STARTING MATERIALS:

MAMMALIAN CELL CULTURE

BioProcess systems in vaccine production

High throughput capture with MabSelect

Fast Trak help to Phase II trials

Amersham Biosciences
In this issue

3 GlaxoSmithKline Biologicals takes delivery of its 50th BioProcess system
Close co-operation, an open working relationship and comprehensive support add up to 50 BioProcess systems at GSK Bio, Belgium.

4 Diabetes vaccine gets a FastTrak start to Phase II trials
Just five years ago, Fast Trak Purification Services assisted in the development of a chromatographic process for a protein intended as a vaccine against diabetes. The expert help received during those early stages has enabled this process to advance rapidly through pre-clinical trials, scale-up and on to a Phase II clinical trial.

6 Fast capture of MAb from large volumes with MabSelect
MabSelect is new protein A affinity medium with a novel base matrix that has high throughput capabilities. New fermenter volumes in the order of 10 000 litres or more can be easily processed in a working day.

9 Starting materials and downstream processing
Starting materials play a crucial role in the downstream processing strategy for biomolecules intended for therapeutic use. Their expression levels and complexity impact the number of purification steps, regulatory issues, and the economics of the process.

10 Industrial downstream processing of proteins expressed in mammalian cells
Mammalian cell culture is one of the main expression systems for the production of biopharmaceutical proteins. This article gives a general overview of the approaches to purifying protein from this starting material, with examples focused on monoclonal antibodies.

Meeting reports
19 The scalability of EBA for the capture of antibody fragments from E. coli.
21 Purification of insulin from crude feedstock by employing a novel ligand for specific adsorption.
23 Charge engineering of a protein domain to allow efficient ion-exchange recovery.

Forthcoming Meetings

The front cover shows a cartoon representation of an intact IgG molecule. Structural data were obtained from the Protein Data Bank entry ligt. [Harris, L. J., et al. Nature 360, 369 (1992)]

Correction: An error occurred on page 16 of Downstream 32. Under Cell culture, the product titre should read 500 mg/litre.

BioProcess, BPG, CHROMAFL low, Downstream, Fast Trak, FineLine, HiTrap, HPrev, INDEX, MabSelect, PhastGel, RESO URCE, Sephrose, SOURCE, STREAMLINE, UNICORN are trademarks of Amersham Biosciences Limited or its subsidiaries.

Amersham is a trademark of Nycomed Amersham plc.

Cytopilot is a trademark of Vogelbusch GmbH.

DiAmy is a trademark of DiAmyTherapeutics AB. Synagis is a trademark of MedImmune Inc.

Enbrel is a trademark of Immunex Corp.

Rituxan is a trademark of IDEC Pharmaceuticals.

Enbrel is a trademark of Immunex Corp.

Synagis is a trademark of MedImmune Inc.

Diamyd is a trademark of Diamyd Therapeutics AB.

Phage Display is owned by Dyax Corp. and is covered by US Patent Nos: 5,213,683 and 5,282,973 and foreign equivalents.

STREAMLINE is covered by US Patent 5,522,993 and foreign equivalents.

Amersham Biosciences AB
Björkgatan 30, SE-751 84 Uppsala Sweden

Amersham Biosciences UK Limited
Amersham Place, Little Chalfont, Bucks, England HP7 9NA

Amersham Biosciences Inc
800 Centennial Avenue, Piscataway, New Jersey 08855, USA

Amersham Biosciences AB
Postfach D-79021 Freiburg Germany

Amersham Biosciences K.K.
Sakuen Building, 3-25-1, Hiyakunincho, Shinjuku-ku, Tokyo 169-0073, Japan

All goods and services are sold subject to the terms and conditions of sale of the company within the Amersham Biosciences group which supplies them. A copy of these terms and conditions is available on request.

The views expressed by contributors and correspondents are their own and do not necessarily reflect the views of Amersham Biosciences Limited.

© Amersham Biosciences UK Limited 2001 – All rights reserved

008
Of over 800 BioProcess™ systems delivered, 50 have gone to GlaxoSmithKline Biologics (GSK Bio) in Belgium. To commemorate this, Arne Forsell, deputy CEO, and Peter Ehrenheim, vice president Separations, presented a gift to Josée Wille, the production manager of a new vaccine unit where the 50th system will reside and operate.

**Service and support**

The group from the vaccine unit was in Uppsala in the autumn of 2000 to perform factory acceptance testing on the new systems before shipment to Belgium. There the systems will be successively brought into operation after installation and validation procedures have been carried out. “This time we hope to be able to do a lot of the installation work ourselves”, said Josée. “After so many systems and so much support from Amersham Biosciences, we are well-trained”. Josée feels that the service and support received is one of the main reasons for the long-standing co-operation between the two companies. “The systems are very reliable, but when we do need assistance, help is just around the corner. And because we have so many systems, we have our own store of spare parts for routine maintenance and service, as well as access to a dedicated engineer from Amersham Biosciences”, she added.

**Open communication**

This close co-operation between the two companies began in the early nineties when GSK Bio was looking for a reliable supplier of chromatography systems for its vaccine production processes. GSK Bio had purchased some of the first generation systems from Amersham Biosciences in the mid-eighties, about the time that Josée Wille joined the company, and was now looking to modernize some of its hardware. The company opted for the BioProcess system concept. Josée is not surprised that so many of these systems have been installed over the last five years. “It’s a reflection of the professional, open communication between the two companies, and the personal working relationships that have developed”.

The majority of the 50 BioProcess systems, all UNICORN™ software controlled, are in operation in FDA-approved vaccine production processes, the others are in process development or scaling up facilities.

GlaxoSmithKline Biologics specializes in the research, development, production and commercialization of human vaccines, and is one of the world leaders in this field, both in volume (number of doses) and in value (sales). In 1999, the company delivered 770 million vaccine doses to people in 162 countries. In order to ensure its leadership in vaccinology, GSK Bio is investing considerable funds in research and development, and in new facilities and sophisticated equipment. Company headquarters are in Rixensart, Belgium where over 2 400 people are employed.
Diabetes vaccine gets a Fast Trak start to Phase II trials

In 1996, Downstream™ reported how a small start-up biotech company, then called Synectics Biotechnology AB, Sweden, had approached Fast Trak™ Process Development Services for help with defining a scalable chromatographic purification scheme for a recombinant human protein. The company, established in 1994, was in the early stages of developing a vaccine against Type 1 (or insulin-dependent, or autoimmune) diabetes mellitus. (Downstream 22, 18-1118-56).

The company is today called Diamyd Therapeutics AB and is starting a Phase II clinical trial in Sweden with its vaccine, Diamyd™. The vaccine is an isoform of the GAD (glutamic acid decarboxylase) protein, GAD65, and is expressed in cultured insect cells. Over the last five years it has been discovered that 10% of individuals diagnosed with Type 2 diabetes have a latent form of the autoimmune Type 1 disease that destroys insulin producing beta cells. It is this group of patients, Latent Autoimmune Diabetes in the Adult (LADA), on which the Phase II trial will focus.

Baculovirus expression
Prevention of autoimmune diabetes by GAD65 administration to a special breed of mice prone to the disease was reported in Nature (1,2) in 1993 by two independent groups of scientists in North America. Not long after these reports, Diamyd Therapeutics secured the exclusive rights to the cDNA sequence for hGAD65, and then re-cloned the gene to obtain a proprietary clone. This clone is used to produce rhGAD65 in a baculovirus expression system (BVES) infected with an insect cell line. The BVES was chosen because it was capable of producing the enzymatically active and immunologically native rhGAD65 protein. Although insect cell expression is among the less frequently used systems for biopharmaceuticals, it does offer several advantages, including possible secretion of product, mimicking posttranslational modifications of mammalian cells, high expression levels of product, as well as reducing the risk of viral contamination normally associated with mammalian systems.

Fast Trak - fast start
The initial downstream recovery strategy developed by Diamyd Therapeutics gave promising results, but required developing with regard to further purification and scale up; that's where Fast Trak stepped in. "We really needed specialist input at that critical time. Because we selectively outsource all tasks for our product development, we found it easy to collaborate with Fast Trak. The transfer of the technology proceeded very smoothly, and the group was very quick to adapt to our needs", confirmed Dr John Robertson, Director of R&D at Diamyd Therapeutics. "We turned to Amersham Biosciences because it is known for its expertise in chromatographic methods. We wouldn't have reached this stage so quickly without the help of Fast Trak. Their understanding of the complexity of this type of manufacture, their expertise in large-scale chromatography and their flexibility were instrumental in putting us in the fast lane" added John Robertson. The collaboration defined a commercially scalable manufacturing process with an overall product recovery of 80% and a purity meeting the requirements for a potential clinical vaccine.

