

Novel Ways of Looking at Old Problems

Protein Therapeutics Tests the Mettle of Characterization Techniques and Tools

K. John Morrow Jr., Ph.D.

The exponential increases in yields of recombinant proteins by various expression systems continue to place demands on the downstream end of the process. Proteins generated at high densities can take on undesirable properties as the molecules crowd together.

These changes must be carefully described and monitored if the investigator has any hope of tracking down and eliminating the problem. Moreover, alternative platforms are beginning to compete with mammalian cells, long the mainstay of the industry. As their properties are different from one another, their expressed proteins require serious analysis and description. A number of companies are currently considering these challenges.

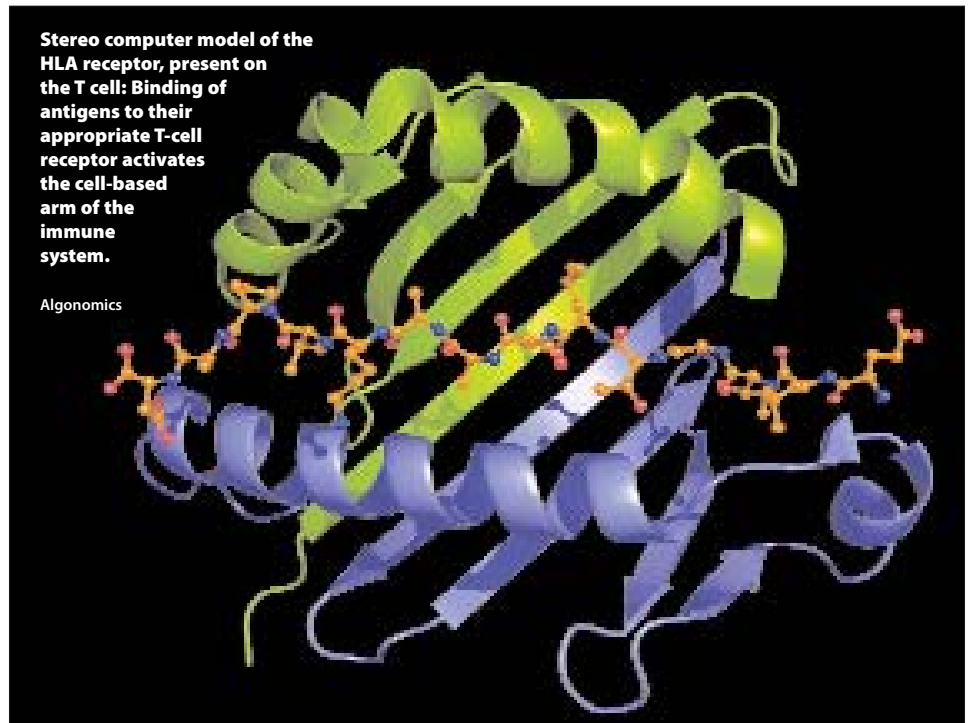
Jesús Zurdo, Ph.D., head of advanced protein technologies at Lonza (www.lonza.com), is knowledgeable about protein engineering approaches to obviate aggregation of recombinant products. According to Dr.

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Zurdo, “proteins by and large are unstable molecules, subject to rapid degradation, of which aggregation is one of the most intractable. By following a Quality by Design strategy we can make modifications in the molecule as we develop the process, increasing our chances of success.”

Aggregation is a significant problem in protein expression and purification, causing reduced half-life, altered activity, and loss of bioavailability and affinity. In addition, aggregated proteins possess increased immunogenicity and toxicity toward the host.

In an intact organism, aggregation is usually not an issue since a number of natural barriers exist, including chaperones and other proteins that act to stabilize the products. But biologics are produced outside the



natural environment and likely have been substantially modified through genetic engineering, a practice that usually focuses on performance rather than durability. After the process is complete, a further layer of difficulty is added, since the proteins may be stored under highly non-physiological conditions. Because it is difficult to define, aggregation may require sophisticated methods of characterization in order to monitor and resolve the problem.

“In developing the parameters that reflect aggregation propensity, it is necessary to have the physical descriptors of the proteins,” Dr. Zurdo says. “These include the physico-chemical properties of the amino acid sequences comprising the protein, the sequence patterns that could prevent self-assembly, as well as the solvent accessibility.”

