The Second International Conference on Accelerating Biopharmaceutical Development was held in Coronado, California. The meeting was organized by the Society for Biological Engineering (SBE) and the American Institute of Chemical Engineers (AIChE); SBE is a technological community of the AIChE. Bob Adamson (Wyeth) and Chuck Goochee (Centocor) were co-chairs of the event, which had the theme “Delivering cost-effective, robust processes and methods quickly and efficiently.” The first day focused on emerging disruptive technologies and cutting-edge analytical techniques. Day two featured presentations on accelerated cell culture process development, critical quality attributes, specifications and comparability, and high throughput protein formulation development. The final day was dedicated to discussion of technology options and new analysis methods provided by emerging disruptive technologies; functional interaction, integration and synergy in platform development; and rapid and economic purification process development.

March 10, 2009
Day 1, Emerging Disruptive Technologies and Cutting-Edge Analytical Techniques
Janice M. Reichert
Tufts Center for the Study of Drug Development; Boston, MA USA

The meeting was opened by Bob Adamson (Wyeth) who remarked that it is the responsibility of biological engineers to develop technologies that will produce drug products rapidly and cost effectively. On average, protein therapeutics cost more than small molecule drugs. However, technological advances can help to drive down the cost of these products. For example, penicillin was scarce in the 1930s and 1940s because of production issues, but this drug is easily and cheaply obtained today.

Recent developments in biosimilars, a potentially disruptive group of products, were discussed by Rob Garnick (Lone Mountain Biotechnology). He first noted the various names by which biosimilars are known. While the term biosimilars is favored in Europe, the US Food and Drug Administration (FDA) uses the term ‘follow-on biologics,’ and Health Canada prefers the phrase ‘subsequent entry biologics.’ The term biogenerics is not usually used because this word implies that the products are identical to approved innovator biologics. The European Medicines Agency and Health Canada have issued regulatory guidances for approval of biosimilars, and some of these products have been approved in Europe. However, the process has gotten stalled in the US for various reasons, including questions surrounding the reliability of sourcing, magnitude of price reduction, need for clinical trials done against a national comparator and comparability issues. On the other hand, the global economic problems have focused political attention on healthcare reform and unsustainable increases in the cost of healthcare.

Dr. Garnick noted that in the US the general consensus is that the Drug Price Competition and Patent Term Restoration Act of 1984, also known as the Hatch-Waxman Act, has been successful in promoting generics while still providing financial incentives for research and development by innovators. In addition, Congress believes that scientific issues surrounding biosimilars are addressable and so a regulatory pathway can be established for approval of biosimilars. Due to the progress in defining a regulatory pathway, some major pharmaceutical firms, including Pfizer, AstraZeneca, Novartis and Merck, have recently indicated that they will develop
these products. Biosimilars development is attractive because the success rates should be 100% if the products are developed correctly, the manufacturing processes are well-understood and can be out-sourced, and the markets are potentially large. With a global market over US $5 billion, rituximab will certainly be targeted as a biosimilar. As with the Hatch-Waxman Act, the key to success of any US biosimilars legislation will be the maintenance of incentives to innovate.

There are still numerous scientific and legal problems to address, including the exact nature of legislative, patent issues, design of clinical trials, substitutability, interchangeability, safety and post-approval surveillance. The challenge lies in the details, e.g., establishing product specifications and test methods, and defining comparability. Dr. Garnick noted that cautionary tales on comparability come from the experience of a number of innovator companies. For example, efalizumab produced by XOMA was found to have differences when compared to efalizumab produced by Genentech. The differences, which included minor changes in acidic forms, galactosylation, charge heterogeneity and an increase in C-terminal processing, were expected to be inconsequential, but translated into different clinical study results. This experience suggests that a combination of written procedures, training, analytical testing and regulatory agency inspections are needed to control the production of biological products. Quality control release tests need to be supported by rigorous product and process characterization and process control.

Outside the US, the reality is that biosimilars are being marketed in Europe, India and China as well as other countries. Marketed biosimilars span a broad range of complexities and include monoclonal antibodies. Reditux, a rituximab biosimilar, was approved in India in April 2007 for non-Hodgkin lymphoma and rheumatoid arthritis. However, the clinical trials included relatively few patients and limited analytical data has been made available. In conclusion, Dr. Garnick remarked that the world is gaining experience with biosimilars, and the products will likely become a reality in the US by 2009. FDA will need input to develop effective guidance documents for assessment of biologics. Immunogenicity will be a key concern of regulators. Comparability studies will be required, product differences will need to be investigated and appropriate clinical studies must be done, but biosimilars will come to market.

Global trends in antibody development by innovator companies were presented by Janice Reichert (Tufts University). Clinical development of protein therapeutics is on the rise worldwide. Approximately 120 recombinant proteins and 240 monoclonal antibodies (mAbs) are currently in clinical studies. While recombinant proteins have historically entered the clinic at a rate of fewer than 20 candidates per year, mAbs are now approaching the 40 candidate per year mark. Recombinant proteins have somewhat higher success rates on average (approximately 30% vs. 20% for mAbs) and have been studied in a wider array of therapeutic categories compared to mAbs, but, because of their versatility as therapeutics, mAbs are clearly the focus of the biopharmaceutical industry’s attention. A total of 22 mAbs are approved in the US, and eight of these products have global markets over US$1 billion. Six additional candidates are currently undergoing regulatory review.

Dr. Reichert noted that the ascendency of mAbs is due to technologies that addressed immunogenicity, affinity, specificity, stability and production challenges. While murine versions dominated in the 1980s, the less immunogenic humanized versions comprised 45% of the total mAbs in clinical study in the 1990s. In the 2000s, the human versions have comprised the largest contingent. Historically, mAbs have not been discontinued while in regulatory review. Assuming the six mAbs in review are approved, then cumulative FDA approval success rates for humanized and human mAbs will be nearly identical (19 and 18%, respectively). mAbs are commonly studied as either anticancer or immunological treatments. There are currently nine anticancer and ten immunological mAbs approved in the US. These products have taken approximately the same length of time for clinical development (6.5 years). The length of the FDA review period was found to vary depending on whether the product was given priority or standard review (average 6.9 or 20.4 months, respectively).

Looking forward, Dr. Reichert suggested that antibody fragments and modified versions (pegylated, alternate glycosylation, Fc engineered) are likely to enter the clinical pipeline in increasing numbers. The focus is likely to remain on human IgG, but designed protein scaffolds and domain antibodies will also be included in company pipelines.

The meeting then turned to discussion of potentially disruptive science and technologies. Stefan Wildt (Merck) reviewed the development of glycoengineered yeast, which he described as a versatile glycoprotein expression platform. He first emphasized the importance of glycosylation, which affects circulating half life, tissue distribution, potency and immunogenicity of therapeutic proteins. Any new bioprocesses need to be scalable, portable, and provide analytically comparable protein at all scales. The primary biomanufacturing platforms are bacterial (e.g., E. coli), fungal (e.g., Pichia pastoris), and mammalian cell culture (e.g., CHO cells). Fungal platforms are currently used for production of common industrial enzymes, but have not been used extensively for production of therapeutics because the yeast glycosylation pathway yields products that are potentially immunogenic in humans. GlycoFi Inc., a wholly owned subsidiary of Merck & Co., Inc. has developed Pichia with humanized glycosylation to circumvent this problem.

In yeast, the carbohydrate processing occurs sequentially in the secretory pathway, like an assembly line. The enzymes act one after another and their actions are separated in time and space. As a consequence, humanized yeast produce proteins with human glycans that are highly uniform. In contrast, traditional mammalian cell production systems produce functional glycoproteins that are heterogeneous and contain non-human glycoforms. As reported by Hamilton et al., GlycoFi eliminated yeast-specific glycosylation in Pichia pastoris and introduced 14 heterologous genes; this process yielded yeast capable of producing complex glycoproteins with greater than 90% terminal sialylation. Candidate protein
can be produced in a bioreactor process that takes three to seven
days, which is somewhat shorter than the time for production in
mammalian cells.

For example, Dr. Wildt discussed MK2578, which is a pegylated
erthropoietin that is terminally sialylated with N-glycans. The
candidate is currently in Phase 1 studies as a potential treatment
for anemia. The glycosylation fidelity from the Pichia platform
is retained when protein is produced at laboratory scale to up to
2,000 L. The yeast can also be used to produce antibody as IgG1.
Compared to CHO cell produced IgG1, candidates produced in
yeast were found to be more potent in inducing ADCC and could
bind antigen as well. In preclinical studies, yeast-produced mAb
glycovariants demonstrated good results in PK studies in Rhesus
monkeys and C57BL mice. In conclusion, Dr. Wildt remarked
that the selection process, which includes screening for titer,
fermentability, glycosylation and protein quality, can result directly
in production strains. Yield is approximately 1.4 grams per liter
for antibody candidates, but yields up to 2 grams per liter can be
achieved.

Annie De Groot (EpiVax) presented information on methods
to reduce protein immunogenicity by design through deimmuni-
ization and tolerance induction. She noted the parallels between
vaccine use, when an immune response is desired, and immuno-
genic therapeutic proteins, which elicit an immune response that
is not desired. In both cases, a payload coupled with a delivery
vehicle and an adjuvant determine immunogenic potential. T-cell
epitopes are a key contributing factor. Like proteins, antibodies are
processed by antigen presenting cells. The activated T-cells in turn
activate B-cells; in the absence of T-cells, no antibody formation
is observed.

EpiVax has developed an array of in silico tools and techniques
to predict whether proteins will be immunogenic. These include
EpiMatrix, in which overlapping 9-mer peptide frames are evalu-
ated for binding potential to eight common class II HLA alleles.
The ClustiMer algorithm can be used to find regions of high
immunogenicity. Using these methods, an overall immunogenicity
score can be estimated. These in silico results can be validated
in vitro and in vivo (e.g., HLA transgenic mice).

The approach has been clinically validated. Koren et al.9 reported
on use of EpiMatrix analysis of a recombinant fusion protein that
predicted promiscuous T-cell epitopes in the C-terminal region. In
a phase 1 study of 76 subjects, 37% developed antibodies after one
injection of the protein candidate. EpiMatrix correctly predicted
the immunogenic region and the likelihood that the protein would
be immunogenic in the clinic.

These results suggest that the technology might be useful as
part of an overall strategy for assessing antibody responses in
non-clinical and clinical settings.9, 10 In addition, rational modifi-
cation of epitopes identified using the technology could effectively
‘deimmunize’ protein candidates. Dr. De Groot also discussed
the discovery of ‘Tregitopes’ (highly conserved regulatory T-cell
epitopes) that are promiscuous, high affinity HLA binders found
in IgG. She noted that there is a correlation of antibody immu-
nogenicity with the presence of Tregitopes. Dr. De Groot and
co-workers have demonstrated that co-incubation of peripheral
blood mononuclear cells (PBMCs) with the Tregitopes can lead to
suppression of immune response to other antigens. This suggests
that the engineering of Tregitopes into antibodies or other proteins
might lead to the development of less immunogenic candidates.

Modular IMMune In vitro Constructs (MIMIC), which is an
in vitro biomimetic human immune system developed to accu-
rately model the immunotoxicity and immunogenicity of drug
candidates, was reviewed by William Warren (Vaxdesign). The
MIMIC system is designed to serve as a ‘clinical trial in a well’
by providing predictive HTP in vitro immunology assessment of
drug candidates. Primary human donor cells are used to simulate
human responses to agents such as vaccines and drugs. The system
consists of three modules: (1) Simulation of innate immunity with
a peripheral tissue equivalent (PTE) module; (2) Simulation of
adaptive immunity with the lymphoid tissue equivalent (LTE);
and (3) a functional assay or disease model. The PTE module
comprises one monolayer of endothelial cells grown over a 3D
collagen matrix. Human PBMCs from donors are used to seed the
module; monocytes extravasate through the endothelial cells and
differentiate into antigen presenting cells. The PTE module can
be used to assess reactogenicity and immunotoxicity responses.
The LTE module functionally reproduces the environment of a
human lymph node. Within the module, T-cells, B-cells, antigen-
presenting cells or follicular dendritic cells interact, leading to
immune stimulation that results in activation of lymphocytes,
cytokine generation and antibody production. The activated
lymphocytes, cytokine profiles and antibodies are then character-
ized using various methods.