Outsourcing - a key
Dr Robertson described how the 'virtual' company of Diamyd has thrived on outsourcing. "Selective outsourcing has been the key to our development strategy partly because it enables us to utilize input from experts in different fields at every step in development, wherever they may be in the world. We turned to Fast Trak for manufacturing because of their established expertise in downstream purification and process scale-up. After defining the process, we chose a contract manufacturer with expertise in the BVES for production of the gram quantities required for preclinical studies. We subsequently outsourced the Phase I clinical trial to a contract research organization in the UK, and now have production of material for Phase II at a contract manufacturer in North
America in facilities compliant with cGMP (current Good Manufacturing Practice). However, it is important to point out that when working in this kind of way, with different contract manufacturers and research organizations around the world, good communication and timing are critical. Each step must meet its goals and time schedules in order to move forward and to obtain necessary funding. What is really crucial is to invest well at the beginning, by selecting reliable and knowledgeable partners that will ensure that your manufacturing process is efficient, robust, and scalable. If you fail at this stage, you may not only lose time but also investors confidence and never get to clinical trials”.

Secure start
Since the involvement of Fast Trak the process has been fine-tuned (using Amersham Biosciences materials), scaled up by contract manufacturers, and made cGMP compliant. Further scale up from 50 litres to 500 litres will ensue over the next three years.

Over 300 million sufferers
Diabetes is today a major killer and large consumer of healthcare budgets around the world. From an estimated 30 million sufferers in 1985, the number had shot up to 135 million ten years later. Now WHO predicts a rise to an alarming 300 million by 2025. Finding a cure or prevention is a high priority in Europe, the US and Japan. To Diamyd Therapeutics and companies like them, we wish them all success.

How Diamyd works

Insulin-dependent diabetes, type 1 diabetes, usually begins in childhood or young adulthood and is thought to be hereditary or triggered by environmental factors such as viruses. It results from destruction of pancreatic insulin-producing beta cells by autoreactive T cells. When approximately 80% of these cells have been destroyed and insulin production is insufficient, diabetes presents itself, with lifelong dependency on exogenous insulin. For several rodent models of autoimmune diseases it has been possible to target autoreactive T cells therapeutically and block their pathogenic activity by using autoantigens themselves. In this way a selective “tolerization” of T cells is achieved, thereby avoiding the need for general immunosuppression. The therapeutic potential of GAD65 tolerization to prevent diabetes has been clearly demonstrated in the non-obese diabetic (NOD) mouse in which pathogenesis closely resembles that of the human disease. NOD mice spontaneously develop diabetes at approximately 20 weeks. The protective effect of GAD65 has been documented in several reports. An additional important finding was that administration of GAD65 well after the onset of beta cell destruction could interrupt cell killing and prevent the onset of insulin dependence.

References

Further reading
Fast capture of MAb from large volumes with MabSelect

MabSelect has been designed to meet customers' increasing needs for processing large volumes at high flow rates. The new cross-linking technique used in the agarose matrix has resulted in high throughput. Optimal performance is achieved when it is packed to a bed height of 20 cm. This can give a saving on investments in larger-diameter columns to process increased volumes.

Current developments in fermentation and cell culture techniques are leading to high expression levels of target molecules. At the same time fermenter volumes are increasing. Crude feedstock volumes of 10 000 litres and more will soon be the industry standard. Demands from biopharmaceutical manufacturers are putting pressure on downstream processes to handle larger volumes in a shorter times, and with improved product recoveries.

High productivity
Our new BioProcess™ affinity medium, MabSelect™, has been developed in collaboration with leading manufacturers of monoclonal antibodies (MAbs) to meet these kinds of challenges. It is characterized by very high throughput and high dynamic capacity at high flow rates, in other words high productivity. At flow velocities of 500 cm/h and a packed bed height of 20 cm, the dynamic capacity of MabSelect is typically 30 mg polyclonal antibody/ml medium. The combination of high speed, high capacity and relatively high bed height is designed to save you time and investment. Under optimized conditions, MabSelect can process fermenter volumes in the order of 10 000 litres in a single working day, in columns with inner diameters of 600–800 mm. In addition, there are few regulatory concerns with MabSelect because the ligand is produced and purified in the absence of mammalian derived products.

New matrix
The introduction of MabSelect is part of a programme to develop matrices for use in very large processes. The matrix of MabSelect is based on a novel technique that increases the rigidity of the agarose beads and the available pore volume, without increasing the matrix volume, which would normally be expected. This increase in rigidity and pore volume gives the matrix very high dynamic capacity at high flow velocities. The new matrix can typically handle large volumes at flow rates five times faster than conventional cross-linked agarose of a similar porosity.

Customer driven
The project to develop MabSelect was driven by customers requiring a robust and optimized, high throughput protein A affinity medium for the production of MAbs. Thanks to the concurrent engineering approach used in our pilot plant facilities, recommendations from customers could be quickly incorporated into the process at the same time that it was being scaled up. Such an approach reduces product development time. Now we have in place a validated production process for a high throughput protein A affinity medium that more than meets our customers' needs.

Enhanced binding
The MabSelect ligand is a recombinant protein A expressed in E. coli, fermented in animal-free soy media, and purified through multiple steps of chromatography. The protein A has been engineered to include a C-terminal cysteine, favouring an oriented thioether coupling. This provides a single point attachment of the protein A, see Figure 1; and enhances the available binding capacity for IgG, see Figure 2 where breakthrough curves of human IgG for different commercially available protein A matrices are compared. Coupling of the
protein A to the matrix is via epoxy chemistry and ensures low ligand leakage. The entire manufacturing process for MabSelect is validated and completely free of contact with products from animal or human sources. See Table 1 for characteristics of MabSelect.

Fig 1. C-terminal cysteine favours oriented thioether coupling.

Fig 2. Comparison of the dynamic capacities at 10% breakthrough (QB10%) at different flow rates for protein A affinity media based on high rigidity agarose (MabSelect, Amersham Biosciences), synthetic polymer (Supplier A) and porous glass (Supplier B). At 10% breakthrough (20 cm bed height, 500 cm/h flow velocity), binding capacity was ca 32 mg hIgG/ml bed volume for MabSelect, compared with 25 mg/ml for Supplier A, and ca 22 mg/ml for Supplier B.

Fig 3. Pressure/flow curves for MabSelect packed to 20 cm bed height in Chromaflow 400 (i.d. 400 mm), BPG 100 (i.d. 100 mm), and BPG 300 (i.d. 300 mm).

Optimal performance at 20 cm bed height
Following method optimization to establish the conditions that will bind the highest amount of target molecule, in the shortest time and with the highest product recovery, the process is scaled up. We recommend a bed height of 20 cm so that the high capacity of MabSelect can be used at high flow rates. Figure 3 shows the pressure/flow curves for MabSelect packed in three standard columns. MabSelect performs well in all our standard column ranges: Chromaflow™, BPG™, FineLINE™ and INdEX™.

Low ligand leakage
In pharmaceutical production processes any traces of protein A must be removed from the final product. Leakage of recombinant protein A from MabSelect is low, thanks to the epoxy-based coupling chemistry. Any trace amounts can be removed efficiently from the IgG-containing fraction using gel filtration or ion exchange chromatography. Typical leakage values found in the IgG-containing eluates after purification of a human polyclonal IgG and a MAb on MabSelect are shown in Figure 4. Leakage data for rProtein A Sepharose Fast Flow are included for comparison.

Table 1. Characteristics of MabSelect.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Composition</strong></td>
<td>highly cross-linked agarose</td>
</tr>
<tr>
<td><strong>Particle size</strong></td>
<td>40-130 µm (d50v ≈ 85 µm)</td>
</tr>
<tr>
<td><strong>Ligand</strong></td>
<td>recombinant protein A (E. coli)</td>
</tr>
<tr>
<td><strong>Coupling chemistry</strong></td>
<td>epoxy</td>
</tr>
<tr>
<td><strong>Binding capacity, dynamic</strong></td>
<td>typically greater than 30 mg human IgG/ml medium</td>
</tr>
<tr>
<td><strong>Chemical stability</strong></td>
<td>stable in all aqueous buffers commonly used in protein A chromatography: 100 mM H3PO4 (pH 1.6); 10 mM HCl (pH 2); 10 mM NaOH (pH 12); 1 M sodium citrate/HCl (pH 3); 6 M GuHCl; 20% ethanol; 2% benzyl alcohol</td>
</tr>
<tr>
<td><strong>Recommended pH working range</strong></td>
<td>3–10</td>
</tr>
<tr>
<td><strong>Cleaning-in-place</strong></td>
<td>2–12</td>
</tr>
<tr>
<td><strong>Recommended working flow velocity</strong></td>
<td>500 cm/h</td>
</tr>
<tr>
<td><strong>Temperature stability</strong></td>
<td>4–40 ºC</td>
</tr>
<tr>
<td><strong>Delivery conditions</strong></td>
<td>20% ethanol</td>
</tr>
</tbody>
</table>

1 d50v is the median particle size of the cumulative volume distribution.
2 Determined at 10% breakthrough by frontal analysis at a mobile phase flow velocity of 500 cm/h in a column with a bed height of 20 cm.
3 No significant change in chromatographic performance after 1 week storage or 100 cycles normal use at room temperature, which corresponds to a total contact time of 16.7 hours.
4 In BPG 300 column, bed height 20 cm, operating pressure <2 bar, 20 ºC, H2O as test solution.
5 Recommended long term storage conditions +4 to +8 ºC, 20% ethanol.