This analysis is aided by Aggresolve™, a software program that yields in silico predictions of the behavior of the protein. These aggregation predictors allow the investigator to discard sequences that appear risky and to design modifications in the protein that can be confirmed by in vitro validation. Dr. Zurdo and his colleagues analyzed a number of Fab antibody fragments with different sequences in silico, and then confirmed their behavior in the real world.

As predicted, those sequences that were at high risk for aggregation in the in silico model had a much higher propensity for aggregation, as shown by their migration patterns on acrylamide gels. When whole antibodies were subjected to in silico and in vitro analysis, it was possible to achieve substantial improvements in their aggregation potential by eliminating aggregation hot spots.

“We are moving toward an in silico formulation design,” says Dr. Zurdo. “The sequence will dictate the properties and behavior of the protein and enable us to specify purification requirements.”

These productivity predictors can

allow the modification of the target protein in a fashion that optimizes performance and will allow better and safer pharmaceutical products.

Antibody Immunogenicity

Philippe Stas is CEO at Algonomics (www.algonomics.com), a firm that looks at both sides of the immunogenicity question. “With an ever-increasing number of protein therapeutics reaching the patient, the specific challenges of this class of drugs are under scrutiny,” he states. “A key issue, immunogenicity, is driven by aggregates as well as by the protein sequence.”

On the one hand, the company is exploiting methods to increase the immunogenicity of the HIV GAG protein, in order to develop a more effective vaccine for this disease. According to Stas, Epibase® software allows predictions of immunoreactivity based on the binding of the GAG peptides to the T-cell receptor.

By replacing weakly immunostimulatory amino acid sequences with more reactive ones, it should be possible to build a more effective vaccine. The HLA receptor present on the T-cell has been extensively studied, and accurate 3-D modeling of antigen binding is possible. So far it has not been possible to design an HIV vaccine that provokes both arms of the immune system, the humoral and cell based, so Algonomics’ approach may represent an important step in resolving this puzzle.

The other side of the question is the suppression of the immune response, essential to the engineering of effective protein-based therapeutics. Algonomics has addressed this problem, taking into account regulatory guidelines formulated by the EMEA. At present, no systems are available to characterize human antidrug antibodies at a pre-clinical level. Animal studies are not always predictive of response in humans, so other

News Bioprocessing Highlights

S AFC Biosciences Expands Media Capability for CHO Cell Lines

S AFC Biosciences (www.safcbiosciences.com) has expanded its portfolio of products and services for use in Chinese hamster ovary (CHO) cell line applications with the introduction of EX-CELL™ CD CHO Fusion, the company’s next-generation chemically defined media formulation, and the CHO cGMP Media Library, an on-the-shelf, media screening library of diverse formulations.

EX-CELL CD CHO Fusion media has been specifically developed to work as a scalable platform media formulation for those cell culture applications requiring a chemically defined animal-component-free media option that can meet production requirements across multiple CHO cell lines, the company reports.

Xendo CTM Opens Manufacturing Facility

Xendo Clinical Trials Material (Xendo CTM; www.xendoctm.com) officially opened its GMP-certified biopharmaceuticals manufacturing facility. The new company is a joint venture between the University Medical Center Groningen (UMCG; www.umcg.nl) and Xendo Manufacturing (www.xendo.nl), and has been established to manufacture biologics for preclinical and clinical trials.

Xendo CTM is positioning itself as a

one-stop-shop for early-phase drug development, offering project management, process development, analytical and formulation development, validation, QA/QC, registration, and the execution of clinical trials. The company will focus on the use of disposable cell culture and fermentation systems, and will offer BioSMB, a scalable, disposable chromatography platform in development by Tarpon Biosystems (www.tarponbiosystems.com) and Xendo. BioSMB will be available to Xendo CTM’s clients later this year.

ReNeuron Automates ReN001 Manufacturing Process

ReNeuron (www.reneuron.com) reported successful automation of the manufacturing process for its ReN001 neural stem cell line, which is in early clinical trials for the treatment of stroke. Through a research project carried out in collaboration with Loughborough University, ReNeuron adapted its standard cell manufacturing process and transferred it, with minor modifications, to The Automation Partnership’s (www.automationpartnership.com) Compact SelecT robotic cell culture system.

ReNeuron said automation of the ReN001 process will allow optimization of the cell culture process, result in lower costs and increased yield, and provide for more reliable scalability for later-stage

methodologies are greatly in demand.