Dr. Warren discussed use of the modules to measure the
magnitude and quality of T-cell response to vaccines. He noted
that primary CD8 T-cell response, in vitro humoral and B-cell
response, antibody titer, and microneutralization can be assessed.
A correlation analysis of MIMIC response versus serum titer in
hepatitis B and influenza vaccination has been performed. Results
suggest that the MIMIC system can be used to predict whether a
vaccine would be efficacious before going to the clinic. In addi-
tion to immunogenicity, the system acts as a biomimetic for
evaluation of immunotoxicity and can be used as an inflammation
model or in vitro infectious disease model. Use of the system has
the potential to accelerate the entire drug development timeline,
and decrease failures by providing better data for evaluation of
preclinical candidates.

Karyn O’Neil (Centyrex, a Johnson & Johnson Internal
Venture) described alternative scaffolds that are being used as new
biotherapeutic platforms by Johnson & Johnson. She started by
wondering whether mAbs are always the best choice since there
are reasons to develop alternatives. For example, desirable epitopes
might be immunologically silent, alternatives to injection delivery
are a challenge, full-size antibodies penetrate tissue and tumors
poorly, and royalties might be due on numerous phases of the
mAb discovery, screening, development and production process.
However, requirements for a next-generation platform, which
include the expansion of the range and possibility of targets, lower
cost of development and manufacturing complexity, novel delivery,
elimination of cold storage, clear freedom to operate and no intellectual property issues, are difficult to meet. Alternative scaffolds do meet many of the aforementioned requirements, and these molecules have favorable biophysical characteristics. Alternative scaffolds can be readily formatted into multi-specific binders with relevant biological activity. For example, Lu et al. combined variable regions of two antagonistic antibodies to produce a human IgG-like bispecific antibody that could strongly inhibit the growth of two different human tumors in HT29 xenografts in vivo.

Johnson & Johnson’s strategy toward the use of alternative scaffolds involves development of both Centyrins™ and DARPin™, which are viewed as complimentary molecule types. Both are small (10 to 18 kDa) single domain proteins that have high affinity (low picomolar to femtomolar range) and high selectivity for their targets. They are compatible with technologies that improve serum half-lives and seem to have low immunogenicity and low toxicity. They are also very stable and can be expressed at high levels in soluble form. DARPin™ have flat wide surfaces that are better suited for disrupting protein-protein interactions, whereas Centyrin™ have extended loops that can interact in protein clefts, enzyme active sites and protein channels.

DARPin™, which are being developed as part of a collaboration between Molecular Partners and Johnson & Johnson, are selected from in vitro display of very large (10^{12}) libraries. The method uses PCR and affinity maturation, and candidates with slow off-rates can be selected. In this way, high affinity, neutralizing DARPin™ can be selected within weeks. Melting properties can be used for selection, resulting in DARPin™ candidates with good biophysical properties. Small scale (2 mL) expression of DARPin™ can yield approximately 1 mg each for additional characterization.

The Centyrin™ scaffolds have loops that are analogous to the CDRs of antibodies. The molecules have excellent biophysical properties (>100mg/mL expression, >170mg/mL solubility, >82°C melting temperature, low predicted immunogenicity, stable in serum for more than one month), and can be engineered for improved stability. An in vitro display system licensed from Isogenica utilizes CIS-display technology for Centyrin™ selection. This is proven technology for peptide display. Libraries are potentially quite large (10^{13}). The CIS-display allows rapid panning and selection of binders with a PCR step that allows for in vitro evolution of binders. Rational design of library diversity can improve scaffold properties. A green fluorescent protein solubility/folding reporter assay is used to assess library quality. The unique properties of alternative scaffolds can be exploited in numerous areas, such as bispecific molecules, medical device, encapsulation, novel formulation and delivery, drug/toxin or radionuclide conjugation, imaging, biosensor, purification technologies and intracellular expression.

Cutting-edge analytical technologies became the focus of the meeting in the afternoon session. Steve Cohen (Waters Corporation) discussed chromatographic analysis for biopharmaceuticals with an emphasis on current trends and future prospects. He first discussed ultra performance liquid chromatography (UPLC) utilizing sub-2 micron particle packing (1.7 μmeter with either 130 angstrom or 300 angstrom pores). Compared to HPLC with 3.5 μmeter particle packing, UPLC gives sharper peaks, and can improve resolution of samples in the same run time or achieve comparable resolution and selectivity in a reduced run time (80 versus 120 minutes). Dr. Cohen then presented results of LC/ultraviolet (UV) analysis of a reduced monoclonal antibody, and a murine monoclonal IgG reduced and alkylated standard run at elevated temperature. He noted that the high temperature (80–90°C) is absolutely required for reasonable chromatography. Analytical methods for monitoring glycosylation of mAbs are important because bioprocess conditions can cause variation in high mannos type, truncated forms, reduction of tetra-antennary and increase in tri- and biantennary structures, less sialylated glycans and less glycosylation.

Dr. Cohen also reviewed new separations technologies such as monolithic materials and chip based nanoscale separations. He presented an example of the use of a ceramic microfluidic UPLC system and the software tool BiopharmaLynx 1.2 to perform humanized peptide mapping. Three dimensional structure analysis using amide hydrogen exchange was also discussed. In a continuous labeling experiment, labeling occurs at 25°C, pH 7, and aliquots are removed and quenched at 0°C, pH 2.5. The protein sample can be subjected to HPLC/UPCL directly, or subjected to online digestion and then HPLC/UPCL. Electrospray mass spectrometry then provides information about isotope pattern and deuterium content that can be used to determine exchange rates. Use of UPLC will provide sharper peaks and improved spectral quality. The technique can be used for quality control or comparability of samples, e.g., differentiation of correctly folded protein from incorrectly folded protein.

Tom Laue (University of New Hampshire) discussed advances in analytical ultracentrifugation (AUC) and analytical electrophoresis (AE). Dr. Laue remarked that AUC provides a framework for thinking about concentrated solutions and proximity energies. AUC can be used to characterize proteins in high concentration formulations. Proximity energies at high concentrations may be positive or negative, and are dependent on such factors as distance, orientation, solvent and time. Potential energy is dependent on forces such as charge-charge, charge-dipole, dipole-dipole, hydrogen bonding, dispersion, dipole induced dipole, charge induced dipole and van der Waals interactions. AUC with fluorescence detection can be used to characterize labeled proteins in concentrated samples such as plasma. For example, mAb interactions in plasma can be observed using fluorescence detected sedimentation. Weak electrostatic interactions will dominate molecular behavior in concentrated solutions.

Dr. Laue then discussed AE as a technique to determine accurate values of protein charge. Interestingly, monoclonal IgGs have an actual charge that is aberrantly low compared to the calculated value (e.g., 2 versus 24). The low charge leads to problems with poor solubility and high viscosity. Dr Laue speculated that the mAb charge suppression may have some housekeeping function such as weakening charge interactions with anionic plasma proteins, or altering co-operativity for Fc FcR binding or other functions. The low charge may be due to a combination of pKa shifts, anion binding (territorial or site), and carbohydrate
involvement. Many of the viscosity and solubility problems encountered during processing may be traced to the low charge on IgGs. He urged attendees to measure the charge and not to rely on calculated charge estimates (e.g., from isoelectric point measurements).

Kermit Murray (Louisiana State University) discussed coupling microfluidic chips to matrix-assisted laser desorption ionization (MALDI) mass spectrometry. The chips can speed proteomics by serving as a single platform for automated cell culturing, digestion, separation and sample deposition. The system is based on synthetic polymer microfluidic devices, with chip components fabricated on a poly(methyl) methacrylate plate using the hot embossing method and off-line MALDI analysis. A key component of the system is a trypsin microreactor. Assembled chips are processed using a pressure-driven or electrokinetic flow; the system utilizes a Dionex LC and a Probot MALDI plate for the former. Digested peptides are coaxially mixed with a MALDI matrix solution and deposited on a MALDI target. Chips are stable for about one month.

Dr. Murray presented experimental results from analyses of cytochrome c under various flow rates. A flow rate of 1 μL/min, with a residence time of approximately 24 seconds within the reaction bed, provided 67% sequence coverage, which increased to 72% when residence time was increased to 48 seconds. Use of the 1 μL/min flow rate resulted in sequence coverages of 35%, 58%, and 47% for 10 μM samples of bovine serum albumin, myoglobin and phosphorylase b, respectively. The digestion efficiency was improved using an electrokinetically driven microreactor using a micro-post structured chip. Using the micro-post system, sequence coverage of 10 μM cytochrome c was 89%; sequence coverage decreased when protein concentration of the sample was lower. Whole bacterial cells can be analyzed using the system. Digestion and deposition of E. coli resulted in identification of the aminoglycoside 3’-phosphotransferase type 1, with 57% sequence coverage.

A two-chamber chip developed by Dr. Murray and colleagues can also be used to provide MALDI MS results for bacteria. It has applications for analysis of sepsis, pneumonia, tuberculosis, blood supply QA/QC, and environmental pathogen samples. The cell culture chamber has sample and media inlet, as well as outlet, channels; the culture chamber itself has a 3 mm diameter and 300 μm depth. The system uses a PMMA chip and PDMS cover. The channel surfaces are sterilized with UV. After assembly, the chamber is filled with nutrient broth, approximately 4,000 E. coli cells are added and the reservoirs are closed. The bacteria are cultured for 24 hours at 37°C. One μL of E. coli is then deposited on the MALDI target plate. In an experiment using ATCC#9637, #11303, or #11775, some cellular protein peaks were found. The results suggest that such on-chip culturing could be used for fingerprint analysis. Finally, Dr. Murray discussed preliminary work on a temperature regulated chip with heating and cooling elements.

The topic of mass spectrometry (MS)-based strategies to study protein architecture, dynamics and binding was reviewed by Igor Kaltashov (University of Massachusetts, Amherst). He first noted that biopharmaceuticals have higher order structure and conformational heterogeneity, and various perturbations or changes in the production process can result in alterations of the primary or higher order structure that can have deleterious effects on the efficacy, immunogenicity or stability of the protein. MS has been applied to the structural characterization of recombinant protein pharmaceuticals and specifically therapeutic antibodies. The tertiary and quaternary protein structure can be characterized directly in solution by electrospray ionization (ESI) MS.

As an example of the use of MS in structure characterization, Dr. Kaltashov discussed studies done on alkylated interferon β-1a (IFN-β-1a). Alkylation at Cys-17 of the protein results in 50–90% reduction of the antiviral activity. He remarked that ‘classical’ biophysical techniques such as size exclusion chromatography, fluorescence, far-UV circular dichroism (CD) and near-UV CD were not very informative regarding conformational changes between the alkylated and unmodified forms. Two complementary MS-based techniques, analysis of ionic charge state distribution and hydrogen/deuterium exchange (HDX), were used to monitor conformational changes. The analysis of the ionic charge distributions indicated a decrease in conformational stability in the alkylated form; the partial unfolding was revealed by the presence of protein ions in the ESI mass spectra with significantly higher charge density compared to the unmodified version. In HDX MS, measurements can be carried out under conditions closely mimicking the formulation buffer, and thermodynamic information is derived from biophysical measurements. Global HDX MS revealed higher flexibility of alkylated IFN. Backbone flexibility was observed to be distributed unevenly across the polypeptide sequence. Structural studies suggest that the loss of antiviral activity of the alkylated form is due to destabilization of a region of IFN that binds with its low affinity receptor (IFNAR1), and disruption of ternary complex formation.