Fig 4. Leakage of rProtein A in the antibody eluate expressed in ppm during purification of a monoclonal antibody (Mab 1) and human polyclonal IgG (hIgG). MabSelect and rProtein A Sepharose Fast Flow were loaded to 25 mg of antibody per ml packed bed. MAb 1 was loaded in CHO cell culture supernatant (feed concentration 1 mg/ml) and hIgG was loaded in equilibration buffer. The eluate was collected in five column volumes of low pH buffer neutralized with Tris-buffer. Aliquots of the buffers, antibody samples and eluates were then analysed by ELISA for their rProtein A content. The only fractions that contained rProtein A were the eluates.
Cleaning and sanitization

MabSelect can easily be cleaned and sanitized. To remove precipitated or denatured substances we recommend a solution of guanidine hydrochloride, or 10 mM NaOH, or 100 mM H₃PO₄. To remove hydrophobically bound substances, use a solution of non-ionic detergent or ethanol. The medium can be sanitized with 0.1 M acetic acid/20% ethanol, or 2% hibitane digluconate/20% ethanol. See Data File or Instructions for details.

In clinical trials

Roche Diagnostics in Penzberg, Germany, is in Phase II clinical trials with a process for a MAb intended for therapeutic use within oncology. The company had been looking to modify its existing process with a higher capacity protein A medium. After extensive comparisons with other commercially available protein A media, Roche chose MabSelect because of its high capacity and stability, and the ease with which it is packed.

The target protein is an IgG₁ expressed in transfected hybridoma cells of B-lymphoid origin, grown using serum-free media. After filtration, cell-free supernatant is applied to a FineLINE column packed with MabSelect to a bed height of 20 cm. Sample load is approximately 30 mg/ml and flow velocity approximately 100 cm/h (flow velocity was limited only by the system in use). Following wash and elution, the eluate is immediately neutralized with potassium phosphate to increase the pH to between 6.8 and 7. Recovery was >90% and purity was >95% as determined by gel filtration, see Figure 5.

Prior to delivery, the packing method was verified in Uppsala, Sweden. A FineLINE 200 column (i.d. 200 mm) was packed to a bed height of 20 cm with MabSelect at 1 bar. The calculated plates/metre was approximately 8 000, corresponding to a reduced plate height of 1.5. This was an excellent result for a medium with this particle size distribution. The method was then transferred to the larger customized FineLINE column.

Tomorrow’s media today

The rigid base matrix, designed for handling large volumes at high flow velocities, is part of our developing platform for a new generation of high throughput media. MabSelect is just the first, and it’s ready to meet tomorrow’s challenges today!

For further information, please ask for the following Data File: MabSelect, Code no. 18-1149-94.

New products

HiTrap Benzamidine FF (high sub)

Ready to use 1 ml and 5 ml HiTrap columns are prepacked with Benzamidine Sepharose™ 4 Fast Flow (high sub) for the removal of proteolytic activity from a protein or peptide preparation, or purification of trypsin and trypsin-like serine proteases such as thrombin and enterokinase.

HiPrep 16/10 IEX FF columns

HiPrep™16/10 IEX FF (20 ml) columns are available prepacked with the following Sepharose 6 Fast Flow ion exchangers - Q, SP, DEAE, and CM, or with ANX Sepharose 4 Fast Flow (high sub). These columns provide fast, reliable, and reproducible preparative ion exchange separations.

HiPrep 16/10 Heparin FF

HiPrep 16/10 Heparin FF is a 20 ml column prepacked with Heparin Sepharose 6 Fast Flow for purification of restriction endonucleases, DNA binding proteins, plasma coagulation factors and other plasma proteins, protein synthesis initiation factors and related proteins, nuclease enzymes, and steroid receptors.

More details in Data File 18-1139-38.

More details in Data File 18-1143-22.

More details in Data File 18-1142-98.
Starting materials and downstream processing

Successful isolation and purification of a target molecule from its source material is a complex task tackled by a well-defined downstream processing strategy. The strategy developed will depend chiefly upon the intended use of the molecule, i.e., its final purity requirements as a therapeutic, diagnostic or enzyme, and on the characteristics of the source material. The source material will also influence validation requirements and the overall economy of the process.

Although several proteins like albumin and immunoglobulins are still obtained from natural sources - human tissue or body fluids - most intended for biopharmaceutical use are produced via recombinant technologies from yeasts, bacteria and mammalian cell cultures. (There are currently some biopharmaceuticals in clinical trials produced from insect cells, transgenic animals and plants). The system chosen to produce the proteins will determine not only the quality and quantity of the product expressed, but also the type and quantity of contaminants that have to be dealt with. For example, if the target molecule is expressed intracellularly in E. coli, it may be located in inclusion bodies. Disruption of the E. coli cells may release proteases. If the product is secreted into the periplasm, the levels of endotoxins may be high. On the other hand, if the protein is a product of mammalian cell culture systems, then adventitious viruses might be present. Host cell proteins, additives, and leached compounds are contaminants common to many systems.

The downstream processing strategy must be carefully designed to remove all contaminants. The degree of complexity and number of steps required to recover a target molecule will directly impact the economics of the downstream process. Each potential expression system must therefore be evaluated for its ability to produce economically the maximum amount of biologically active product and assessed for its impact on validation and regulatory requirements.

The most suitable starting material or expression system for a particular product is therefore dependent on several variables: the end use and value of the final product, the economic constraints of the downstream process and regulatory requirements.

In this issue, Downstream™ looks at mammalian cell culture as an expression system and starting material for the production of recombinant proteins, particularly monoclonal antibodies, and will consider some of the typical approaches to downstream processing of these antibodies.

**Downstream processing strategy**

A typical downstream strategy consists of two or more stages, each stage consisting of one or more steps.

**Capture** is the recovery of the target molecule from crude or clarified feed, and initial purification. Here the product is concentrated, (water being the main ‘contaminant’) and transferred to an environment which conserves potency/activity. Significant purification from soluble contaminants is the main goal.

**Intermediate Purification** consists of one or several steps and may be necessary to remove remaining bulk impurities and critical contaminants such as DNA, viruses and endotoxins to achieve safety of the product. Depending on the properties of the starting material and the efficiency of the capture step, some processes may omit this stage.

**Polishing** removes trace contaminants and any remaining specific impurities, thereby delivering the product at the required purity and safety levels.

The details of this three-phase strategy will depend upon many factors: the characteristics of the starting material, the final product purity requirements, the economic framework of the process, etc. Current developments in cell culture, molecular engineering and purification techniques, as well as pressures to get product to market quickly, are tending to reduce the number of steps in the downstream process.
Expression of protein with the correct folding and posttranslational modifications is a major advantage of mammalian cell culture systems. These inherent characteristics can now be better exploited for the large-scale production of complex proteins due to recent advances in molecular engineering and cell culture techniques. The renewed interest in MAbs is a case in point. In this article, Downstream gives an overview of the route from expressed protein to final product and some of the considerations along the way.

Protein expression

Culture systems

Mammalian cells are grown in two types of culture systems, in suspension or attached to a surface or support. The chosen system will determine the reactor configuration. Suspension cells are grown in hollow fibre reactors, fluidized bed reactors like Cytopilot™ and stirred-tank reactors. Anchorage-dependent cells are grown in hollow fibre reactors and fluidized bed reactors. Cells vary in their susceptibility to mechanical forces, so the reactor type should take into account the fragility of the cells. Stirrers, impellers, spargers, etc. must not cause stress and damage to cells as this can result in protease release. Proper nutrient and oxygen supplies are critical for growth, any variations can cause cell death.

