Engineering protein stability

By Lev Osherovich, Senior Writer

Two American teams have proposed distinct strategies to improve the stability of recombinant proteins. The techniques could be used to increase the manufacturing yield, shelf life and potentially the duration of efficacy of biologics, including antibodies and therapeutic cytokines.

Efforts to improve a protein's stability typically require the production of hundreds of variants and characterization of their biophysical properties *in vitro* in order to choose the best variant. The two new techniques are expected to be applicable to a range of proteins and could stabilize antibodies and therapeutic cytokines more quickly and efficiently than current methods (see Figure 1, "Two approaches for improving protein stability").

A team at **The Scripps Research Institute** uncovered an amino acid sequence that, when placed near glycosylation sites, helps stiffen the protein's backbone.¹ This stabilizing amino acid sequence proved transplantable into non-glycosylated proteins, allowing them to become glycosylated and stabilized.

Pfizer Inc. already has obtained a license to the approach.

Meanwhile, a group at the **Massachusetts Institute of Technology** devised an enzymatic scheme to join the two ends of small cytokines like interferon- α_2 (IFNA2; IFN- α_2), G-CSF (CSF3) and erythropoietin (EPO) and tag them with the stabilizing molecule polyethylene glycol (PEG). The resulting pegylated circular proteins retain all of their biological activity but are more stable than their parent molecules.²

Spicing with sugar

Jeffery Kelly, professor of chemistry and chairman of the Department of Molecular and Experimental Medicine at Scripps, said that increasing the stability of therapeutic proteins could lead to easier manufacturing and dosing.

"One key challenge for making biologic drugs is to have a long half-life, which leads to good patient compliance," said Kelly. "The other key challenge is manufacturing stable proteins. There's very strong evidence in the literature that the stability of protein-based drugs correlates with their half-life" on the shelf and *in vivo*.

Taking a rational approach, Kelly and Evan Powers, an associate professor of chemistry at Scripps, set out to crack the protein sequence code that would explain the stability caused by glycosylation, a post-

translational modification naturally found in many useful proteins, including mAbs.

Glycosylation involves the addition of a sugar-rich glycan chain to specific sites containing an asparagine residue next to glycine and threonine residues. Previous studies have shown that blocking glycosylation destabilizes naturally glycosylated proteins, so it is widely believed that glycosylation improves stability.

Indeed, researchers have long attempted to increase the stability of nonglycosylated proteins by engineering glycosylation sites into them. Although the glycan is usually easy to add, the problem is that improvements in stability are rare. Thus, figuring out where to put the glycosylation site to improve stability required painstaking, hit-or-miss protein biochemistry.

"Before, people did this by trial and error, marching around the protein looking for a suitable position to glycosylate," said Kelly. "That method mostly results in error."

The Scripps team hypothesized that there are additional structural features near naturally occurring glycosylation sites that help the glycan stabilize a protein.

To uncover these features, the group worked with a model protein whose structure resembles an antibody. The team started with an unglycosylated fragment of the Cd2 protein from rats, added a glycosylation site and then made a series of mutations and tested their effect on the protein's stability *in vitro*.

They found that introduction of a phenylalanine two amino acids before the glycosylated asparagine increased the protein's stability compared with that of wild-type proteins. When

this stabilized protein was expressed in cell culture, the yield increased compared with that of wild-type protein, likely reflecting the protein's improved stability.

The researchers next engineered the same phenylalanine-enhanced glycosylation site into two unrelated proteins—human muscle acylphosphatase (ACYP2) and protein peptidylprolyl *cis/trans* isomerase NIMA-interacting 1 (PIN1)—and saw comparable increases in stability. That finding suggests that the phenylalanine-enhanced glycosylation sequence could stabilize a range of proteins, potentially including therapeutically relevant ones.

The findings were reported in *Science*.

"It's clear that sugars aid the thermodynamics and kinetics of protein folding," said Jonathan Weissman, professor of cellular and molecular pharmacology at the **University of California, San Francisco**. Weissman's lab is focused on protein folding.

The Scripps team "showed that there's a specific interaction of the sugar with phenylalanine that's highly stabilizing," said Weissman. "This can be thought of as a modular stabilization domain."

The findings "add to our toolbox to engineer proteins for industrial applications," said David Brems, executive director of process development at **Amgen Inc.**

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**—Jeffery Kelly,
The Scripps Research Institute**

Figure 1. Two approaches for improving protein stability.

Culyba *et al.* and Popp *et al.* have devised two strategies to improve the stability and manufacturing yield of recombinant protein therapeutics.

Culyba *et al.* dissected the amino acid sequence of an immunoglobulin-like model protein and found sequence motifs that increased the stabilizing effect of asparagine-linked glycosylation, a common type of post-translational modification in eukaryotic cells.

Wild-type protein, which included a naturally occurring asparagine (N), glycine (G) and aspartic acid (D) residue, **[a(1)]** was unstable *in vitro* and poorly expressed in cell culture. A glycosylation sequence (N-G-T (threonine)) **[a(2)]** was created by mutating the D to T, which allowed the addition of an *N*-acetyl-D-glucosamine (GlcNAc) moiety but did not substantially improve stability or expression. By substituting a nearby leucine (L) with phenylalanine (F), which is two amino acids away from the glycosylated N, a three-way interaction between the GlcNAc and the F and T side chains occurred that increased the protein's *in vitro* stability and cell culture expression yield **[a(3)]**.

Popp *et al.* used enzymatic circularization to improve the stability of recombinant interferon- α_2 (IFN α_2 ; IFN- α_2), which is marketed by at least eight companies to treat viral infection, cancer and autoimmune disease.