Dr. Kaltashov concluded by suggesting that ESI MS can be used to characterize highly heterogeneous systems and presented findings from a study of heparin, which is very heterogeneous, and difficult to characterize by MS. With colleagues, he has developing a mass spectrometry-based strategy for characterization of anti-thrombin interaction with low molecular weight heparin and heparin oligomers.

Genentech’s use of high throughput (HTP) methods in bioprocess development was discussed by Judy Chou (Genentech). She described analytical methods as the ears and eyes of the production process. Use of a high throughput platform is directly related to the need for rapid analysis of bioprocess samples. The need for speedy analysis, which enables new products to get to patients in a timely fashion, has to be balanced with the need for extensive sample characterization that might be time-consuming. The aim is to leverage new technology, especially in HTP purification and automation to increase the number of experiments while reducing resources required and shortening timelines. The HTP approach involves scaling down cell culture, use of protein A well-plate purification (PAWP), purification in plate (PiP), HTP impurity analysis and an at-line reverse phase high performance liquid chromatography (RP-HPLC) method.
In the PAWP method, 24- or 96-well plates with protein A are used. The plates can be subjected to centrifugation or vacuum procedures, then samples are directly analyzed by HPLC, liquid chromatography-mass spectrometry (LC-MS), capillary electrophoresis (CE), or image capillary isoelectric focusing (icIEF) techniques. The method allows product quality tests to be expedited, and allows the company to address product quality issues early on and reduce resource and time cost later. Use of the PAWP method was directly compared with use of a standard purification procedure (protein A column). Samples subjected to both methods gave similar results in an array of tests (SEC IEC, CZE, icIEF analysis, CE-Glycan assay, peptide mapping).

RP-HPLC rapid monitoring can be used as a fast method to monitor mAb fragments in both the purified samples and the cell culture fluids without any sample preparation procedure. It provides a powerful tool to look into antibody reduction issues and helps to monitor as well as to develop bioprocess to mitigate the risk of losing product quantity and quality. Furthermore, the on-line RP-HPLC-Mass Spectroscopy (MS) enabled the understanding of the new peaks identified in the cell culture fluids and increased the process knowledge during the development and operation phases.

HTP impurity assays were also developed as a tool to quickly narrow down purification conditions. A CHO protein Meso Scale Discovery (MDS) impurity assay that utilizes electrochemiluminescence was incorporated in an HTP process. Plates can be prepared and stored for up to nine months. The MSD assay was compared to ELISA at various purification steps and the difference was found to be less than 15%, whereas only 10% of resources are used to perform assay and the total assay time was only 2.5 hours for ~400 samples. In addition, a leached Protein A HTP assay based on MSD technology was also developed. In order to prevent the signal masking introduced by the products, a novel approach of acidification combined with effective blockers was implemented. The new method is generic to all the molecules tested so far and only takes three hours for ~400 samples with limited amount of resources needed.

A novel TEACAN system that puts all the relevant analytical assays as well as the PiP and High throughput Formulation development in an assembly line is currently being used. Dr. Chou mentioned that the details of the methods she described will be published soon.

References

1. Scheider CK, Kalinke U. Toward biosimilar monoclonal antibodies. Nat Biotechnol 2008; 26:985-90.
2. Dudzinski DM, Kesselheim. Scientific and legal viability of follow-on protein drugs. New Engl J Med 2008; 358:843-9.
3. Reichert JM. Monoclonal antibodies as innovative therapeutics. Curr Pharma Biotechnol 2008; 9:423-30.
4. Reichert JM, Rosensweig CJ, Faden LB, Dewitz MC. Monoclonal antibody successes in the clinic. Nat Biotechnol 2005; 23:1073-8.
5. Reichert JM. Monoclonal antibodies in the clinic. Nat Biotechnol 2001; 19:819-822.
6. Reichert JM, Valge-Archer VE. Development trends for monoclonal antibody cancer therapeutics. Nat Rev Drug Disc 2007; 6:349-56.
7. Hamilton SR, Davidson RC, Sethuraman N, Nett JH, Jiang Y, Rios S, et al. Humanization of Yeast to Produce Complex Terminally Sialylated Glycoproteins Science 2006; 313:1441-3.
The meeting continued with sessions on accelerated cell culture process development strategies [chairs: Timothy Charlebois (Wyeth) and Rohini Deshpande (Amgen)]; critical quality attributes, specifications and comparability [chairs: Reed Harris (Genentech) and Paul Tsang (Amgen)]; and high throughput protein formulation and development [chairs: David Volkin (Centocor) and Bruce Kerwin (Amgen)].

The first speaker, Florian Wurm (ETH-Lausanne) discussed the potential of transient gene expression (TGE) as a large scale manufacturing technology for recombinant protein therapeutics. He first reviewed the transfection process, explaining the differences between stable and transient transfections, then provided an example of how transient transfection studies were performed as many as 20 years ago for improving clot lysis activity in human tissue plasminogen activator at Genentech.1 In this particular case, truncation of 24% of the light chain sequence was detected through liquid chromatography-mass spectroscopy and cDNA sequencing. With further RT PCR analysis, they realized that cryptic mRNA splicing was responsible for the deletion observed. This was remedied with a silent mutation in the target sequence. Thus, implementing a risk assessment strategy at the early stage of cell line generation successfully eliminated higher risk cell lines from further development.

Dr. He described two strategies that could be used for determining the suitability of a cell line in terms of productivity: phenotypic assessment and genetic characterization. At Lilly, phenotypic assessment is performed by aging cells via serial passaging, and thereafter determining productivity through fed-batch studies across different generations, typically generations 30, 45 and 60. However such an analysis is time and resource intensive. In addition, growth rates change as cells age, which leads to non-ideal feeding strategies that affect antibody production. This would not then be a true measurement of cell line stability.

To improve the process of cell line suitability assessment, Lilly has developed a novel approach based on genetic characterization. Dr. He presented a quantitative analysis of transfected genes in recombinant single cells derived from the clonal cell lines. This method applies three parameters to the evaluation of cell line suitability: the percentage of the population in which the genes are not detected, the distribution of mAb gene copies within a generation and the consistency of the distributions across generations. The suitability of more than ten cell lines was evaluated by this approach, and they observed that most of the unstable cell lines displayed various degrees of mAb gene copy number heterogeneity. In addition, about one-sixth of the cells in the populations of two cell lines had completely lost mAb genes at relatively early generations. Dr. He noted that the results were reproducible and independent of process conditions. However, she also cautioned that this analysis may not be sufficient to identify all unsuitable cell clones. She concluded by remarking that such an approach in the cell line generation process leads to a significant reduction of the time and manpower required to eliminate higher risk cell lines from further development.

Denny Kraichely (Centocor Research and Development, Johnson & Johnson) presented a novel fluorescent Protein G methylcellulose screening assay that could potentially accelerate...
the identification of high-producing clones. After a brief review of the key steps involved in cell line development, he discussed some of the challenges associated with this process. In general, the aim is to obtain a cell line with high productivity and cell density, while maintaining the desired product quality. However, this must be achieved within certain logistical limitations, and tight timelines, thus highlighting the importance of an efficient clone screening process.

Potential limitations of Centocor’s internally developed ‘Halo’ screening protocol necessitated development of an improved antibody secretion detection assay. The objective was to establish an assay that allowed for efficient selection of high-producing clones, while eliminating any animal components and reducing the overall cost. To this end, Dr. Kraichely and his colleagues first examined alternate reagents available for antibody detection, including Protein A, Protein G and Protein L, and assessed the specificity of these reagents towards the target antibody molecule. Their procedure entailed plating a mixture of antibody producing and host CHO cells in methylcellulose, subsequent addition of Protein G Alexa Fluor and examination of the correlation between fluorescence intensity and antibody titers.

Once they established that this assay specifically detected antibody producing clones and the concentrations of reagents had been sufficiently optimized, the procedure was applied to creation of parental and sub-cloned cell lines. A strong relationship between fluorescence intensities and expression levels (at shake flask levels) of both the parental and the sub-clones was observed (correlation coefficient value of approximately 0.8). Importantly, cell lines with the highest fluorescence intensities were the best producers.

The last developmental phase consisted of automation of the screening assay with the ClonePix FL and Hamilton StarPlus systems, which allowed for automatic clone picking and clone manipulations up to the shake flask level, thus minimizing manual operations. Dr. Kraichely concluded his presentation by noting that this cost-effective assay was quantitative and predictive, enabling easy isolation of high-producing cell lines, and that this novel assay had the potential of shortening the cell line development timeline by as much as one month.

**Gregory Hiller (Wyeth)** discussed efforts to achieve cell culture yield improvements through modifications to the platform bioreactor process. Dr. Hiller noted that while there have been impressive increases in titer over the past 10–15 years, most improvements have been as a result of greater integrated viable cell densities (IVCD). For a process where specific productivity is relatively constant, the titer is directly dependent on the IVCD attained.

With the objective of enabling production of Phase I clinical material at a smaller scale, a set of platform process alterations, termed PIPES (Phase I Platform EnhancementS), were made at Wyeth to boost IVCD while maintaining the specific productivity. These modifications included a 3–5 fold increase in inoculum cell density, increases in nutrient content in the basal media and an augmented feeding strategy with more concentrated, frequent and higher volume feeds. Dr. Hiller described how these process changes enabled elevated cell densities in culture and subsequent improvements in titer of up to two fold.

In their experimental studies directed towards improving IVCD, Dr. Hiller and colleagues observed that the accumulation of lactate in culture was most likely to inhibit cultures from achieving high cell densities, although other factors such as the lack of nutrients and the buildup of ammonia, alanine and carbon dioxide sometimes played a role. Suppression of lactate accumulation through culture manipulations such as a reduced glucose feed during the exponential growth phase was observed to negatively affect cell growth. Concerns have also been raised about potential effects on product quality, e.g., altered glycosylation patterns, when adopting a controlled, low glucose level.

An alternate method to reduce lactate accumulation using a strategy of feeding glucose based on pH was successfully adopted. Lactate carryover from the n-1 seed reactor was used in place of glucose as the source of sugar during cell inoculation. As the lactic acid was consumed by cells, elevated pH levels warranted slow addition of glucose through the feed medium. Subsequent lactate production and consumption in culture as detected by pH levels were then followed by another cycle of glucose feeding. This strategy ensured that lactate never accumulated to high levels, and thus high cell densities, higher cell specific productivities, and consequently increased titers were achieved. Since the limited glucose feeding only occurred during the growth phase of culture (when little protein is produced), concerns regarding aberrant glycosylation are significantly reduced.

Dr. Hiller concluded by noting that significant increases in cell mass and productivity achieved through PIPES for Phase I clinical material production should help to free up large scale bioreactor capacity for technology development, support of process changes, or commercial supply.

**Paul Motchnik (Genentech)** opened the next session, which focused on critical quality attributes (CQAs), specifications and comparability, by describing a risk ranking and filtering approach towards identifying the CQAs of mAbs. Having defined CQAs as a physical, chemical, biological or microbiological property or characteristic that should be within an appropriate limit, range or distribution to ensure the desired product quality, he then described multiple aspects considered while developing a process for identification of these attributes at Genentech. Primary amongst these factors was the evaluation of a number of risk assessment tools, including Preliminary Hazards Analysis, Failure Mode and Effects Analysis and Risk Ranking and Filtering. The Risk Ranking and Filtering tool was ultimately selected because it allowed capture of multiple factors involved in the risk, while enabling identification of CQAs independent of process capabilities. These factors could then be combined into a single relative risk score, which could be used for ranking risks. Filters, in the form of cut-offs for risk scores, could be included to fit the risk ranking to management or policy objectives.

Using this tool, the risk associated with an attribute was identified based on potential impact to product safety and efficacy, and the uncertainty in assigning this impact. Impact was assigned a score on a 2–20 scale, based on available knowledge about...
biological activity, pharmacokinetics, immunogenicity and safety. As an example, Dr. Motchnik assessed the impact of specific variants on immunogenicity. In the absence of clinical data, the immunogenicity impact of mAb variants was assessed by occurrence of analogous plasma-derived IgG variants, e.g., removal of the C-terminal lysine from circulating IgGs. Applied to a product candidate, such an event would be a non-CQA with a low impact score, whereas the presence of aggregates would have a high impact score, and consequently a high risk associated. Clinical experience would be necessary to make a final assessment.