Culture media

The culture medium in which cells grow is a complex mixture of proteins, polysaccharides, lipids, steroids, vitamins, salts, amino acids, phenol red and growth-factors and may contain viruses. If serum supplements are added they may introduce contaminants such as prions (transmissible spongiform encephalopathy), which will need removing. Traditionally, approximately 10% foetal calf serum was added to the culture medium to promote cell growth. Today, serum-free media supplements are more typical, with the trend towards protein-free media to increase safety. These kinds of supplements are manufactured for certain cell lines, providing just the right nutrients the cell line needs. This ensures that the growth medium is well defined in composition, facilitating the subsequent downstream purification.

Industrial downstream processing of proteins expressed in mammalian cells

The preferred system for the production of recombinant eukaryotic proteins for biopharmaceutical use is mammalian cell culture. Its major benefit is the expression of the desired protein, even large, complex proteins like Factor VIII, directly into the culture medium with the correct folding and posttranslational modifications for biological activity. For high value products, this benefit easily offsets the relatively high fermentation costs, slow growth, relatively low expression levels, and potential risks for viral contamination. Expression levels will depend on product type, cell type, culture conditions and fermentation method, but they are typically in the range of tens to hundreds of milligram product per litre of culture per day. (For monoclonal antibodies (MAbs) from Chinese Hamster Ovary (CHO) cells expression levels can reach 1–2 g/l.)

Mammalian cell cultures are today being used for the expression of biopharmaceuticals such as tissue plasminogen activator (tPA) — the first drug to be produced commercially by mammalian cell culture — and MAbs for therapeutic use. The potential of MAbs as therapeutic antibodies, once termed “magic bullets”, was recognized 25 years ago, but the stumbling block to widespread use has been their immunogenicity. The renewed focus on MAbs as biopharmaceuticals and target-specific drugs for cancer and other diseases is partly due to advances in molecular engineering to prevent immune response in patients.

The most commonly used cell line for expression of biopharmaceutical protein is CHO cells. These cells multiply quickly, are relatively hardy, and grow well in culture. Genetically engineered CHO cells are currently used for production of Factor VII and Factor VIII. Other epithelial cell lines used for recombinant proteins include Baby Hamster Kidney (BHK), African Green Monkey (COS and CV-1) and hybridoma cells.
Downstream of the fermenter
At industrial scale it is common practice to express the native protein without any attached fusion protein or tag so as not to interfere with the intracellular in vivo folding of the protein. (Proteins are sometimes expressed with an IgG Fc region, but this is for reasons of functionality). As a result, no generic capture procedures have emerged and different purification approaches have been reported for different proteins. Below follows a brief description of the chromatographic steps that can be used for MAbs - a major product group of mammalian cells.

Considerations for purifying MAbs
Most monoclonal IgGs are stable and soluble at medium to high pH and low conductivity, a condition often used during capture. However, some MAbs act as cryoglobulins with reduced solubility below 37°C. Strongly basic MAbs form stable ionic complexes with polyvalent anions (phosphate, citrate, sulphate, borate) resulting in aggregation. Buffers should always be selected with care. MAbs also complex with nucleic acids; the reaction can be reversed in presence of 0.3-1.0 M NaCl. The resulting change in the net charge of the molecule leads to destabilization. If foetal calf serum has been added to the culture medium, the purification strategy should include removal of bovine albumin and bovine IgG.

Capture
Since mammalian cells secrete product into the culture fluid, there are basically two approaches to retrieve it see Figure 1. A traditional approach to capture uses centrifugation and/or filtration to separate cells from the supernatant and reduce volume before application to a packed bed chromatography column for purification. An alternative and relatively new approach at industrial scale is expanded bed adsorption (EBA). This technique allows the entire contents of the fermenter to be applied directly onto an expanded bed of adsorbent particles, completing the capture step in one operation. Following EBA, one or more chromatographic techniques will ensue to achieve the required purity of the final product.

Affinity chromatography (AC) with protein A is the purification principle best suited for the capture of IgG. Except for IgG₃, all subclasses of human IgG bind to protein A at the Fc region at neutral to alkaline pH, and at low to high conductivity. Generally, no or only slight adjustment of the supernatant is needed for application and binding. The binding is also unaffected by the nature of the glycosylation. Elution of the MAb is performed by decreasing the pH. The addition of additives such as 30-60% ethylene glycol or 1-2 M urea to the elution buffer is sometimes used to elute the protein at values up to pH 8. If foetal calf serum is present in the culture medium, bovine IgG may amount to more than 50% of the IgG eluted. Also, as no chromatographic medium is leakage-free, very small amounts of leached protein A ligand may be present. A new development in protein A media for capture of MAbs is MabSelect™, see article on page 6.

Ion exchange (IEC), cation and anion exchange chromatography, is a commonly used technique suitable for all MAbs. It has relatively high resolution, capacity, and recovery, and effectively concentrates the product. Its concentrating effect makes it very suitable for capture, particularly when water is a major contaminant. Binding conditions and choice of ion exchanger will depend on the stability and the pI of the antibody.

Hydrophobic interaction chromatography (HIC) is less common as a capture step because of the high salt levels required for adsorption. However, in cases where the supernatant already has high ionic strength and is quite “clean”, HIC can provide the capacity for an effective capture step.

Expanded bed adsorption with STREAMLINE™ Protein A offers the advantages of direct capture by combining the separate steps of centrifugation, clarification and filtration into one operation. The expressed product is handled very gently. The technique is based on creating a stable, uniform, expanded bed of adsorbent particles through which the contents of the fermenter pass. The target molecule is captured, whilst cells, cell debris, contaminants, etc. pass through the column. Mammalian cells can be fragile and very sensitive to shear forces (high shear forces will disrupt the cells, releasing proteases). In a more traditional approach the sensitive cells are easily damaged by shear from the equipment used for filtration. STREAMLINE, on the other hand, does not generate shear, but allows both the molecules and the cells
Intermediate purification

Depending on the efficiency of the capture stage, one or more steps may be required to remove remaining contaminants, endotoxins, nucleic acids, viruses, etc. The chromatographic techniques used, IEC or HIC, will depend upon the characteristics of the target protein. Bead size will be in the range 30-90 µm. For some processes this stage may be omitted, with polishing following directly after capture.

Polishing

In the final stages of the downstream purification process the feed volume is low, while product concentration and value are high. However, some contaminants may still remain in trace amounts, for example dimers and other conformers, and leached compounds. To achieve the required purity for therapeutic use, high resolution techniques on small diameter (10-30 µm) particles are needed. An example of this is separation on high resolution SOURCE™ 15 IEC or SOURCE 15 HIC media. Gel filtration (GF) is also an effective technique for polishing, but it can be limited by the feed volume. Reversed phase chromatography (RPC) is less applicable for MAbs because of the high risk of denaturation in the organic solvents normally used for elution.

Removal of viruses and contaminants

Mammalian cell lines are prone to contamination because of the complexity and duration of their culture. Also, they can host many of the adventitious agents that human cells do. The addition of growth factors to the culture medium may introduce contaminants from bovine sources, such as prions, and the purification media used might leach compounds.

Removal and monitoring of all potential contaminants must be designed into the purification process and clearance factors documented. Regulatory authorities require the integration of at least two specific virus inactivation and removal steps, such as the solvent/detergent method, nanofiltration, heat treatment, etc. In addition to the specific steps mentioned above, the chromatographic techniques themselves contribute to virus inactivation/removal. A rough estimate of the capability of different purification methods to remove adventitious agents is given in Table 1. A suggested quality control programme is outlined in Figure 2.

Table 1. Removal of adventitious agents from human monoclonal antibodies by different chromatographic principles. Data are from Gagnon P (1996). The table describes the impact of different industrial purification principles on the removal of adventitious agents. Variations may occur depending on the purification conditions applied.