The C-terminus of IFN- α_2 was engineered to contain cleavage sites recognized by bacterial sortase (SrtA) enzymes **[b(1)]**. *In vitro*, sequential treatment of the purified protein with SrtA enzymes from *Streptococcus pyogenes* and *Staphylococcus aureus* followed by chemical ligation of polyethylene glycol (PEG) **[b(2)]** led to a circular form of IFN- α_2 **[b(3)]** that was more stable than wild-type protein and had comparable biological activity.

The team obtained similar stabilizing results with G-CSF (CSF3) and erythropoietin (EPO).

Historically, he noted, "new glycosylation sites have been engineered into proteins to improve properties such as blood pharmacokinetics, solubility, stability, immunogenicity and expression levels."

According to Brems, engineering glycosylation sites into proteins is already a part of industry practice. He cited the addition of a glycosylation site to EPO to yield Amgen's anemia drug Aranesp darbepoetin alfa, which has a long half-life and thus requires less frequent administration than the company's Epogen EPO.

Designing Aranesp took painstaking work, and the Scripps team's technique might accelerate this aspect of drug development, Brems said.

"The next step is to see if this technique improves the yield of proteins" under industrial-scale manufacturing conditions, said Weissman.

The other question is which biologics could be improved by adding a stability-improving glycosylation site.

The Scripps team found that the stability boost occurs when the phenylalanine and the glycosylation site interact with a protein's peptide backbone in a distinctive structure called a β -bulge turn.

Brems recommended looking at structural models of therapeutically relevant proteins for suitable β -bulges.

"The most obvious application would be for those proteins that already possess a [β -bulge] turn that would readily accommodate the necessary amino acid sequences to achieve the stabilizing interaction," he said. "A survey of the frequency of this type of turn needs to be done with clinically important proteins."

Weissman said antibodies would be the most obvious candidates for stabilization using the Scripps team's technique.

Scripps has filed for a patent on the protein engineering technique, and Kelly said the method has been nonexclusively licensed to Pfizer.

Kelly noted that the glycosylation method is unrelated to the technology of FoldRx Pharmaceuticals Inc. The protein engineering company, which he cofounded, was acquired by Pfizer last year.

Squaring the circle

Whereas the Scripps group opted for glycosylation engineering, a team led by Hidde Ploegh, professor of biology at MIT and a member of the **Whitehead Institute for Biomedical Research**, have proof-of-concept data that certain proteins can be stabilized by linking their two ends together.

The method uses a pair of bacterial enzymes to join the C- and N-terminus of small therapeutic cytokines and to add a PEG group near the junction.

The addition of long, hydrophilic PEG polymers is a widely used method of increasing the solubility, immunological tolerability and *in vivo* half-life of biologics. But conventional pegylation chemistry is hard to control, and the biochemical purification needed after pegylation adds expense and complexity.

Ploegh's technique hits two birds with one stone, generating a stabilized, circular protein with a distinct sequence site that can readily be conjugated to PEG.

The MIT team started with IFN- α_2 , a biologic marketed by numerous companies to treat viral infections and cancer. The C- and N-terminus of IFN- α_2 are physically close to each other when the protein is properly folded, making the protein a good candidate for circularization.

The team inserted a cassette containing binding sites for two versions of sortase A, a bacterial enzyme that moves pieces of proteins from one place to another.

In the first step of the *in vitro* reaction, the team used *Staphylococcus aureus* sortase to replace the engineered IFN- α_2 's C-terminus with a synthetic peptide containing both a PEG acceptor site and a binding site for sortase A from a different bacterium, *Streptococcus pyogenes*.

In the second step, the team used *Streptococcus* sortase A to link the modified C-terminus to the N-terminus, forming a circle.

Finally, PEG was added using conventional aniline-catalyzed oxime ligation.

The resulting circular, pegylated IFN- α_2 had higher stability *in vitro* and greater antiproliferative activity in a cell culture assay than a linear, unpegylated form of the protein. When injected into mice, the circularized IFN- α_2 also had a longer half-life than a linear, unpegylated control protein.

The team replicated the findings with two other biologics: G-CSF and EPO. Amgen markets a pegylated form of G-CSF as Neulasta pegfilgrastim to treat neutropenia in a range of hematological, infectious and cancer indications.

Data were published in the *Proceedings of the National Academy of Sciences*.

UCSF's Weissman said that Ploegh's method could have broad applications in the production of recombinant proteins.

"A lot of proteins have their N- and C-termini close to each other,"

said Weissman. "This is a nice, simple way to circularize proteins."

For now, Weissman said, the circular cytokines engineered by Ploegh's team "are likely the best candidates" for further development, but a next step would be to test the method with larger, more complex proteins.

For Ploegh's technique to take root in industry, the benefits of circularization and single-site pegylation would have to greatly exceed those of standard pegylation techniques, said Charles Squires, VP of discovery and external partnership at **Pfenex Inc.**, a protein manufacturing company.

Pfenex, a spinout of **Dow Chemical Co.**, engineers bacterial strains to optimize the yield of recombinant proteins.

"This is a slick technology, but it depends on two enzymatic steps," said Squires. He noted that at an industrial scale, the added complexity of using two sortase enzymes might offset the improved stability and yield from Ploegh's method.

Squires also expects that circularized versions of IFN- α_2 and G-CSF would need to undergo fresh regulatory review, which he said is a commercially daunting proposition for molecules facing competition from generic biosimilars.

MIT has filed for patents on Ploegh's method, and the IP is available for licensing.

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