Using a 0–7 point scale, an uncertainty score was also assigned; this score ranked the relevance of knowledge used to assign the impact score. An overall risk severity score that reflected the relative risk associated with an attribute was obtained by multiplication of the impact and uncertainty scores. Attributes with a score of 13 or higher were categorized as CQAs.

Before applying such a strategy, Dr. Motchnik and colleagues classified product quality attributes for antibodies into the following categories common among antibodies: 'always CQAs' that do not require assessment, i.e., pH, particulate content; 'attributes requiring risk assessment', i.e., charge and size variants, glycan variants; and 'generically assessed attributes', i.e., host cell protein content, leachables. Dr. Motchnik also discussed the timeline for identification of CQAs. CQAs should ideally be reviewed during the pre-IND period, and then updated at the end of Phase II, at marketing application submission and at product approval. Dr. Motchnik concluded by cautioning that this approach, though robust, requires refinement based on experience.

Sally Anliker (Eli Lilly) discussed how CQAs could be used to develop control strategies for bioproducts, and convey these to health authorities. She first reviewed what patients require from pharmaceutical therapy, e.g., safety, efficacy, value for money, good performance characteristics and convenience. Although patient needs for biopharmaceuticals are the same as for small molecule drugs, biologics involve greater process and product complexities, and thus these products require different control strategies.

CQAs, defined by Dr. Anliker as the most important attributes to achieving desired product performance in the intended patient population, could be viewed as the connection between patient needs and product quality. Common CQAs can be identified for all sterile drug products, including identity, appearance, strength formulation, delivery functionality and purity. Bioproducts may require an additional CQA for biological activity. As various aspects of purity are managed by different control strategies, purity as a CQA may be expanded into several purity-related attributes including product and process related impurities, e.g., host cell proteins; post-translational modifications, e.g., glycosylation; adventitious agents and microbial purity. CQAs could thus be used to drive the development of both product and process, as well as associated control strategies. They can also be utilized as a framework for comparability assessments, and for communicating manufacturing control strategies in regulatory submissions.

Dr. Anliker concluded by citing examples of when the failure to understand CQAs, and the consequent lack of appropriate control strategies, led to undesirable outcomes. In one such example, a master cell bank was recloned, manufacturing was subcontracted, and changes were made to the fermentation medium, fermentor configuration and harvest procedure in efforts to improve yield for a candidate that had completed Phase 3 studies, but was not yet marketed. The candidate exhibited amino acid substitutions and altered glycosylation patterns when produced using the new process and could not be marketed.

Izydor Apostol (Amgen) provided an analytical perspective on product specification lifecycle during development of biotechnology products. The focus of the presentation was alignment of the emerging analytical platforms with a product specification strategy across the lifecycle of product development. Protein specification components include product attributes (e.g., purity), parameters (e.g., molecular weight, isoelectric point, charge, heterogeneity), analytical methods and limit or acceptance criteria. The specifications for drug substance and product are defined through a combination of clinical experience, published literature on similar products, variants or impurities, and results from non-clinical studies, e.g., binding assays, in-vivo and cell-based assays. There would also be contributions from process capability with respect to variability observed in manufactured lots. Analytical capabilities will depend on the methods selected and the limits established.

Dr. Apostol noted the challenges associated with method validation and the appropriate extent of characterization for products at different stages of their lifecycle. The performance characteristics that need to be assessed during qualification and validation include specificity, range, precision, linearity, accuracy, and limits of detection/limits of quantification. He stressed that method precision was the most important performance characteristic relevant to setting specification limits, and governing uncertainty of results. Dr. Apostol pointed out an alternative approach in assessing the precision of purity methods by examining signal to noise ratio.

In addition, the problem of understanding the biological relevance of impurities and product variants was discussed. The example of monitoring microheterogeneity in clinical samples was used by Dr. Apostol to illustrate the need for assessment of biological significance of quality attributes. Microheterogeneity is observed in IgG2 samples because this type of antibody can exist in different forms/variants, separable by NR-RP-HPLC, that vary in the arrangement of disulfide bonds. Conversion of variants was found in clinical samples. Disulfide bond rearrangement was found to cause the distribution of isoforms change over time, where over 13 days, the percentage of IgG2-B was increasing and IgG2-A decreasing. In concluding, Dr. Apostol noted that understanding method performance characteristics and the science governing the uncertainty of analytical results is critical for setting specifications limits, assessing process variability and reporting results. Specifications should focus on critical quality attributes, and the maturity of specifications should evolve with increased understanding of analytical methods and the product.

Patrick Swann (Center for Drug Evaluation and Research, US Food and Drug Administration) discussed the use of Quality by Design (QbD) concepts to categorize biotechnology product quality attributes. He first defined QbD as a systematic approach...
to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management.

While listing the key steps in implementation of QbD for a biotechnology product, he emphasized the importance of risk assessment in the identification of CQAs. CQAs are also instrumental in defining product and process design space. Dr. Swann provided an historical overview of public proposals for potential CQAs for biotechnology products, which included potency, sterility, adventitious agents, impurities and formulation components. However, not all biotechnology product attributes are universally identified as critical. For example, two companies might choose to identify different attributes as CQAs. Dr. Swann then outlined key questions and observations from his review of proposals to systematically categorize attributes, such as the level of confidence or type of study needed to identify an attribute as non-critical. The process of analyzing the resultant effects of molecule modifications can be challenging, as was illustrated in an example involving posttranslational modifications of an antibody where $10^8$ combinations were theoretically possible.

Dr. Swann also outlined the distinction between product-related substances and product-related impurities based on properties comparable to those of desired product with respect to activity, efficacy and safety. The underlying theme of the presentation was that systematic methods for defining CQAs should consider the quality target product profile and clinical experience. There can be no single list applicable to all antibodies.

The final session of the day featured presentations on high-throughput protein formulation and development.

**Albert Lee (Genentech)** presented his company’s evaluation of high-throughput formulation screening using rapid stress methods. He first noted that one of the main objectives of formulation science in the biologics industry is to identify and optimize various parameters within the formulation design space, including pH, buffer species, salts and sugars, to ensure product quality and stability until it is delivered to patients. However, these robust formulations need to be achieved within tight process development timelines.

Dr. Lee then discussed how automation was leveraged to replace labor intensive formulation operations and accelerate biopharmaceutical formulation development at Genentech. The typical methodology of formulation screening by testing different stress conditions in individual glass vials was replaced by a high-throughput approach of using well plates and direct mixing of components from stock concentrates. Comparability between the two approaches was demonstrated by comparison of results from various analytical methods used to study two different antibodies in six diverse formulations.

Often, elevated temperatures (40°C) on a month timescale are employed for formulation optimization. Once automation was achieved, Dr. Lee and his colleagues explored whether identification of stable formulations was possible through a set of alternative stress methods on a shorter 24 hour timescale. These methods included physical agitation, ultraviolet light exposure and thermal stress at even higher elevated temperatures (i.e., 55°C). Agitation could potentially induce physical degradation, whereas UV light exposure could lead to free radical formation and photo-oxidation and subsequent physical and chemical degradation. Similarly, higher elevated temperatures could increase the rates of degradation and induce conformational changes. Through peptide map analyses, these rapid stress methods were shown to be predictive of more conventional stress methods.

In closing, Dr. Lee suggested that these automatable rapid stress methods, which require less material, can have broad applications ranging from preformulation or formulation development to quick understanding of formulation-dependent and molecule-specific stability.

**Juan Alvarez (Transform Pharmaceuticals, Johnson & Johnson)** discussed the use of high throughput technologies in the development of biopharmaceuticals. He noted that formulation of biopharmaceuticals was challenging due to the complexity of molecules and product requirements, where the molecule must remain physically and chemically stable under even harsh conditions that may occur during transport or storage. He emphasized the vastness of the formulation design combinatorial space and noted that there are no universal formulations for all biopharmaceuticals.

The shortcomings of the traditional formulation design process, which includes limited exploration of a small number of conditions that might result in the selection of a sub-optimal formulation, were then reviewed. To overcome these limitations, Dr. Alvarez and colleagues at Transform Pharmaceuticals have applied high throughput technologies to explore the large formulation combinatorial space, i.e., assess the effects of a large number of formulations on physical and chemical stability and solubility of biotherapeutic molecules. These technologies, with relatively low material requirements, allow for the creation of several thousand formulations which can then be subjected to different stresses and assessed in multiple assays. Through analysis utilizing a powerful informatics back end, formulation parameters can be simultaneously optimized, resulting in a data-driven decision-making process. Multiple examples of representative data were presented highlighting the breadth of information achieved with the system in support of protein solubility and stability screens. The automated high throughput systems permitted evaluation of multi-dimensional input and output data and led to an increased understanding of the molecule and its formulation space with reduced resources and in accelerated timelines. However, Dr. Alvarez cautioned that maximum value would be gained only if a good informatic infrastructure was present.

**Yuhong Zeng (University of Kansas)** represented Professor Russell Middaugh for the next presentation, which focused on an empirical phase diagram (EPD) approach towards stabilization of macromolecular therapeutics. She discussed a rational methodology towards development of stable protein formulations, based on a thorough understanding of the biophysical properties of a therapeutic protein under all conditions of interest. However, the challenge would be to gather as much information as possible and decipher a comprehensive picture under timeline pressures.

An EPD-based high throughput screening approach towards formulation design was developed at Professor Middaugh's
Macromolecule and Vaccine Stabilization Lab. The first step in the approach is systematic and comprehensive biophysical characterization of the target biopharmaceutical molecule in the high throughput mode using a variety of techniques including UV-visible absorption, circular dichroism, fluorescence, Fourier transform infrared spectroscopy, static and dynamic light scattering and differential scanning calorimetry (DSC). Recently, techniques that detect protein dynamic behaviors, such as pressure perturbation calorimetry, high resolution ultrasonic spectroscopy, red edge fluorescence excitation, lifetime fluorescence anisotropy and time correlated single photon counting have also been employed to correlate protein dynamics and stability.

The next step in the process is the creation of the EPD. This involves construction of vector descriptions of the biophysical data obtained, subsequent manipulations of these vectors to create an red-green-blue color density matrix and visualization of the color density matrix to generate the EPD, which is a colored map that depicts biophysical changes of a protein under various environmental stresses. Using the apparent phase boundaries defined by a phase diagram, optimal formulation conditions can be selected. Furthermore, high throughput screening assays can be developed based on the EPD to identify potential stabilizers for the macromolecule. As an example, Dr. Zeng cited the characterization and stabilization of an anthrax vaccine containing recombinant protective antigen (rPA). Using the EPD for rPA, they were able to define pH and temperature conditions associated with various biophysical states of the molecule, including structurally disrupted states, aggregated states, as well as stable states. This allowed them to design a stable formulation with minimal optimization requirements.

Mark C. Manning (Legacy BioDesign) presented criteria for efficient and rational design of stable liquid biopharmaceutical formulations. He first discussed how formulation was necessary in drug development to ensure optimal product performance by the appropriate choice of dosage form, excipients, processes and packaging. Performance denotes the quality of the product in terms of stability, solubility, potency and safety. Lower costs and accelerated timelines necessitate efficient and effective formulation strategies that depend on a broad knowledge database, personal experience, as well as corporate expertise.

Biorecombinates are generally packaged in three different dosage forms: lyophilized powders, frozen solutions and liquid formulations. While rules for the development of lyophilized powders are well-defined, development of frozen formulations is extremely challenging. However, a rational approach for developing liquid formulations (either 2–8°C or room temperature) is emerging.