<table>
<thead>
<tr>
<th>Chromatographic principle</th>
<th>Endotoxins</th>
<th>Nucleic acids</th>
<th>Viruses</th>
<th>Note no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein A (AC)</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>1</td>
</tr>
<tr>
<td>Anion exchange</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>2</td>
</tr>
<tr>
<td>Cation exchange</td>
<td>+</td>
<td>+++</td>
<td>++</td>
<td>3</td>
</tr>
<tr>
<td>Hydrophobic interaction</td>
<td>+</td>
<td>+++</td>
<td>++</td>
<td>4</td>
</tr>
<tr>
<td>Gel filtration</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>5</td>
</tr>
</tbody>
</table>

1) Complexes between monoclonal antibodies and nucleic acids are dissociated at high salt concentrations tolerated in the Protein A application.
2) Nucleic acids bind strongly to anion exchangers. Strongly basic IgGs and IgMs form stable complexes with DNA, especially at low conductivity. Consequently the clearance factor is reduced.
3) Nucleic acids do not bind to cation exchangers.
4) Nucleic acids do not bind to HIC media. Antibody-nucleic acid complexes are dissociated at high salt concentrations. Endotoxins may form micelles or higher secondary structures in aqueous solutions, especially at high salt concentrations. These high molecular weight structures are excluded from the matrix.
5) Nucleic acids form complexes with monoclonal antibodies at low conductivity resulting in a reduced clearance factor.
Advantages
- The protein is secreted into the medium
- Usually expresses and secretes even complex posttranslationally modified proteins in their active form
- Good regulatory track record

Disadvantages
- Slow cell growth
- Relatively low expression levels
- Expensive growth media
- Sensitive to shear forces
- Potential virus/Mycoplasm contamination risk
- Safety concerns about transformed cell lines

Fig. 3. Summary of the advantages and disadvantages of mammalian cell culture.

MAb drive
Despite the known drawbacks of mammalian cell culture as an expression system, see Figure 3, it remains the most important producer of large complex proteins. Moreover, developments in cell culture techniques and advances in molecular engineering are leading to mammalian cell culture systems being more efficient, more cost-effective, and better defined with respect to downstream processing. These developments are, in part, being driven by the renewed focus on MAbs as therapeutics.

Further reading


Acknowledgement
Downstream wishes to thank Kim Ry Højens, L & K Biosciences Aps, Denmark, for help in preparing this article.

The 17th ESACT Meeting
From Target to Market
June 10-14, 2001, Tylösand, Sweden

ESACT is a society which brings together scientists, engineers and other specialists working with animal cells in order to promote communication between European and International investigators, and progress the development of cell systems and products derived from them.

ESACT 2001 will focus on the role of animal cells in drug development, from target to market. Topics will include the route from identification of drugs and drug targets to novel therapeutic approaches and marketed products. For more information, visit our web site: www.esact.org/esact2001.
The atmosphere was lively and enthusiastic at this first meeting in Europe covering issues around the downstream purification of humanized antibodies and fusion proteins. GAb 2000, sponsored by Amersham Biosciences, was well attended with more than 100 participants from countries around the world.

The three-day meeting was opened with a lecture by Andreas Plückthun (University of Zurich) who talked about the evolution of methods for producing monoclonal antibodies (M Abs) - from the classical starting point of obtaining M Ab from hybridoma cells to the exciting possibilities achievable today through genetic engineering. With these technologies we have created many different libraries of molecules, phage display, ribosome display, human combinatorial antibody, etc, to enable us to make any type of antibody. However, despite all this technology, we still do not yet fully understand how they function.

**GAb success stories**

Following this opening address, three speakers presented examples from industry and health-care. James Young (MedImmune Inc.) described the development of Synagis™ a recombinant monoclonal antibody which provides protection against respiratory syncytial virus (RSV) in infants. Synagis is a humanized IgG1, a composite of human (95%) and murine (5%) antibody sequences, that binds to the F-protein of RSV. The drug exhibits neutralizing and fusion-inhibitory activity against RSV. The downstream process neutralizes cation exchange, protein A affinity chromatography and anion exchange.

Enbrel™, indicated for reducing symptoms and delaying structural damage in patients with active rheumatoid arthritis, was presented by Helmut Sassenfeld* (Immunex Corp.).

Enbrel is a recombinant, human tumour necrosis factor receptor (TNF-R) fused to the Fc portion of IgG, thereby creating the active dimer. The protein is expressed in Chinese hamster ovary (CHO) cells. Enbrel acts by binding with TNF to reduce the immune response.

A third case study was presented by Ira Pastan (National Institute of Health, NIH). He described the development of a new treatment for metastatic cancer. Through genetic engineering of a bacterial toxin, Pseudomonas exotoxin A (PE), it is possible to kill cancer cells selectively. PE is a three domain protein composed of 613 amino acids. Anticancer agents are produced by deleting its binding domain (aa 1-252) and replacing it with the Fv fragment of an antibody or with a growth factor that binds to antigens present on cancer cells. These therapeutics are termed recombinant toxins and are produced in E. col.

**New approaches to purification**

Session two included presentations of a new, high performance tangential flow technique that exploits both size and charge characteristics, by Charles Christy* (Millipore Inc), and a description of how membrane filters are used to remove viruses from monoclonal antibody products, by Ernst-Günter Graf* (Pall GmbH). Harish Iyer* (IDEC Pharmaceuticals) described the purification process for the first chimeric monoclonal IgG1 approved by the FDA (Rituxan™) and Jean-François Hamel (MIT) presented an application of STREAMLINE™ expanded bed adsorption. A novel approach which separates biological macromolecules on the basis of pH-dependent behaviour of ionizable dual-mode ligands, called hydrophobic charge induction chromatography, was described by P. Santambien (Life Technologies BioSera). Protein A media are the most widely used adsorbents for M Ab purification. Gunnar Malmquist* (Amersham Biosciences) presented the effect of residence time on binding capacity for a new protein A medium, M abSelect™, comparing the results with four Protein A media already on the market. More details about this new product can be found on page 6.

**Regulatory and quality issues**

Gail Sofer* (BioReliance Inc.) gave an overview of the regulatory considerations for genetically engineered antibodies, focusing on critical aspects such as starting materials, process validation, viral clearance and comparability. Adequate viral clearance is still a major hold up for M Abs in clinical trials. An approach to virus inactivation using a High Temperature Short Time, HTST, microwave heating system was described by Joachim Walter* (Boehringer Ingelheim Pharma KG). This is a non-invasive physical treatment for high value pharmaceutical proteins and enables exposure of protein to elevated temperatures due to its ultrashort residence time in the millisecond range. Viral clearance using chromatography, protein A affinity and anion exchange, was discussed by Stephen Notarnicola* (Biogen Inc.). Their approach is to exploit the power of column chromatography to separate product from virus particles in order to reduce the need for extra viral clearance steps in a process. Development of cryopreservation as a scalable tool for eliminating unwanted changes during the downstream processing of bioengineered products was the title of an interesting presentation by David Sesin* (Integrated Biosystems). The technology is based on the formation of dendritic ice crystal growth which controls the harmful effects of cryococoncentration. Cryopreservation was presented as an efficient, reproducible tool for use in manufacturing processes to freeze/thaw bioengineered products and/or decouple multi-step processes.

**Modifications of molecular structure and functionality**

Bob Lutz (Immunogen) described an approach for developing anticancer drugs that use M Abs to bind to tumour associated markers on the surface of cancer cells. This has led to the creation of a new generation of antibody-drug conjugates that act as Tumour Activated Prodrugs (TAPS). Bernard Malfroy (Eukarion) talked about ways of getting around the inability of monoclonal antibodies to cross the cell membrane.

* Denotes an extended abstract in the Supplement, Downstream GAb abstracts.
membrane and alter intracellular functions. By using a novel process of lipidation and carbohydrate amination to modify the antibodies, they have had some promising results in overcoming these difficulties. If these processes can be applied to humanized antibodies, then this might enable humanized antibodies to be used as novel therapeutic agents in man. Supply of monoclonals for therapeutic applications is typically targeted to acute indications, due to costs and sufficient quantities. Mariangela Spitali* (Celtech Chirosience) described how a team has tried to address this by developing a technology to allow the rapid and economical production of therapeutic antibodies. This can be achieved through high level expression of humanized Fab' fragments in E. coli, to which PEG is attached. The result is an antibody fragment with a prolonged in vivo half-life. J. Bhatia (Royal Free Hospital), described the development of recombinant fusion protein for cancer treatment that is being prepared for a Phase I clinical trial. The fusion protein was first expressed in E. coli, but the addition of a hexahistidine tag and a switch to the yeast Pichia pastoris resulted in higher yields and improved purification. Stability in vivo and in vitro was also confirmed. Jan Feuser* (Boehringer Ingelheim Pharma) closed the session by describing the process for cleaving off the Fc region of a murine MAb, (IgG2b) and the subsequent downstream processing steps for the Fab-fragments so that pure fragments could be applied as diagnostic probes for in vivo detection and treatment of cancer cells.