“Protein aggregates are highly problematic, typically leading to a transient immune response with low affinity anti-drug-antibodies,” Stas notes. “But they can contribute to immunogenicity in conjunction with T-cell epitopes, boosting the system toward a T-cell driven memory response.”

While in the final analysis clinical studies are necessary to determine whether the immunogenicity of a protein therapeutic makes it an unsuitable drug, Algonomics hopes to eliminate the worst of the lot before they reach this stage. In vitro studies may require inordinate amounts of protein, so the in silico approach may be a satisfactory alternative. The strategy is to select a protein with the fewest HLA-binding peptides, on the assumption that these would be the least troublesome.

In later stages of investigation, in vitro T-cell activation studies will provide additional information that may result in the elimination of unsuitable drug candidates. This will allow the most parsimonious selection of final candidates for animal and later human trials.

Diversity and Complexity of Proteins

Aggregation can be a problem even for established protein products, according to Tudor Arvinte, Ph.D., chairman and CEO of Therapeomic. “Proteins aggregate, degrade, bind to cell walls, and generate fibrils,” he states. Because of their dynamic properties, the conventional analytical methods are not well suited to understand how a protein behaves when internalized.

Aggregation of protein therapeutic molecules can trigger a toxicity effect, compromising the effectiveness of the drug. Dr. Arvinte and his colleagues have monitored performance using dyes that specifically stain aggregates or other types of protein-degradation products. One method uses Nile Red, making aggregates visible for qualitative and quantitative analysis, allowing a fast screen of different solutions for their aggregation potential.

Protein aggregate analysis is demanding, requiring the use of various technologies including asymmetrical field flow fractionation, fluorescence spectroscopy, fluorescence microscopy, and transmission electron microscopy. Some procedures are especially informative under particular conditions, so the investigator may need to pick and choose in order to develop an accurate picture of the changes that the molecules undergo.

Dr. Arvinte discussed the formulation problems associated with Herceptin, which is supplied as a lyophilized powder. Dr. Arvinte says that the formulation described in the patient information leaflet is unduly complex and employs a novel chemical entity, apparently required for the stabilization of the Herceptin antibody. His team’s experience indicated that extremely subtle handling issues, down to the speed of injection, can create aggregates.

Following a warning on the label to

refrain from the use of a dextrose diluent, Dr. Arvinte and his coworkers determined that dextrose-containing diluents caused the formation of aggregates and made the preparation clinically unacceptable.

Therapeomics has developed a set of guidelines that eliminate components that trigger aggregation and other sub-optimal formulation options. Because of the diversity of antibody molecules, a wide variety

of individualized formulation options must be designed that take into account antibody flexibility and stability, and no two diluents solutions will be exactly the same.

Dr. Arvinte argues that companies that ignore a thorough investigation of formulation needs during product development do so at their peril. “Whereas the biology of most protein-based therapeutics may be well understood, the biophysical changes that

they may undergo can constitute a profound unknown of critical significance to the success of the final product,” he concluded.

High-Affinity Lamprey Antibodies

A number of alternatives to immunoglobulin-based antibodies have been proposed such as lipocalins, fibronectins, ankyrins, and src-homology domains, but Zeev

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Protein Characterization

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Pancer, Ph.D., assistant professor at the Center for Marine Biotechnology, University of Maryland Biotechnology Institute, proposes another possibility—the lamprey antibody. The jawless lampreys and hagfish are at the base of the vertebrate branch of the evolutionary ladder and have been around for a long time.

The lamprey's immune system is based on somatic rearrangements of diverse leucine-rich repeats within incomplete vlr

genes. The N- and C- terminal ends of the vlr gene are constant, and these repeating units form the lymphocyte receptors, which are the primary recognition sites of the lamprey's immune system. These somatic rearrangements have the potential to generate over 10^{14} unique variable lymphocyte receptors (VLRs), a figure that is comparable to the level of diversity inherent within the mammalian immune system.

"The evolutionary history of these

organisms is fascinating," says Dr. Pancer. "The lampreys are part of the deuterostome lineage that includes modern vertebrates, but their immune systems have diverged dramatically, so their molecular structures are quite different."