Dr. Manning outlined rules for obtaining a stable liquid formulation, including maximizing thermodynamic stability, reducing colloidal instability and preventing surface induced denaturation, slow oxidation and other chemical damage. Thermodynamic or conformational stabilization implies increasing the free energy of unfolding, i.e., shifting the native-state ensemble away from more expanded, aggregation-competent states. Use of an excluded solute reduces the solvent accessibility of partially buried residues that might undergo chemical degradation. This was illustrated by an example of how sucrose addition decreased protein aggregation rates in formulations of interferon-γ and recombinant human granulocyte-colony stimulating factor (rhG-CSF).

On the other hand, colloidal stability can affect both aggregation behavior and solubility. This variable can be measured as the second osmotic virial coefficient. As an example, Dr. Manning presented the case of rhG-CSF, which is more prone to aggregation at pH 5 and above due to strong colloidal stabilization at acidic pH. After elaborating on strategies to achieve interfacial stability and to minimize chemical degradation, he emphasized the importance of mathematical tools in formulation development. He noted that the use of mathematical tools such as chemometrics and phase diagrams allowed extraction of maximal information from product development data sets. He concluded his presentation by elaborating on the hallmarks of a good formulation. A good formulation must be simple, safe, manufacture-friendly while satisfying shelf-life objectives, target profiles and timelines.

In his presentation on a parallel, microscale, automated approach to preformulation and stress test studies for large molecules, Byeong Chang (Symyx Technologies) discussed approaches to systematic screening of the variable space in the formulation development of large molecule therapeutics. Dr. Chang outlined a novel protein formulation workflow in which a sample preparation station was physically integrated with environmental stress chambers and analytical stations. Analytical measurements included size exclusion chromatography-high performance liquid chromatography (SEC-HPLC), dynamic light scattering (DLS), UV-visible and fluorescence spectrometry, lab-chip gel electrophoresis, DSC, osmometry, pH measurements and visual monitoring using cameras. An integrated software platform allowed for automated stress testing of matrix-based formulation experimental designs. Using illustrations, he delved into the process of formulation preparation, analytical measurements and data collection, as well as sample preparation procedures for different analytical methods. A case study involving protein surfactant screening was presented in which the principal objectives included formulating a representative protein with several surfactant compositions and concentrations, studying the effect of surfactant composition and concentration on protein stability and identifying the best surfactant composition for the protein. The process involved addition of desired amounts of dilution buffer, surfactant and protein, followed by sample agitation. At every time-point, DLS, SEC-HPLC and chip electrophoresis analytics were applied to monitor aggregation (soluble and insoluble) and denaturation of the protein. To ensure formulation robustness, a three-prong process involving forced degradation studies (involving surfactants), high throughput screening and stability studies (involving candidate formulations) was recommended.

The keynote speaker for the second day was John Carpenter (University of Colorado), who presented the causes, control, consequences and challenges associated with the aggregation of therapeutic proteins. He noted that aggregation could be one of the most challenging problems for the development of safe and effective biopharmaceuticals. The consequences of protein aggregation could be quite adverse, with even relatively low levels...
of aggregates potentially causing immunogenicity in patients. Immunogenicity could result in neutralization of the drug or loss of drug efficacy, potentially leading to lethal consequences. However, there is little documented data on protein aggregates and sub-visible particles for products with demonstrated immunogenicity problems. Based on work with vaccines, it has been hypothesized that the most immunogenic aggregates are large and composed of molecules with native protein-like structure. It also appears that subcutaneous injections may be most problematic, with repetitive administration increasing the risk of immune response.

Dr. Carpenter discussed how protein aggregation could be controlled by maximizing conformational and colloidal stability. As charge-charge interactions are an important factor in aggregation, pH is a critical parameter in formulation development. As an example, he presented the case of Protein 'X' where recovery of monomer was attained in solution by optimizing pH and thus conformational stability. However, absence of aggregation was observed with benzyl alcohol-induced structural perturbation of rhGCSF at low pH. This result might have been due to the unfavorable energetics of intermolecular interactions (i.e. colloidal stability) between rhGCSF molecules at this pH.15

While discussing the causes of protein aggregation, Dr. Carpenter noted that recent studies have shown that protein aggregates are formed from partially unfolded protein molecules, which could be part of the native state ensemble of substrates. Even in a greatly stabilizing formulation, aggregation could proceed at significant rates. Furthermore, aggregation could occur due to protein exposure to numerous stresses such as air-liquid or liquid-solid interfaces and light that occur during production, purification, storage and delivery to the patient. Light-induced damage may occur due to photodegradation reactions of tryptophan. This was studied in a model protein: sTNF-R1, where aggregation due to light damage was observed to increase with time and decreasing pH. The presence of sucrose and methionine did not inhibit aggregation, whereas tryptophan was found to accelerate the aggregation process.

Aggregation can sometimes occur due to the presence of foreign micro- and nano-particles that act as nucleation agents. For example, syringe barrels are coated with silicone oil to facilitate smooth movement of plungers. Such treatment could lead to droplets of silicone oil being suspended in the product formulation, while sub-visible particles for products with demonstrated immunogenicity problems. Based on work with vaccines, it has been hypothesized that the most immunogenic aggregates are large and composed of molecules with native protein-like structure. It also appears that subcutaneous injections may be most problematic, with repetitive administration increasing the risk of immune response.

In discussing challenges associated with protein aggregation, Dr. Carpenter explained how factors critical in governing aggregate-induced immunogenicity in patients were not known. Questions about types of proteins and aggregates that cause immunogenic reactions, dosing and comparability between animal models and humans for study of immunogenic effects still remain. Also, analytical approaches presently in use for protein aggregation studies may not be appropriate. Even though size exclusion chromatography is commonly used for quantifying protein aggregation, this technique could under-report aggregate levels. Analytical ultracentrifugation may be useful, but requires expert and careful sample preparation and data analysis.

Dr. Carpenter noted that even though sub-visible particles may be most important for immunogenicity, they are not analyzed properly. One reason may be that the amount of protein (e.g., 0.1%) in particles may not be detectable as loss of monomer. Secondly, USP guidelines require quantitation of particles with diameters more than 10 microns. However, there are examples demonstrating the presence of tens of thousands of particles less than 10 microns in diameter, when 10 micron particles are at low levels. One possible method for quantification of sub-visible particles would be use of microflow imaging techniques, but more work is needed to determine the best instruments and protocols for this purpose. Furthermore, relationships between soluble aggregates, insoluble aggregates, sub-visible particles and visible particles are unknown.

Concluding, Dr. Carpenter stressed that each protein had a different aggregation behavior and must be characterized and studied individually.

References
1. Bennett WF, Pooni NE, Keyt BA, Botstein D, Jones AJ, Presta L, et al. High resolution analysis of functional determinants on human tissue-type plasminogen activator. J Biol Chem 1991; 266: 5191-201.
2. Backliwal G, Hildinger M, Chenuet S, Wulhfard S, De Jesus M, Wurm FM. Rational vector design and multi-pathway modulation of HEK 293F cells yield recombinant antibody titers exceeding 1 g/l by transient transfection under serum-free conditions. Nucleic Acids Res 2008; 36; 96.
3. Lee C, Ly C, Sauerwald T, Kelly T, Moore G. High-throughput screening of cell lines expressing monoclonal antibodies. BioProcess Int Cell Line Eng Supp 2008; 4:32-5.
4. Schofield T, Apostol I, Kooler G, Powers S, Stawicki M, Wolfe R. A rational approach for setting and maintaining specifications for biological and biotechnology-derived products—part 2. BioPharm Int 2008; 21:32-40
5. Apostol I, Schofield T, Kooler G, Powers S, Stawicki M, Wolfe R. A rational approach for setting and maintaining specifications for biological and biotechnology-derived products—part 1. BioPharm Int 2008; 21:42-54.
6. Liu YD, Chen X, Enk JZ, Plant M, Dillon TM, Flynn GC. Human IgG2 antibody disulide rearrangement in vivo. J Biol Chem 2008; 43:29266-72.
7. Dillon TM, Ricci MS, Veizina C, Flynn GC, Liu YD, Rehder DS, et. al. Structural and functional characterization of disulfide isoforms of the human IgG2 subclass. J Biol Chem 2008; 283:16206-15.
8. Peak, LJ, Brey RN, Middaugh CR. A rapid, three-step process for the preformulation of a recombinant ricin toxin A-chain vaccine. J Pharm Sci 2007; 96:44-60.
9. Ramsey JD, Gill ML, Kamerzell TJ, Price ES, Joshi SB, Bishop SM, et. al. Using empirical phase diagrams to understand the role of intramolecular dynamics in immunoglobulin G stability. J Pharm Sci 2008; DOI 10.1002/jps.21619.
10. Jiang G, Joshi SB, Peak LJ, Brandau DT, Huang J, Ferriter MS, et. al. Anthrax vaccine powder formulations for nasal mucosal delivery. J Pharm Sci 2006; 95:80-96.
11. Carpenter JF, Pikal MJ, Chang BS, Randolph TW. Rational design of stable lyophilized protein formulations: some practical advice. Pharm Res 1997; 14:969-75.
12. Carpenter JF, Chang BS, Garzon-Rodriguez W, Randolph TW. Rational design of stable lyophilized protein formulations: theory and practice. Pharm Biotechnol 2002; 13:109-33.
13. Chi EY, Krishnan S, Randolph TW, Carpenter JF. Physical stability of proteins in aqueous solution: mechanism and driving forces in nonnative protein aggregation. Pharm Res 2003; 20:1325-36.

14. Ruppert S, Sandler SI, Lenhoff AM. Correlation between the osmotic second virial coefficient and the solubility of proteins. Biotechnol Prog 2001; 17:182-7.

15. Thirumangalathu R, Krishnan S, Brems DN, Randolph TW, Carpenter JF. Effects of pH, temperature, and sucrose on benzyl alcohol-induced aggregation of recombinant human granulocyte colony stimulating factor. J Pharm Sci 2006; 95:1480-97.

16. Carpenter JF, Randolph TW, Jiskoot W, Crommelin DJ, Middaugh CR, Winter G. Overlooking subvisible particles in therapeutic protein products: gaps that may compromise product quality. J Pharm Sci 2009; 98:1201-5.

17. Tyagi AK, Randolph TW, Dong A, Maloney KM, Hitscherich C, Carpenter JF. IgG particle formation during filling pump operation: a case study of heterogeneous nucleation on stainless steel nanoparticles. J Pharm Sci 2009; 98:94-104.
On the final day of the meeting, technology options and analysis using emerging, disruptive technologies [session chairs: Gordon Moore (Centocor), Brian Kelly (Genentech), and Jim Swartz (Stanford University)], functional interaction, integration and synergy in platform development [session chairs: Brendon Hughes (Wyeth) and Patricia Alred (Centocor)] and rapid, economic purification process development [session chairs: Jorg Thommes (Biogen Idec) and Paul Mensah (Pfizer)] were discussed.

The implementation of compact, flexible and modular biomanufacturing technologies using disposable technologies was presented by Sadettin Ozturk (Centocor, Johnson & Johnson). He first noted that only a handful of current commercial products had demands in excess of 1,000 kg. These include adalimumab, manufactured by Abbott at 2,000 kg/yr and bevacizumab, manufactured by Genentech at 2,700 kg/yr. Trastuzumab (Genentech), rituximab (Genentech/Biogen Idec) and infliximab (Johnson & Johnson) all had demands close to 1,000 kg/yr.

Significant advances in cell line engineering and cell culture process development, including media development and feeding strategies have occurred in the last 20 years. Product titers in process development have increased from about 0.5 g/L to 10–13 g/L, as reported by Wyeth and Biogen Idec in conference presentations. In addition, companies such as Percivia and Artelis SA have reported bioreactor specific titers as high as 27 g/L (PER. C6®) and 31 g/L (CHO) using perfusion based systems. The average commercial titer in 1990 was about 240 mg/L, whereas today the range is typically 2–4 g/L. Much of these increases can be attributed to increases in the integral of viable cell concentration (IVCC) from an average of 14 x 10^6 in 1990 to 126x10^6 cells/mL/day, whereas the cell specific productivity (Qp) has not changed dramatically and average reported values are 19–26 pg/cell/day.