**Design of affinity ligands**

Mattias Uhlén* (Royal Institute of Technology, Stockholm) talked about engineering of protein domains capable of specific binding to target proteins. These binding protein domains, or affibodies, are derived from bacterial receptor staphylococcal protein A or streptococcal protein G, and possess high affinity to immunoglobulins. Protein engineering of protein A includes changing the B domain, and in protein G substitution of the asparagine residues. These processes can render the affibody more stable to high pH, and therefore more suitable as affinity ligands in industrial chromatography. Work with monoclonal antibodies for targeting radionucleotides in human bladder cancer patients was the topic for Richard Smith* (University of Nottingham). Purification is by affinity chromatography using epitopes/ mimotopes. Suitable mimotopes were identified by screening immobilized whole antibody using a T7 bacteriophage library displaying 415 copies of an encoded peptide on the T7 capsid head. Chris Lowe (University of Cambridge) outlined how synthetic ligands can be refined and optimized by making small changes in their structure. He went on to describe the construction of an 88-member IgG-binding ligand library synthesized by a solid phase assembly procedure on an agarose matrix, using a modified mix and split procedure to enable rapid identification and evaluation of chemical leads.

**Poster display**

Posters supporting the four session topics were displayed in an adjoining room. Here many ideas and experiences were exchanged in the breaks around the oral presentations.

This was very successful first meeting with everyone’s interest held right up to the closing remarks. The only question without an answer was the location of the second conference in 2002.

---

* Denotes an extended abstract in the Supplement, Downstream GAb abstracts. 

**Downstream GAb abstracts**

"Reports from GAb 2000"

**Code number:** 18-1150-47.

This booklet, containing extended abstracts from many of the presenters at the GAb 2000 meeting, is available via your local Amersham Biosciences office.
This four day conference, organized by the Australian Biotechnology Association continues to offer a forum which attracts scientists, industrialists, policy makers, intellectual property specialists, financiers and service providers to the Biotechnology industry, primarily for Australia and New Zealand.

The programme consisted of 140 oral presentations, and 53 poster presentations. Presenters were from Europe, USA, and more locally Asia, New Zealand, Japan, and Australia. Topics covered in the scientific area included metabolic engineering, bioinformatics and genomics, drug design, miniaturization and robotic design, discovery of new bioactives, biomaterials, vaccines, and scale up strategies.

Finance and patents
Financial topics included biopartnering, venture capital case-studies, economic developments, and aspects of managing the commercialization and risks in the corporate world.

In the area of patenting, papers were given by Jodi Lawler (IP Australia) on patentability of biotechnology in Australia, and by Friederike Stolzenburg (Vossius & Partner, Germany), and Daniel Altman (Knobbe, Martens, Olsen & Bear, USA) on the recent developments in patenting biotechnology in Europe and USA respectively.

Session topics
Papers on tissue approaches to disease and dysfunction, included "Dendritic cell vaccination for melanoma" (Ranjeny Thomas, University of Queensland), "Haematopoietic tissue engineering" (Terry Papoutsakis, Northwestern University, USA), "Vaccine production in plants" (Ian Polkinghorne, Queensland Agricultural Biotechnology Centre), and "Self-assembled membranes for protection of foreign tissue transplants" (Lars Nielsen, University of Queensland).

New technologies papers looked at membrane fluidity in microbial populations during bioprocesses, developments in biotechnology statistics, and potential virulence of viable-but-noncultural bacterial pathogens.

CEO Roundtable
Each day commenced with CEO Roundtable breakfast discussion, covering financing, managing technology platforms and intellectual property, startups and spinouts. Site visits were also offered on the final day. Reports from these discussions are available on the ABA web site at www.aba.asn.au. Topics for discussion at future meetings for CEO Roundtables were Financing a start-up, Listing a company – when & how, and Management: human resources issues at start-up.

Biotechnology and public attitudes
Inline with the growing public debate on food biotechnology and its place in society, a session was dedicated to consumerism and biotechnology. Presentations here looked at changing consumer attitudes to biotechnology. Another session in this vein looked at public communication strategies, including how can scientists enhance the societal acceptance of agricultural biotechnology. (CS Prakash, Tuskegee University, USA).

Presentations included one from an Australian museum looking at the place of biotechnology exhibits for public consumption. (Bronwyn Terrill, Museum Victoria)

An opportunity was also given for the general public to attend with a session entitled “Biotechnology in society - a community awareness forum”. This was held during an evening, and was a highly interactive and entertaining session generating lively debate among the panelists and audience.

The general mood of the conference was very positive and vibrant, indicating an excellent forum for the meeting of the broad range of professional skills involved in the biotechnology industry. The conference showed the healthiness of the biotechnology industry in this region.
Following the success of the inaugural Plasma Product Biotechnology Meeting held on Daydream Island, Queensland, Australia, no one was left in doubt that a follow-up meeting was highly desirable. The focus of PPB 2001 is “Real issues, real interaction” and the programme details will develop on the web site as participants register their interests. This approach will ensure that PPB 2001 deals with the topics that matter today. The main scientific programme is outlined below. Sessions will cover key issues along the road from discovery to routine production, rather than specific plasma products.

If you work with plasma product R&D, manufacture, economics, safety, or any other relevant issue, and want to make an active contribution to PPB 2001, you can register your interests, follow developments, and make your own views known on www.bo-conf.com/ppb2001.

Visit the site and bookmark the address today or contact the Secretariat at the address below:
PPB 2001, Secretariat
B.O. Conference Service
P.O. Box 10078
S-750 10 Uppsala
Sweden
Tel: + 46 18 61 20 177
Fax: +46 18 30 40 74
E-mail: osterlund@bo-conf.com

Keynote address by Juhani Leikola, Finnish Red Cross

<table>
<thead>
<tr>
<th>Session topics</th>
<th>Chairman</th>
<th>Affiliation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Research and novel technologies</td>
<td>Jan Over</td>
<td>CLB, Amsterdam, The Netherlands</td>
</tr>
<tr>
<td>Development</td>
<td>David Naveh</td>
<td>Bayer Corp., Berkely, USA</td>
</tr>
<tr>
<td>Clinical issues</td>
<td>Darryl Maher</td>
<td>CSL Ltd, Australia</td>
</tr>
<tr>
<td>Regulatory perspectives</td>
<td>Albert Farrugia</td>
<td>Therapeutics Goods Administration, Australia</td>
</tr>
<tr>
<td>Manufacturing</td>
<td>Johan Vandersande</td>
<td>Baxter Healthcare Corp., Los Angeles, USA</td>
</tr>
<tr>
<td>Safety considerations</td>
<td>Bernard Horowitz</td>
<td>New York, USA</td>
</tr>
</tbody>
</table>

Amersham Biosciences builds on plasma purification know-how

Developing countries will now be able to purify plasma to make Factor VIII for haemophiliacs, helping moves towards self-sufficiency. Amersham Biosciences announced today a deal with HemaSure Holding A/S, Denmark, acquiring exclusive technology know-how rights in plasma purification. The deal will enable Amersham Biosciences to offer customers the ability to start up safe plasma purification to make Factor VIII, a life-sustaining product for haemophiliacs.

Plasma is currently used to produce several major products including albumin, polyvalent gamma globulin, Factor VIII and Factor IX. Factor VIII is commercially valuable with a worldwide market of $US 1.8 billion, including product from recombinant sources.

Amersham Biosciences already offers the chromatographic technology to purify albumin and gamma globulin. The new deal allows the company to complete their offering with an approved production-scale chromatography method for Factor VIII and a laboratory-scale method for Factor IX.

The majority of the world’s supply of Factor VIII is currently produced by patented methods by large pharmaceutical companies in the US and Europe. Amersham Biosciences’ technology, which incorporates two virus inactivation and removal steps, will allow blood transfusion centres and related organisations in the developing world to have confidence that they can successfully start up safe development of their own product - utilizing locally donated blood.

Established methods for production include so-called cryoprecipitation to separate Factor VIII from plasma. Amersham Biosciences’ chromatography method avoids this and uses gel filtration and ion exchange to produce a high purity product. The company can provide a complete process based on Sepharose® ‘Fast Flow and Sepharose High Performance media, including all equipment and know-how.
Anti-CD18 is a humanized F(ab')2 antibody expressed in a soluble form in the cytoplasm of E. coli, at a concentration greater than 1 mg/ml. The initial purification process involved the production of a frozen cell paste and the subsequent resuspension of the paste in a large volume of buffer. Following cell disruption, the cell extract was clarified by centrifugation, which had a yield of approximately 80%. The clarified cell extract was then purified over a series of packed bed chromatography columns. The first step in the series was an initial capture step that used a mixed bed ion exchange (IEC) medium. This ion exchange step required that the clarified cell extract be greatly diluted to reduce the conductivity to bind the antibody to the medium. The antibody was eluted using a gradient elution and fractions were collected. The initial capture step gave a reasonable yield and good purification of the antibody; however it was not suitable for scale up to 10,000 litres due to the large dilution of the load, the gradient elution and the collection of fractions to determine the pool.