In a manner similar to the mammalian immune system, lamprey secrete VLRs in their plasma. The animals respond to repeated injections of antigen by mobilizing a hearty immune response of low-

affinity antibodies followed by high-affinity antibody idiotypes. Several investigators have already developed monoclonal antibodies using the lamprey system, and they were found to bind antigen with high avidity.

Dr. Pancer argues that the lamprey system offers a unique opportunity to generate antibodies that could recognize mammalian antigens invisible to immunoglobulin-based antibodies due to self-tolerance. Moreover, the VLRs are modular, single-chain polypeptides, which makes them highly amenable to molecular engineering. Dr. Pancer cautions that lamprey antibodies may not be good therapeutic candidates since their sequences could be immunoreactive to mammals, but they could serve a variety of important functions in the diagnostic sphere, including protein chips, flow cytometry, immunohistochemistry, and ELISAs.

Furthermore, there is a spirited search under way today for better affinity purification tools, and the lamprey antibodies could fill the bill. So while the lamprey system is very old, it is hardly out of date.

Accelerating Antibody Production

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"The PA 800 plus Pharmaceutical Analysis System automates SDS-gel, isoelectric focusing, and glycan analysis of monoclonal antibodies and other therapeutic proteins," according to Mark Lies and Hans Dewald, capillary electrophoresis product managers at **Beckman Coulter** (www.beckmancoulter.com). The platform integrates quantitative, qualitative, and automated solutions for analysis of protein purity, charge isoform distribution, and glycan structure.

"We developed the PA 800 plus based on feedback from the industry," notes Lies. Designed with the biopharmaceutical analyst in mind, the instrument routinely handles demanding SDS-gel applications that require high-viscosity buffers, he adds. Software guides users through set up and operation with the aid of large icons to provide navigation guidance and on-screen cues monitoring both system progress and system requirements.

Since data variability is an area of particular concern to the biopharmaceutical industry, the system was designed to provide consistent results from run to run, lab to lab, and location to location, the managers report.

"Beckman Coulter has marketed capillary electrophoresis for twenty years," adds Dewald, "and we have introduced new systems, methods, or chemistries every year. The PA 800 plus builds on this history and combines industry feedback with validated applications and new technologies, including advanced cIEF methods and new synthetic pI markers."



Researchers at the University of Maryland Biotechnology Institute are investigating the lamprey antibody as an alternative to immunoglobulin-based antibodies.

tion platform of fusion products,” states Nathalie Forster, product development and support manager for R&D at **Maine Biotechnology Services** (MBS; www.maine-biotechnology.com). With the Multipure platform, one can normalize assays, perform biotin conjugations, and measure epitope compatibility for 96 or more clones simultaneously, she says.

In an effort to generate monoclonal antibodies to equine luteinizing hormone (eLH) suitable for the veterinary reproductive diagnostic market, MBS recently embarked upon a collaborative effort with James Weber, Ph.D., DVM, of the University of Maine. LH triggers ovulation and is critical for the reproductive process, which tends to be tenuous in horses and unresponsive to conventional reproductive technologies.

Accurate measurement of eLH levels would allow optimal timing of breeding and favor successful fertilization. It would also provide insight about when to terminate the process, as conventional tools reveal a broad window of several days, which adds to the expense. The goal of this project was to develop a prototype matched-pair sandwich assay sensitive and accurate enough to detect physiological eLH levels.

According to Dr. Forster, the success of the antibody-development project is contingent upon the integrity of the immunizing and screening reagents, the ability of the screening strategy to discern fusion products of interest, and the capability to characterize the resulting antibodies for use in the end application.

The ability to analyze the biological and physical characteristics of the fusion products at an early stage in the hybridoma process allows critical decisions to be made concerning choices for subcloning, production, and purification. This approach is ideal for the development of antibodies destined for diagnostics and matched pair applications. The protocol takes advantage of a 21-day rapid immunization strategy with a fusion done on day 28. The fusion screening typically occurs approximately 14 days post-fusion.

The MultiPure purification platform allows many clones of interest to be simultaneously grown and purified, with normalization of production rates so affinities can be compared.

By narrowing down a large number of clones and making pairwise assessments it was possible to isolate the highest performing combinations of monoclonal antibodies to be used in a diagnostic assay, Dr. Forster adds.

The resultant antibody pair and prototype assay were used to evaluate daily serum samples from mares taken through the course of one estrous cycle. In parallel, measurements of the dominant follicle and progesterone levels were assayed using a

commercially available test kit. Serum samples were run on a quantitative MBS prototype eLH assay. The results on the serum samples provided insight as to what the optimum breeding time would have been, validating the assay and concept.