In addition to these advances, the availability of disposable bioreactors up to a 2,000 L scale from companies such as GEHealthcare (500 L), Hyclone (2,000 L) and Xcellerex (2,000 L) has enabled the widespread use of disposable bioreactors even for clinical manufacturing. Dr. Ozturk presented data on culture growth, titer and product quality (peptide map and glycosylation profiles) using a 1,000 L Hyclone single use bioreactor (SUB), and demonstrated scalability compared to a 30 L stainless steel bioreactor. He made a strong case for the feasibility of using 1,000 L scale SUBs to meet commercial product demands. Assuming 6 x 1,000 L SUBs per suite, a 300 day operation and 70% overall purification yield, he showed that coupled with optimized titers, a product demand of 1,000 kg/yr could be met with 20 batches/yr, or just 3–4 perfusion batches, with cost of goods (COG) reduced from over $300/g to about $100/g product. In addition to the options available for upstream processing, several purification technologies are emerging that should facilitate the use of fully disposable suites. Such facilities not only have the advantage of a smaller footprint compared to traditional stainless steel plants (e.g. SIP and CIP piping) but also offer benefits of modularity, flexibility and reduced build-out time.

In summary, Dr. Ozturk noted that disposable bioreactor platforms when combined with high efficiency processes and downstream disposable technologies can transform the manufacturing paradigm, and he could foresee future manufacturing needs being met by more modular, compact and cost effective manufacturing facilities using disposable technologies.

Brian Hubbard (Amgen) reviewed future directions in purification technology and the drivers for their introduction in commercial monoclonal antibody (mAb) processes. Recent increases in mammalian cell culture titers present significant challenges to downstream manufacture of biologics. The increase in product mass that results often exceeds the design basis of existing manufacturing facilities. Using a Monte Carlo simulation, Dr Hubbard systematically analyzed and addressed the bottlenecks for each unit operation and outlined filtration area for clarification, viral and ultrafiltration (UF)/diafiltration (DF), product pool volumes and buffer volumes as typical bottlenecks in existing plants when processing cell culture feedstocks up to 10 g/L.

High cell mass that enables higher titers can result in decreased centrifugation performance, which in turn can lead to decreased depth filter performance that may not be sustainable below 100 L/m². Alternative strategies to improve harvest performance (e.g., increased depth filter throughput) include the use of floculants2 such as poly(ethylenimine), chitosan, calcium chloride or low pH to improve removal of contaminants during centrifugation. Although significant advances have been made in the mass capture step using Protein A in terms of increased binding capacity, base-stable protein A, improved hydrodynamics and excellent COG value (direct cost approximately $2/g),1 further increases in Protein A capacity are needed to overcome pool volume limitations. In collaboration with vendors, several next generation Protein A resins with significantly higher binding capacities (>55 g/L target operating capacity) are in early development.

In the near term, some process improvements to reduce pool volumes include loading resin closer to breakthrough capacity and optimizing elution conditions and peak collection criteria. Pool volume limitations can also occur at each unit operation, and some of the mitigation options can include increasing column loadings, use of steeper gradients, minimizing load dilution for conditioning, and optimized peak collection. Continuous or tandem processing also offers significant benefits in terms of eliminating pool tanks, reducing processing time and the potential to increase facility throughput, and reducing fixed COGs. However, this has to be balanced against considerations such as increased automation, virus validation process validation and robustness requirements.

Regarding polishing steps, a two step process as adopted by some companies, including Wyeth and Amgen, offers benefits in terms of...
reduced processing time and buffer volumes as well as process tank limitations. Again, these considerations have to be balanced against virus validation requirements and process robustness particularly in removal of high molecular weight (HMW) species. Dr. Hubbard described efforts in high throughput screening (HTS) of multimodal anion exchange (MMA) libraries to select resins offering enhanced selectivity to remove high molecular weight species using several mAbs. Non-product binding membrane adsorbers with high membrane permeabilities and minimal mass transfer limitations can also be used for this purpose.

Another facility bottleneck can be the filter area required for virus clearance, which is also a major component of downstream COGs. Issues can be addressed by installing more filter housings, batch splitting and connected processing. Other strategies to increase viral clearance and throughput can include the use of virus preparations of higher purity in virus spiking studies for filterability and the use of next generation filters (e.g., Vpro from Millipore) with improved throughput. Options for removing UF constraints can include increasing membrane loading, purchasing a larger UF skid and performing multiple UF cycles. Recently, a novel single pass – tangential flow filtration (SP-TFF) process that offers an “in-line” 30-fold concentration of mAbs has also been reported by Pall Life Sciences as a potential solution to intermediate tank limitations.

Finally, Dr. Hubbard discussed some new directions in purification technologies, including use of non-chromatographic methods such as precipitation as a replacement for Protein A and crystallization as a polishing step. The latter is simple, inexpensive and can be operated under safe operating conditions and has been used successfully with several mAbs and Fc fusion proteins (dissolution rates to >50 g/L, product quality maintained), with yields >90% achievable in less than one day in some cases. In the near term, facility limitations can be overcome by a combination of operational or engineering approaches and process improvements (current technology or those easily implemented in existing facilities). Chromatography and filtration are not technology limiting up to the 10 ton scale. Longer term advances in expanded bed adsorption and simultaneous multi-column chromatography systems (SMCC), new resins for Protein A and polishing steps may offer benefits. The drivers for change include increased plant throughput, process productivity and facility utilization to enable more product mass per unit time and a reduction in COGs.

Have state-of-the-art mAb production processes already succeeded in disrupting our industry? This was the key question that Brian Kelley (Genentech) posed in his thought-provoking presentation. Twelve years ago there was only one licensed recombinant mAb (rituximab), novel processes yielded low titers and capacity concerns led to the development of alternate hosts and installation of large (12,000L) productions tanks. Today, over 20 mAbs are approved, many plants can produce multiple products (25kL max) and all marketed mAbs as well as Fc fusion proteins are derived from mammalian cell cultures with 2–5 g/L titers.

Production batch sizes utilizing platform processes range from 50–100 kg and capacity concerns have been addressed by the installation of tanks up 25,000L (total two million L in 2006, with projections of four million L in 2013). Upstream and downstream processes have converged on common platforms employing up to >12kL fed-batch and downstream unit operations. A single cycle of cell line development using mammalian hosts is a common goal for many companies; cell culture processes typically generate 2–5 g/L and gene to investigational new drug (IND) application filing timelines are converging. The downstream process, typically capable of processing 50–100 kg batches has also evolved to common unit operations encompassing centrifugation, depth filtration, two or three column processing, viral filtration and UF/DF. It appears that mAb drug substance production processes have become industrialized, and converged to a common process flow-sheet employing mature and low-risk technology.

Using facility fit modeling tools, Dr. Kelley demonstrated that different purification bottlenecks (e.g., buffer concentration and volume, pool volumes, harvest filter areas, number of chromatographic cycles, viral filtration duration, UF/DF recycle tank volume, bulk storage) exist for legacy products for different plant capabilities within Genentech’s manufacturing network. Many of these bottlenecks can be addressed by optimizing the process across sites, which may require different operating targets. Quality by design (QbD) concepts can be helpful in moving away from a single point target for key parameters and toward a range that the sites can select to optimize with their equipment or operations. For example, a 25,000 L bioreactor yielding a 5 g/L titer generates a batch size of approximately 100 kg. Most modern facilities are capable of handling such batch sizes with some process optimization and little technology innovation, effectively meaning there is no purification bottleneck.

Based on a cell culture titer of 2g/L, the total worldwide capacity translates into the ability to manufacture 65 and 130 tons/year in 2006 and 2013 respectively. The global commercial demand is estimated at seven tons/year in 2009 (with a mean mAb demand of 167 kg/year) and the 2012 unadjusted demand is approximately 25 tons/year. Capacity limitations are very unlikely for many years. In addition, the commercial demands of many products (>50) is estimated to be less than 50 kg/year. An analysis of industry-wide bulk requirements of commercial and clinical pipeline candidates taking into account target indication, likely dosage, expected market penetration and likely productivity estimate, the capacity utilization in 2013 will only be 65% (utilizing 2.7 million liters out of the four million available).

In terms of drug substance COGs, a reasonable estimate is approximately $100/g, with raw materials representing about $10/g. With a minimum sale price of $2,000/g for commercial mAbs and Fc-fusion proteins, and a median price of $8,000/g, COGs is not a substantial percentage of sales.

Dr. Kelley then posed the question: “What are the drivers for higher titer processes or kg/batch?” It does not appear to be driven by capacity or COGs. He wondered whether a competitive advantage would be produced even if a “biosimilar factory of the future” reduced COGs substantially (even to $0/g for arguments sake). Production technology, capacity and COGs do not appear likely to determine the outcome of competition with biosimilars. Currently, there are over 20 commercial processes that are
operating worldwide with relatively similar unit operation platforms and equipment. Introducing new process technology, even when justified in existing plants, can be difficult because of the requirement of capital expenses, plant shutdowns and more complex scheduling and should be considered perhaps for the highest volume demand products. Proposed changes must be enabling, not marginally better.

The maturing production technologies are efficient, portable, and low-cost, and allow predictable development, scale-up, technology transfer and production with acceptable product quality and overall low risks. In other words, mAb production processes have become industrialized like other commodities such as antibiotics. How should biological engineers and scientists respond to this situation? Dr. Kelley suggested consideration of a strategic shift in technology development efforts away from enabling high titers and debottlenecking toward development of new tools or databases and fundamental understanding of processes. Such technologies could include high-throughput techniques to study clone selection, media development, design space, facility fit analysis and manufacturability assessments, development of metabolic flux models and -omics tools.

**Tom Ransohoff (Bioprocess Technology Consultants)** brought a different perspective to the need for development and implementation of new technologies in bioprocesses, which he suggested was critical for continued improvement of product quality, reduction of development and production costs and acceleration of process development. The obstacles for introduction of new technologies are high, especially when existing platform processes meet requirements, and include technical, regulatory (comparability) and business (cost of change) hurdles. He also suggested that even if current processes meet today’s needs, improvement opportunities still exist and that the external environment will continue to create driving forces for change.

The drivers for innovation include rising healthcare costs, of which biopharmaceuticals are a rising share, emergence of biosimilars and follow-on biologics, changing regulations and standards, competition from lower-cost geographical regions, pressure to improve time-to-market and return on capital (reduce or defer capital and increased speed of deployment) and improved product knowledge. Other factors that may warrant innovation include efforts to improve product quality or safety, process simplification or robustness and increased production throughput or capacity.

Several case studies were presented to illustrate how process innovation has been used in bioprocess development. An example is the evolution of Protein A use in downstream purification processes, which has grown by more than 5-fold over the past decade, mirroring the growth of monoclonal antibody and Fc-fusion therapeutics. Innovations over the past 20 years include: *Staphylococcus aureus* production complemented with recombinant protein with the availability of animal-derived free Protein A ligands; improvements in media and immobilization technologies that support higher pressure drop ins and flow rates and reduce leaching; the development of a novel alkali-tolerant ligand (GE Healthcare’s MahSelect SuRe ligand); and increased capacities of media up to 50 g/L. Another example mentioned was the selection of polypeptide sequences for protein purification from phage display libraries. In screening for ligands that bind to target with desired selectivity and other properties, it was shown that losses in load and wash were negligible with similar elution profiles, product quality attributes, impurity clearance compared to immunoaffinity media. A final case study presented involved use of disposable bioprocessing equipment, including SUBs and use of a disposable simulated moving bed (SMB) in downstream processing to replace large stainless steel facilities and conventional chromatographic unit operations.

Tom Ransohoff concluded that to enable implementation, new technologies must: provide a solution or significant and competitive improvement in an area of compelling need (e.g. decrease costs and increase product quality and capacity utilization). A clear path towards implementation is required and the technology should be scalable to production scale and have appropriate quality and regulatory support. Close collaboration and development is required between suppliers and end users, and demonstration of success with “early adopters” is critical to successful implementation of technology innovations. Companies that are most successful in this regard are those that think strategically and define critical needs, manage change effectively, empower individuals to take risks and budget resources for technology evaluation and feasibility assessment.