Expanded bed adsorption - an alternative

Expanded bed adsorption (EBA) chromatography was considered as a possible alternative method for direct capture of the antibody. It was hoped that EBA could handle the high solids content of whole cell broth, increase the yield by reducing the number of steps and perhaps decrease the costs of production at scale. However, the EBA column would have to operate within some process constraints - the maximum dilution of the load to the column (2.4 fold) was pre-determined by the tank capacity in the plant. This dilution of the broth would lead to a solids content of approximately 15% in the load. Given the restrictions on the dilution of the feedstock, the conditioning parameters of pH and conductivity were tested for binding capacity to both STREAM LINES™ SP and SP-XL media. The process was tested for scalability using a prototype EBA column and compared to the traditional packed bed process. The processes were compared in terms of product yield, purity, quality and process robustness.

There were 3 goals to the experiments:
1. To minimize the product binding to the debris
2. To minimize the debris binding to the adsorbent, and
3. To maximize the product binding to the adsorbent.

In the first of these, we found that at low pH and conductivity, the product binds to the debris in the broth and tends to precipitate upon centrifugation. In order reduce the antibody-debris interactions some salt is required, particularly at lower pHs. At increasing pH and conductivity, the recovery of the antibody increases as can be seen from Figure 1. However, at the higher pH and conductivity, the capacity of the adsorbent decreases. In order to bind to the cation exchange adsorbent, there needs to be a fine balance between low pH and conductivity.

Fig. 1. Anti-CD18 binds to debris at low pH and conductivity.
In the absence of adsorbent under the conditions used the product associates with the debris, and will precipitate out if centrifuged. In the presence of adsorbent, the product appears to have a higher affinity for the adsorbent than for the debris, and the adsorbent tends to 'extract' the product from the debris-product complex. If the feed is diluted without maintaining the conductivity, less product is recovered, presumably because it is more tightly bound to the debris and is not captured by the adsorbent (Table 1).

Table 1. The balance between conductivity and recovery.

<table>
<thead>
<tr>
<th>Fermentation #</th>
<th>Dilution factor</th>
<th>Salt added (mM)</th>
<th>Conductivity (mS/cm)</th>
<th>Recovery (g/l broth)</th>
</tr>
</thead>
<tbody>
<tr>
<td>201</td>
<td>2.3</td>
<td>0</td>
<td>5.2</td>
<td>1.2</td>
</tr>
<tr>
<td>195</td>
<td>2.4</td>
<td>0</td>
<td>5.2</td>
<td>1.2</td>
</tr>
<tr>
<td>195</td>
<td>3</td>
<td>8</td>
<td>5.2</td>
<td>1.2</td>
</tr>
<tr>
<td>201</td>
<td>3</td>
<td>0</td>
<td>4.3</td>
<td>0.7</td>
</tr>
</tbody>
</table>

**Maintained performance at larger scale**

In order to test the operating conditions at a larger scale, Amersham Biosciences kindly lent us a prototype EBA column that was 600 mm in diameter, 1.7 m in height. The scalability of the process was assessed using the criteria of yield, product quality, product purity and overall process performance. The column was run twice and each time a small-scale (25 mm diameter x 1 m height) column was run in parallel as a control. STREAMLINE SP-XL was packed in each column to a final bed height of 20 cm. The disrupted whole cell broth was diluted 2.4 fold, and adjusted to a pH of 5.1, with a final conductivity of 5.2 mS/cm. Anti-CD18 was loaded to 28 g/l adsorbent (as determined by a specific HPLC assay). The column was loaded at a flow rate of 150 cm/h and washed and eluted at 300 cm/h. The pool was made by OD and was less than 1 total column volume (approximately 560 litres). The pool was tested for product quality and purity (E. coli proteins). The yields, product quality and product purity were comparable for the small (25 mm) and large (600 mm) scale columns (Table 2). In terms of the performance of the EBA column at the larger scale, the chromatography performed as well as it had done at the smaller scale.

**Comparing processes**

The original packed bed process was also run at small scale in order to compare the product quality, purity and yield. The pools from both first steps were taken downstream in order to assess whether the final products of both processes were indeed comparable (Table 3). The purity of the final pools was highly comparable for EBA and packed bed products, 800 and 300 ppm ECP respectively, as was the product quality, 86 and 87% main peak by CE-SDS, and 85 and 80% main peak by HP-IEC. Overall yield was higher for the EBA process, 36% compared with 27% for the packed bed process.

**Confidence for manufacturing**

In our experience, the performance of the small-scale EBA column was predictive of the performance of the larger EBA column in terms of yield, product quality and product purity. From the comparison between the EBA process and the packed bed process, it can be seen that the EBA process gave a higher yield of antibody, while maintaining the same product quality and purity. These data can be used to give us confidence that EBA can be scaled up for use in a manufacturing facility and that the final product of the process is comparable in terms of quality and purity to the product from a packed bed process.

**Table 2** Comparison of scale: 600 mm vs 25 mm diameter EBA columns.

<table>
<thead>
<tr>
<th>Load</th>
<th>Yield (%) n=2</th>
<th>%F(ab')2 * n=2</th>
<th>ECP (ng/mg) n=2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Load</td>
<td>29</td>
<td>77</td>
<td>571,000</td>
</tr>
<tr>
<td>600 mm column</td>
<td>90</td>
<td>77</td>
<td>570,000</td>
</tr>
<tr>
<td>25 mm column</td>
<td>89</td>
<td>76</td>
<td>574,000</td>
</tr>
</tbody>
</table>

* by HPLC assay

**Table 3** Comparison of EBA and packed bed processes (PB).

<table>
<thead>
<tr>
<th>Step</th>
<th>Yield (%) EBA</th>
<th>ECP (ng/mg) EBA</th>
<th>CE-SDS (% mainpeak) EBA</th>
<th>HP-IEC (% mainpeak) EBA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capture Step</td>
<td>86</td>
<td>563000</td>
<td>72</td>
<td>N D</td>
</tr>
<tr>
<td>2nd Column</td>
<td>84</td>
<td>220000</td>
<td>70</td>
<td>N D</td>
</tr>
<tr>
<td>3rd Column</td>
<td>48</td>
<td>80000</td>
<td>86</td>
<td>85</td>
</tr>
<tr>
<td>Total Yield</td>
<td>35</td>
<td>27</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N D = Not determined.

PB = Packed bed process.

*CE-SDS Non-gel sieving (CE-SDS) measures the amount of fully intact (Fab')2 present as a % of the main peak.

**High Performance Ion Exchange (HP-IEC) measures the % of fully intact F(ab')2 in each pool.**
Purification of insulin from crude feedstock by employing a novel ligand for specific adsorption

M. Asplund, R. Bhikhabhai, M. Westerfors, R. Haglund, Amersham Biosciences, Uppsala, Sweden
E. Cohen, G. Mchel, T. Ransohoff¹, Dyax Corp. Cambridge, MA, USA
¹ Current address: TranXenoGen Inc., Shrewsbury, MA, USA

Summary of a presentation at EBA 2000

A protein can be purified using affinity chromatography on the basis of its biological function or individual chemical structure. This allows for a very effective technique for the capture stage of a downstream process. The limited availability of robust, cost-effective, process-suitable ligands has, however, restricted the use of affinity chromatography for the capture stage. A collaboration between Amersham Biosciences and Dyax Corp. was established to develop a new platform for affinity adsorbents and thus address this limitation.

The collaboration includes a project that combines the advantages of Dyax's phage display ligand discovery technology - an effective method for rapidly identifying specific ligands that bind to molecular targets - with the benefits of STREAMLINE™ expanded bed adsorption, the technology that allows direct capture of target molecule from crude feedstocks without any clarification of the sample.

Encouraging start

Previously, a novel ligand with high specificity for human insulin, derived from a phage display library, was coupled to Sepharose™ 4 Fast Flow. In the packed bed capture step, insulin was recovered from clarified yeast homogenate with a purity greater than 95%. Prompted by requests for a customized affinity ligand, and the need to make affinity media more applicable for bioprocessing, this model was transferred to STREAMLINE expanded bed adsorbents.

One among 67 million

The source for the insulin ligand was Dyax's Protein G/Ig Binding Domain helix library that contains more than 67 million variants of phage-displayed ligands. This library was screened under the desired binding conditions for candidate ligand compounds. The selected insulin ligand had a molecular weight of 8291, a pI of 6.8, and six primary amines. The ligand was expressed in E. coli host, and purified to 95%. Following characterization, the insulin ligand was coupled to STREAMLINE quartz base matrix using NHS-activation. Ligand density, determined by amino acid analysis, was 12 mg ligand/ml adsorbent.

