not prohibited under this Convention, as long as the types and quantities are consistent with such purposes” (OPCW, 1993). The CWC verification regime also requires the declaration and monitoring of two placeholder toxins: ricin and saxitoxin. But distinguishing between legitimate and illicit activities is difficult, and often comes down to an assessment of intent.

To help prevent the misuse of protein toxins while preserving their beneficial applications, we propose several policy options. The first is to restrict certain types of defensive military research involving toxins. Reportedly, the US biodefence programme includes ‘laboratory threat-characterization’ studies in which scientists engineer putative toxin-warfare agents to guide the development of counter-measures, such as vaccines, antidotes and detectors (Petro & Carus, 2005). Because the potential modifications of protein toxins are almost unlimited, however, such experiments have little practical value and could be perceived by other countries as offensive in intent, thereby sparking a renewed biological arms race. In light of this risk, unless a country has clear evidence that an adversary has engineered a certain toxin for military use, there is no reason to develop a putative agent for the purposes of threat assessment.

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Second, countries that conduct research involving lethal toxins should pass legislation requiring all academic institutions and private companies that work with such materials to be registered and licensed. In addition, scientific personnel who are planning to work with toxins should undergo a

security vetting process before being authorized to do so. This screening should include, at a minimum, periodic interviews by the laboratory supervisor to assess the mindset, psychological stability and reliability of the scientist or technician.

Third, the scientific community should promote professional codes of conduct and other forms of self-regulation. Scientists conducting basic or applied research on protein toxins should educate themselves, and the next generation of researchers, about their legal and ethical obligations under the BTWC and the CWC. All researchers, including students and post-doctoral fellows, should be encouraged to alert the appropriate authorities if they become aware of suspicious activities that would increase the lethality or military value of toxins. Moreover, 'whistleblowers' from any nation or institution should be protected from retribution if their allegations prove to be accurate.

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Fourth, every country that is engaged in the engineering of protein toxins or the development of fusion toxins should establish a national biosecurity board to review and oversee the proposed experiments. This board should have the legal authority to block the funding of specific projects, or to constrain the publication of sensitive scientific results, whenever the dangers to society clearly outweigh the benefits. Because of the global nature of toxin research, regulations governing the registration and licensing of facilities, the security vetting of laboratory personnel and the oversight of dual-use research should be harmonized internationally. This task might be undertaken by a technical working group established under the auspices of the World Health Organization (Geneva, Switzerland), with the active participation of other international scientific organizations and societies.

As outlined above, the prospect that states—and, in the indefinite future, technically sophisticated terrorist groups—could misuse protein-engineering techniques to create new biological weapons is not as far-fetched as it might

initially appear. Just as certain areas of research on human and animal pathogens pose biosafety and biosecurity risks warranting regulation, the engineering of protein toxins raises similar concerns. To encourage the beneficial applications of this powerful technology while limiting its potential for misuse, appropriate review and oversight mechanisms should be established.

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