A key outcome from application of development platforms is a better balance between resource investment (time, full-time equivalent resource and facility utilization) and the probability of technical success. There are many company specific factors that influence a particular platform; however, most platforms have converged to a common set of activities and gene to IND timelines. **Timothy Charlebois (Wyeth)** mentioned that Wyeth’s platform process was originally conceived to eliminate barriers for clinical supply and to ensure that Chemistry, Manufacturing and Controls (CMC) activities remained off the critical path wherever possible. The platform is designed to balance the extent of investment and risk and permit continuity over product development and lifecycle. It is important to note that the timeline may not be the fastest possible to first-in-humans (FIH) administration. However, the platform must ensure predictability of process performance and product quality, emerging schedules must be achievable and sustainable, and the process and product must be capable of reasonably meeting clinical demand and worldwide regulatory requirements. The platform also makes use of experienced staff who are responsible for Phase 0 through Phase 3 drug substance and product development, and commercial process and product improvements.

All development functions and facilities are co-located at a single site. Wyeth’s platform has evolved over time by optimizing key technologies in bioprocess development, facilities and business processes. Like many other companies, Wyeth’s platform strategy is to have a cell line that has the capability to support a product launch. Dr. Charlebois presented data from nine molecules that showed that the timeline from gene to completion of the Certificate of Analysis (CoA) was 17–18 months on average. The platform activities also allow transparency and alignment.
between projects in terms of duration of resource utilization. The overall benefits of the platform include increased throughput and decreased development timelines for pipeline candidates, predictability and reliability for development activities, simplification and streamlining of process steps, operations and associated business processes, improved ability to manage implementation of innovation, new technology and transparency and a common understanding of the technology and operational approach to bioprocess development.

Wyeth has continued to work on improvements to the Phase I platform. For example, cell culture advances in media development and feeding strategies have led to a two-fold average increase in product titers across a number of early stage molecule candidates. Other advances being incorporated include future use of ClonePIX FL, which combines expression screening with clone selection, better understanding of the factors underlying genetic and epigenetic instability of cell lines that enable high titer cell culture processes and streamlined recovery processes.

With the success of Wyeth’s platform, new questions are being raised about how to enable changes in platform design criteria without causing clinical supply constraints, i.e., how to take advantage of opportunities for further acceleration and streamlining of development activities without risking downstream activities. These questions include: Is it possible to use a non-final clone for toxicology material production? Is it possible to eliminate the process confirmation/scale-up step prior to tox manufacture? Are there opportunities for streamlining the testing and disposition activities? Are quality systems flexible and suitable for clinical production? These considerations enable fast, simple and inexpensive clinical production without exquisite planning because asset utilization is modest and simple. Dr. Charlebois suggested that plants of the future were unlikely to be large stainless steel facilities but could be small, modular facilities employing disposables. Such facilities could easily be used for the production of GMP grade clinical material at relatively small scales (250 L).

**Victor Fung (Amgen)** reviewed the company’s platform process for CMC development, which is optimized for efficiency and speed to FIH. The process is a modality-specific, generic manufacturing processes for toxicology and clinical candidate production that enables consistent and predictable early clinical processes, reduced technology transfer burden, use of similar raw materials, minimal inventory, reduced testing and improved resource management. The platforms are a common starting point for commercial process development (CPD) and the two stages of development are linked and not duplicated. In addition, technology efforts focused on improving platforms are also actively pursued. Continuous improvements to the platform are also implemented by incorporating lessons learned from clinical experience and commercial process development on an annual basis.

Molecule assessment (MA) conducted by Amgen’s Research, Protein Science and Process, and Product Development groups allow the early assessment of product quality, stability and manufacturing when designing or selecting protein lead candidates for commercialization. This assessment involves deliberate design/selection of optimal lead molecules for commercialization from product conception, and emphasizes QbD concepts from project inception. For example, bioactivity of the product and process attributes are considered in lead molecule development and selection, and may require a compromise with manufacturability considerations (e.g., inadequate yields, presence of particles or coloration, high viscosity). Mr. Fung commented that prior to the implementation of MA, 45% of reported issues could have been identified via the process. MA spans activities from the identification and selection of lead molecule to the selection of the final cell line, and includes sequence analysis and engineering, upstream, downstream and formulation platform fits and biochemical analysis. Sequence analysis include identification of non-germline framework residues in the variable heavy and light chains of mAbs, analysis of hotspots in the complementarity determining region and implementation of recommended signal peptides to maximize expression and minimize product heterogeneity.

In terms of platform assessments, product from amplified pools in platform process are used to assess growth and productivity in order to obtain an early read on product heterogeneity, evaluation of yields, in-process pool quality and HMW levels and an assessment of product solubility, stability to freeze thaw and UF/DF processing to ensure appropriate dosage form and drug product shelf life. Biochemical assessments employ a variety of analytical methods (LC/MS, SE-HPLC, SDS PAGE & rCE-SDS) to characterize aggregates, dimers, isoforms, truncations and other post-translational modifications. Product stability is assessed by Near UV CD, FTIR, ANS binding, DSC and dynamic light scattering to identify candidates that may have tendency to unfold and aggregate. These assessments were illustrated in a case study of six molecule candidates that were evaluated as clinical candidates.

The platform processes also allowed Amgen to align five commercial manufacturing facilities, including several legacy facilities. Commercial process guidelines and unit operations platforms were used to define facility capabilities and standardized unit operations, respectively. These efforts created strong alignment between process technologies and facility capabilities and provided significant benefits to commercial operations by streamlining technology transfers, reducing plant reconfiguration costs and down-time, and by enabling the assessment of new technology implementation. Two examples were provided to illustrate this strategy: installation of increased depth filter capacity and changes in downstream buffers to meet site specific waste disposal requirements. In addition to all the benefits associated with platform processes previously mentioned, application of the process also helped to foster a culture of shared ownership, responsibility and connectivity between functional groups.

QbD is a systematic approach to development of biopharmaceuticals. It addresses the interaction of critical process parameters (CPPs) with critical quality attributes (CQAs) to assure robust and flexible manufacturing processes. Patricia Alred (Centocor, Johnson & Johnson) mentioned that QbD is important, but involves a significant amount of upfront investment to achieve the required information for design space. If a product is approved, then the payoff is high in terms of process robustness, yield, better understanding of CQAs and CPPs. However, if the product is not
approved then a significant loss occurs in terms of time and cost. Therefore, it seems appropriate to make a business case for where, when and how to apply QbD in order to minimize risk while maximizing product opportunities.

For high risk products with low probability of technical success (PTS), the approach suggested was to conduct less work upfront in early development and defer work on development of a design space until completion of proof of concept. Two case studies were presented highlighting these scenarios. In the first case, a mAb had a known Mechanism of Action (MoA) in a known indication, competitive products, easier identification of CQAs and links to clinical data. This candidate thus had a high PTS. In this case, extensive efforts in applying QbD for creation of a design space are worth the early investment, although an extensive development effort with rigorously identified CQAs, CPPs and alignment of all functional areas is required. The second case study involved a mAb studied in a novel indication with high unmet need, and time to market was critical. It was difficult to link CQAs to clinical data and analytical characterization was relied upon instead. Overall, the PTS for this molecule was <30%. In this case, the early definition of design space is probably not warranted. Dr. Alred described detailed CMC activities for this molecule, and emphasized the need for a robust platform, alignment of functional activities allowing management of risk and meeting timelines. In addition, appropriate risk mitigation strategies also need to be developed for the process and product (e.g. clone stability, process scalability, product quality, solubility and stability). Dr. Alred concluded by suggesting that each product is unique and the analysis for when and how to apply QbD concepts should consider the appropriate drivers such as desirability of speed to market, the extent of knowledge of the mechanism of action and cost and flexibility of manufacturing operations.

The final session of the meeting began with a presentation by Sam Guhan and Oliver Kaltenbrunner (Amgen). In response to the perceived cell culture industrial capacity deficit, largely driven by low culture titers, significant capital investments were made to build new plants in the last decade (over 1.1 million liter capacity has been added since 20006,7). These plants were designed for low cell culture processes. However, significant advances in process development have led to titers as high as 10–13 g/L in fed-batch cultures. A plant capacity of 100,000 L can generate up to 13 ton product/yr (with 5–10 g/L titer, 70% overall yield, 95% fill/finish yield and 20 bioreactor turnaround/yr), resulting in a potential purification bottleneck. High asset utilization is critical for optimum use of facilities, and to fully realize the economic benefits of higher titers. There are also other factors to consider, including supply chain ease, risk, tax implications and inventory strategy. Manufacturing costs include both fixed (labor, depreciation, property taxes, insurance) and variable costs (upstream and downstream raw materials). Higher titer cell culture processes can reduce the relative cost of upstream processes but, has a negligible impact on downstream costs.

Dr. Guhan used four scenarios to highlight different approaches for managing a 5-fold titer increase. These scenarios were (1) construction of a new flexible facility using smaller bioreactors, (2) not utilizing the full capacity of existing facility, (3) using the full cell culture capacity, but for fewer lots/yr and (4) the use of a multi-product facility using full cell culture capacity and optimized purification process. These different approaches can generate cost savings in the range of 7–31%. Greater savings can be achieved by running more products in a multi-product facility. However, this can also result in increased risk of failure with a small number of lots, potential for additional savings if excess plant capacity could be outsourced or sold, potential increased stress to supply chain. There is also a need to ensure that all products adhere to the platform to ensure standardization and minimize changeover costs. Using a similar approach (Monte Carlo simulation) to that described earlier by Dr. Hubbard, Dr. Guhan included filtration area (clarification, viral, UF/DF), product pool volumes and buffer volumes as typical bottlenecks in existing plants when processing cell culture feedstocks up to 10 g/L. Potential strategies to overcome these bottlenecks include investment in existing facilities to achieve multi-product manufacturing, investigation of good engineering approaches to increase plant utilization and increase throughput (e.g. continuous processing), investment in systems for efficient multi-product manufacturing (cleaning technology, faster change-overs, automation and platform alignment) and continued investment in process improvements and new technologies to maximize plant throughput. While scientifically challenging and exciting, implementation of new technologies is accompanied by significant risk and cost. Current mAb processes are quite robust and capable of delivering high productivities and introduction of new technologies requires a good understanding of costs and benefits.

Quality by design directives have placed a greater emphasis on the creation of a manufacturing design space, which when filed and approved by the regulatory authorities, allows manufacturing flexibility within the design space without additional regulatory approval. Purification process design space can be defined using a statistical design-of-experiment (DOE)-based approach to establish input parameters ranges that assure process consistency and product quality. Experiments based on the analysis of separate unit operations have a disadvantage in that performance of a single unit operation may depend on the quality attributes of the preceding step. Lynn Conley (Biogen Idec) suggested that one way to address this challenge is to forward-link the output quality attributes from the previous unit operation experimental design as input parameters. However, this approach may have disadvantages due to the increased design complexity, number of variables and results interpretation. An alternative approach to forward-linking is the use of partitioned experimental designs, and since this incorporates sequential process steps in a single experimental design, interactions between unit operations can be investigated. Conceptually, this design is similar in approach to a traditional DOE. For both partition and traditional DOE designs a screening design is performed first to identify significant factors. Once significant factors are identified, a partition design across multiple process steps is constructed. This design is used to determine significant effects and interactions within and between process steps and to build a predictive empirical model.
In addition, this approach allows the determination of the impact of process parameters on quality attributes even if they cannot be directly measured.