E. coli and yeast homogenate

The performance of the new STREAMLINE affinity adsorbent for direct capture of human recombinant insulin from crude feedstocks was evaluated using both E. coli and a yeast homogenate as feedstreams. Binding capacity and elution conditions were verified in packed bed mode using a sample load of 0.5 mg human recombinant insulin per ml equilibration buffer. This was fed onto an HR 5/5 column (i.d. 5 mm) packed with 1 ml of STREAMLINE/insulin ligand. The flow velocity was 100 cm/h. From the results in Table 2, it can be seen that a salt-containing buffer had little effect on binding. Consequently, a salt-free buffer (buffer 1) was selected for the equilibrating the column.
Table 1. Packed bed capacity data.

<table>
<thead>
<tr>
<th>Column: HR S/S with 1 ml STREAMLINE/insulin ligand adsorbent</th>
<th>Sample: 0.5 mg human recombinant insulin per ml equilibration buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equilibration buffer 1: 20 mM Na₂HPO₄, pH 7.0</td>
<td>Equilibration buffer 2: 20 mM Na₂HPO₄, 150 mM NaCl pH 7.0</td>
</tr>
<tr>
<td>Elution buffer: 1 M acetic acid</td>
<td>Flow rate: 100 cm/h</td>
</tr>
</tbody>
</table>

Table 2. Effect of salt on binding.

<table>
<thead>
<tr>
<th>Buffer 1</th>
<th>Buffer 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capacity (mg insulin/ml adsorbent)</td>
<td>Capacity (mg insulin/ml adsorbent)</td>
</tr>
<tr>
<td>C/C₀</td>
<td></td>
</tr>
<tr>
<td>0.6</td>
<td>13.0</td>
</tr>
<tr>
<td>0.5</td>
<td>12.0</td>
</tr>
<tr>
<td>0.4</td>
<td>11.3</td>
</tr>
<tr>
<td>0.3</td>
<td>10.5</td>
</tr>
<tr>
<td>0.2</td>
<td>8.5</td>
</tr>
<tr>
<td>0.1</td>
<td>6.9</td>
</tr>
<tr>
<td>0.05</td>
<td>5.4</td>
</tr>
</tbody>
</table>

In a prototype STREAMLINE column, 12 ml of STREAMLINE/insulin ligand adsorbent was expanded approximately 2.5 times its settled bed height with the equilibration buffer. Unclarified homogenized yeast suspension was applied to the expanded bed at a flow velocity of 200 cm/h. Following wash, insulin was eluted in settled bed mode. The purity of the collected fractions was determined by SDS-PAGE and reversed phase chromatography (RPC). The process was then repeated with the E. coli feedstream.

**Efficient capture**

Figure 1 shows the chromatogram and SDS-PAGE for the direct capture of insulin from a yeast homogenate. Recovery was 98% and purity, as determined by reversed phase chromatography, was 97%. Similar results were obtained with the E. coli feedstream under the same running conditions, see Figure 2, RPC analysis.

**Untold opportunities**

Dyax’s phage display technology offers an opportunity to rapidly identify a wide selection of novel and biologically diverse ligands for virtually any biological target. These ligands can also be engineered and synthesized to achieve the desired properties for a specific application. The coupling of these tailor-made ligands to STREAMLINE adsorbents will open many opportunities for the application of affinity chromatography for capture, irrespective of the feed stream composition.

**How does phage display work?**

Phage display, or the surface display of genetic diversity, takes advantage of the ability to insert a family of genes of interest into a bacteriophage genome, vector, in such a location that its product, a protein, is expressed on the viral surface as a fusion protein with a viral coat protein. Generally Dyax uses the gene 3 protein of the M13 filamentous bacteriophage, of which there are five copies displayed.

Libraries of hundreds of millions of genes can be inserted and displayed on phage. These libraries can be produced on a DNA synthesizer (synthetic), or may be cloned from particular organs or cells (genomic).
Charge engineering of a protein domain to allow efficient ion exchange recovery

Sophia Hober, Torbjörn Gräslund, M y Hedhammar and Mathias Uhlén
Dept of Biotechnology, Royal Institute of Technology (KTH)
SE 00 44 Stockholm, Sweden

Summary of a presentation at EBA 2000

Ion exchange chromatography (IEC) is a widely used protein separation technique. Its widespread use is due to the fact that ion exchange media (i) are robust and relatively cheap, (ii) can withstand cleaning-in-place conditions and (iii) are well documented in small and large-scale separations.

Adsorption to an ion exchanger is dependent on the physical characteristics of the target protein, for example pI and charge distribution. IEC has the potential to result in high-resolution separations, but performance is dependent on the amount of contaminants with the same adsorption characteristics as the target protein. One way to improve the purification of a recombinant target protein is to change its charge distribution to enable either stronger adsorption to the ion exchanger, or adsorption under conditions unique to the target protein.

The aim of our work was to create protein domains with extreme surface charge that can be used as purification tags for facilitated capture by IEC. Using an existing protein domain as a scaffold, we added charges to create variants that could be purified by cation exchange chromatography at high pH values, where there is potentially little adsorption of contaminating E. coli host cell proteins.

Scaffold

We used the staphylococcal protein A-derived Z domain (Zwt) as a scaffold to create two mutants with highly charged surfaces. Both Zwt and the two mutated protein domains are compact 58 amino acid three-helix bundles that contain no cysteines and are highly soluble. Also, Zwt has been shown to help in solubilizing fused target proteins in vitro, and is stable against proteolysis in a number of different hosts.

Zbasic1 and Zbasic2

By using Zwt as a starting scaffold, two mutants, Zbasic1 and Zbasic2, were constructed by exchanging surface exposed amino acids in helix one and two. In Zbasic2, 10 amino acids were targeted for mutagenesis and six of those were changed for lysines or arginines. In Zbasic1, 11 amino acids were replaced with arginines (Figure 1). The two mutants were successfully produced in E. coli at high levels and with similar structure, as analyzed by measuring the circular dichroism spectra between 190 and 250 nm (Figure 2). The double minima for the three proteins at 222 and 209 and a high peak around 195 are characteristic for proteins with high α-helix content. Based on the collected spectra it could be concluded that both Zbasic1 and Zbasic2 have a high α-helical content, but lower than that of the parental molecule.

Cation exchange experiments

Purified samples of the three proteins were loaded on a cation exchange column at pH 3, followed by washing and elution with a linear NaCl gradient. At this pH all three proteins bound quantitatively to the adsorbent. Zwt eluted first at the lowest conductivity followed by Zbasic1 and Zbasic2. An overlay plot of the chromatograms is shown in Figure 3. In a corresponding experiment performed at pH 9, Zwt was collected in the flowthrough, whereas Zbasic1 and Zbasic2 were still quantitatively adsorbed. These results show that Zwt was positively charged at pH 3 and negatively charged at pH 9. The two mutants were positively charged at both pH 3 and pH 9.
Expression and purification of Z\textsubscript{basic2}Klenow polymerase

In order to evaluate the chromatographic properties of Z\textsubscript{basic2} fused to a target protein, a plasmid encoding Z\textsubscript{basic2}Klenow polymerase was transformed into E. coli. The cells were grown in a fed-batch fermentation and harvested by passage through a high-pressure homogenizer. The pH of the homogenate was adjusted to 7.5 and loaded onto a STREAMLINE\textsuperscript{TM} SP expanded bed cation exchange column equilibrated at the same pH. The entire purification procedure was monitored at 280 nm (Figure 4). The flowthrough was collected, and after extensive washing bound proteins were eluted with 1.0 M NaCl. The eluted peak was collected and analyzed by SDS-PAGE and also for Klenow polymerase activity. In this single purification step, product yield was greater than 85% and purity was greater than 90%.

Conclusion

The study demonstrates the potential of charge engineering to create domains with desired properties. By exchanging up to 11 amino acids on the surface of the Z domain, two mutants were created with structural properties similar to the parental molecule. The modified molecules can be produced as full-length products in E. coli and successfully purified from an E. coli homogenate by cation exchange chromatography. Results show that both mutants bind to a cation exchange adsorbent at pH values in the range 9 to 11. In addition it was also shown that Z\textsubscript{basic2} could be genetically fused to a target protein, expressed in E. coli and purified from a homogenate by STREAMLINE SP expanded bed adsorption. The properties of the variants suggest that they could be useful for further investigation as fusion partners for cation exchange chromatography purifications.