“With the successful outcome of this study we are making our MultiPure technology available as a service to our Hybridoma customers,” Dr. Forster says.

Biotech companies keep returning to the problems of narrowing down candidate diagnostics and therapeutics, a process that can entail substantial costs and extended time frames. The approaches discussed here entail new ways of looking at old problems and applying newly developed hardware and software to resolving them. Venture capital funds have virtually disappeared for all but the furthest late-stage products, so biotechs could profit from the opportunity to speed preclinical development to completion. **GEN**

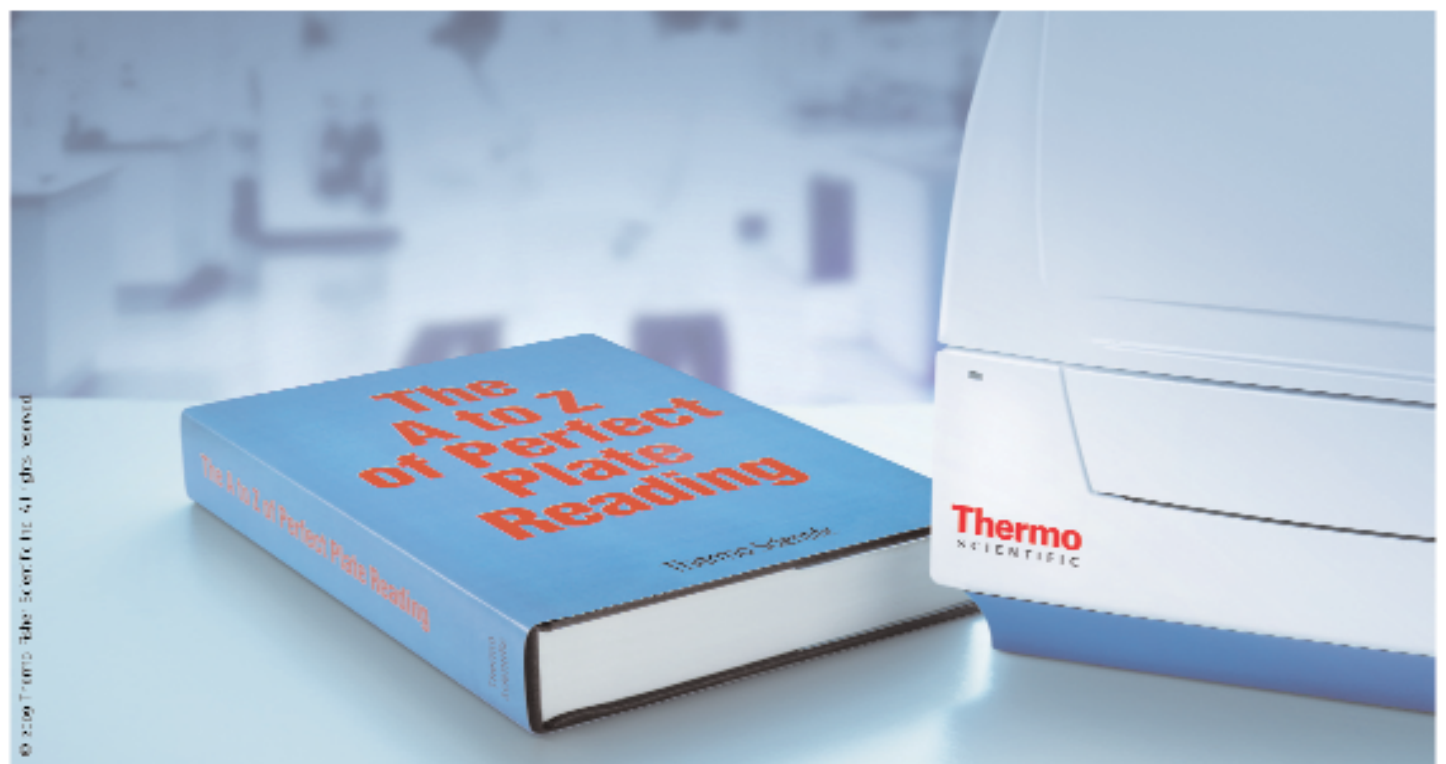


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