Partition designs have only been described theoretically in the literature using simulated models. The purpose of the study Mr. Conley presented was to examine the feasibility of using this type of design to link three unit operations (harvest, protein-A and CEX) in a mAb purification process and assess its ability to identify significant factors affecting process performance. The product quality attribute that was followed through the partition design was host cell protein (HCP) because previous design space studies using traditional DOE with forward linking had demonstrated that HCP concentration from each preceding step in the process had a significant influence on the ability of the subsequent process step to remove HCP. Significant factors that affected HCP clearance were identified during screening studies and used in the partition design. These included harvest pH (4.5–7), protein-A wash conditions, elution velocity and load capacity, and CEX wash volume, wash pH and ionic strength and load capacity. Each partition was a full factorial design generating 16 experimental conditions per partition with four center points (20 partition 1: Harvest & Protein-A + partition 2: 20 CEX experiments + three harvest load pools generated) for total of 43 experiments. The overall design across both partitions was a 1/16, resolution IV fractional factorial design. The HCP response was compared to traditional DOE designs for Protein A and CEX unit operations for which a total of 77 experiments were performed (32 Protein-A + 36 CEX experiments + nine load pools generated). The partition design identified the same significant main factors and ranked their relative significance similarly compared to the traditional DOE approach. In addition, the partition design approach identified interactions between unit operations that could not be identified using the traditional DOE approach.

Christopher Gallo (Wyeth) described the development of a late-phase non-platform manufacturing process within a standard platform development timeline. He first described Wyeth’s platform process, which employs cell lines capable of supporting product launch and averages gene to IND timelines of about 17 months. He described use of automation technologies for rapid clone screening (300 clones/h, Protein A purification rate of 336 samples/2 h), two-column purification conditions (400 conditions/day) and formulation screening (solubility and stability). The case study he described involved in-licensed, early stage program in which the CMC development and drug substance (DS)/drug product (DP) clinical manufacturing had been subcontracted. This Phase I/II process resulted in average titers of approximately 0.45 g/L, a 50–60% aggregate level in the harvest cell culture fluid, 17% overall yield and 4.6% aggregate level in the final product.

Wyeth had no prior experience with this type of protein. Extensive comparability studies were required to support the proposed Phase III process changes, and greater risks were undertaken to meet project timelines. The goal was to develop a process with a 2-fold increase in titer and significantly streamlined downstream processing that would enhance product quality, improve process yield, reduce product- and process-related impurities, increase DS/DP concentration and optimize formulation. These goals under aggressive timelines were met by pursuing multiple development efforts in parallel, whilst applying platform development approaches where possible.

The best option found for the cell culture process was one that employed a Wyeth-derived cell line and culture medium. The total time from transfection to 190L pilot scale-up was about 5.5 months. The process was scaled to 6,000L with an average titer of 2.1 g/L for clinical manufacturing. Challenges involved in purification included reduction of HMW species, comparability of the Phase 1/2 to the Phase 3 material, which in turn required redevelopment of many release and characterization assays. Rapid purification development allowing identification of operating windows was enabled by early HTS of potential chromatography options. Using this approach and DOE, AEX operating conditions were identified for HCP removal. However, identification of operating conditions for AEX or CEX capable of significantly reducing aggregate levels proved to be more challenging. The HTS method was used to identify ceramic hydroxyapatite that could reduce HMW species and HCP levels to <2% and 100 ppm respectively.

The final Phase III process was capable of generating 2.1 g/L, with a simplified downstream process (three columns + four membrane unit operations, compared to ten unit operations for the original process), overall yield of approximately 28%, and HMW species of 0.8%. Overall, these efforts represented a 8-fold increase in productivity. A flexible, lyophilized dosage form and placebo were also developed. A comprehensive comparability program was used to demonstrate structural and functional equivalence of the Phase I/II and Phase III DS/DP. In summary, a complex manufacturing process with low productivity was replaced by a high yielding and efficient Wyeth process while keeping to similar platform development timelines.

Despite significant efforts to develop predictive tools, chromatographic purification processes of biopharmaceuticals are generally conducted using an empirical approach that involves large screening experiments. The first step in the development of ion exchange chromatography is selection of the loading pH, which is usually selected on the basis of the isoelectric point (pI) of a molecule. A protein is generally assumed to bind to anion- or cation-exchange resins at one pH unit higher or lower, respectively, of its pI. Tangir Ahamed (SynCo Bio Partners B.V.) noted that this assumption holds true for acidic and basic proteins, but not for neutral proteins. He mentioned that the rule-of-thumb is often misused because the actual binding behavior of proteins to ion exchange resins is determined by the titration curve (defined as net charge vs pH) rather than the molecule’s pI. Net charge of proteins varies very little in pH range 5.5–8.5, and therefore neutral proteins are not sufficiently charged to bind AEX at pH ≥ (pI+1) and CEX at pH ≤ (pI-1).

To illustrate the point, Dr. Ahamed used the example of a protein with a pI value of 7.3 that was unable to bind to S and SP-ligated cation exchangers at ≥pH 5.0 and Q-ligated anion exchangers at ≤pH 9.5. The net charge of the protein was found to be close to zero in the pH range 5.0–9.5. If the titration curve of the protein cannot be calculated accurately due to the unavailability
of its amino acid sequence or significant post-translational modifications (e.g., glycosylation, phosphorylation, cysteinylination, metal binding properties), then pH-gradient ion exchange chromatography can be a powerful tool for optimization of IEX parameters. Dr. Ahamed and colleagues observed that optimal pH loading in AEX and CEX chromatography was 0.3 pH units higher and lower than the elution pH of the product in pH-gradient AEX and CEX chromatography respectively.\(^9\) He concluded that proper use of rules-of-thumb accelerates downstream process development, and may not necessitate the need for high-throughput screening for IEX and CEX operating conditions.

The final presentation was delivered by Karol Lacki (GE Healthcare Bio-Sciences), who described the fast, efficient development and scale-up of a mAb purification process using high throughput process development (HTPD) and compared this process to traditional process development methods. The goals of the downstream process were to deliver a robust process with an overall yield $\geq 75\%$, monomer content $\geq 99\%$, HCP $\leq 10$ ppm and leached ligand $\leq 10$ ppm. One of the main challenges for this process was the high and varying dimer/aggregate levels of between 9–14%. Dr. Lacki described the use of a disposable wave bioreactor\(^\text{TM}\) for upstream processing and pre-packed 96-well format for screening (PreDictor\(^\text{TM}\)) using a Tecan Freedom 200. Analytical assays to support the HTS work included UV measurement for the determination of mAb concentration (96 samples in <5 minutes), size exclusion chromatography (SEC) for aggregates content and a Gyrolab Bioaffy\(^\text{TM}\). Immunobased HCP assay (112 samples in 50 minutes with a sample volume <10 μl). The method used two 3 mL Superdex\(^\text{TM}\) 200 5/150 GL columns in series and an autosampler for 96-well plates (15 minutes/sample).

Among interesting results it was shown that although the binding capacity measured in each well of a microtiter plates used in HTS studies will always represent a static situation, more sophisticated studies on protein uptake curves and on dynamic binding capacity can be done with the correct experimental set-up.\(^9\) Results of dynamic binding capacity (DBC) versus residence time using MabSelect SuRe\(^\text{TM}\) compared favorably to column-derived values. Although the binding capacity in each well will always represent a static situation, more sophisticated studies on protein uptake curves and on dynamic binding capacity can be done given the correct experimental set-up. HTS methods were also used to determine the wash conditions and monomer yield for MabSelect SuRe\(^\text{TM}\), as well as the load, elution conditions, yield and impurity levels using Capto adhere\(^\text{TM}\). The HTS methods allowed screening of nine chromatography media and a total of 1,482 conditions in 63 working days using 1 FTE. Small columns (HiScreen\(^\text{TM}\) and HiTrap\(^\text{TM}\)) using a DOE approach were used for verification, optimization and column performance comparison to the HTS results. The process developed using HTS was tested for robustness at the pilot scale and found to be acceptable. Dr. Lacki also noted that for pilot scale operations, pre-packed, pre-qualified and sanitized columns (ReadyToProcess\(^\text{TM}\) and AxiChrom\(^\text{TM}\)) can enable speed, flexibility, scalability and a greatly reduced change-over time.

References

1. Kelley B. Very large scale monoclonal antibody purification: the case for conventional unit operations. Biotechnol Prog 2008; 23:995-1008.
2. Riske F, Schroeder J, Belliveau J, Kang X, Kurzko J, Menon MK. The use of chitosan as a flocculant in mammalian cell culture dramatically improves clarification throughput without adversely impacting monoclonal antibody recovery. J Biotechnol 2007; 128:813-23.
3. Cell culture manufacturing capacity: trends and outlook through 2013. Bioprocess Technology Consultants, 2008.
4. Kelley B. Selection of polypeptide sequences for protein purification from phage display libraries. CHI Phage Display Conference, Cambridge, MA, Mar. 1999.
5. Kelley B, Blank GS, Lee A. Downstream processing of mAbs: current practices and future opportunities. In: Process Scale Purification of Antibodies, Ed: U. Gottschalk, 2009; 1–23.
6. Molowa DT, Mazanat R. The state of biopharmaceutical manufacturing. Biotechnol Annu Rev 2003; 9:285-302.
7. Farid SS. Process economics of industrial monoclonal antibody manufacture. J Chromatogr B Analyt Technol Biomed Life Sci 2007; 848:8-18.
8. Ahamed T, Chilamkurthi S, Nfor BK, Verhaert PD, van Dedem GW, van der Wielen LA, et al. Selection of pH-related parameters in ion-exchange chromatography using pH-gradient AEX and CEX chromatography respectively.8 He concluded that proper use of rules-of-thumb accelerates downstream process development, and may not necessitate the need for high-throughput screening for IEX and CEX operating conditions.

The final presentation was delivered by Karol Lacki (GE Healthcare Bio-Sciences), who described the fast, efficient development and scale-up of a mAb purification process using high throughput process development (HTPD) and compared this process to traditional process development methods. The goals of the downstream process were to deliver a robust process with an overall yield $\geq 75\%$, monomer content $\geq 99\%$, HCP $\leq 10$ ppm and leached ligand $\leq 10$ ppm. One of the main challenges for this process was the high and varying dimer/aggregate levels of between 9–14%. Dr. Lacki described the use of a disposable wave bioreactor\(^\text{TM}\) for upstream processing and pre-packed 96-well format for screening (PreDictor\(^\text{TM}\)) using a Tecan Freedom 200. Analytical assays to support the HTS work included UV measurement for the determination of mAb concentration (96 samples in <5 minutes), size exclusion chromatography (SEC) for aggregates content and a Gyrolab Bioaffy\(^\text{TM}\). Immunobased HCP assay (112 samples in 50 minutes with a sample volume <10 μl). The method used two 3 mL Superdex\(^\text{TM}\) 200 5/150 GL columns in series and an autosampler for 96-well plates (15 minutes/sample).

Among interesting results it was shown that although the binding capacity measured in each well of a microtiter plates used in HTS studies will always represent a static situation, more sophisticated studies on protein uptake curves and on dynamic binding capacity can be done with the correct experimental set-up.\(^9\) Results of dynamic binding capacity (DBC) versus residence time using MabSelect SuRe\(^\text{TM}\) compared favorably to column-derived values. Although the binding capacity in each well will always represent a static situation, more sophisticated studies on protein uptake curves and on dynamic binding capacity can be done given the correct experimental set-up. HTS methods were also used to determine the wash conditions and monomer yield for MabSelect SuRe\(^\text{TM}\), as well as the load, elution conditions, yield and impurity levels using Capto adhere\(^\text{TM}\). The HTS methods allowed screening of nine chromatography media and a total of 1,482 conditions in 63 working days using 1 FTE. Small columns (HiScreen\(^\text{TM}\) and HiTrap\(^\text{TM}\)) using a DOE approach were used for verification, optimization and column performance comparison to the HTS results. The process developed using HTS was tested for robustness at the pilot scale and found to be acceptable. Dr. Lacki also noted that for pilot scale operations, pre-packed, pre-qualified and sanitized columns (ReadyToProcess\(^\text{TM}\) and AxiChrom\(^\text{TM}\)) can enable speed, flexibility, scalability and a greatly reduced change-over time.