Antibody-based therapeutics are increasingly being developed for use in indications such as oncology, inflammation, and infectious disease. Advances in antibody engineering technologies have facilitated the rapid generation of high-affinity antibodies of defined specificity and have led to the development of a diverse range of antibody-based molecules formatted to address specific applications. High dose requirements for treatment of chronic indications in large markets necessitate the development of high-yielding, low-cost manufacturing processes that consistently deliver high-quality product. Mammalian cell culture is currently the system of choice for producing whole antibodies, whereas the generally preferred expression host for antibody fragments is *Escherichia coli*. The advantages of shorter processing time and increased scale of operation associated with *E. coli* production counter some supply and cost-of-goods constraints inherent in mammalian cell expression. Furthermore, process development times are shorter for microbial systems, allowing more rapid progression to the clinic.

This article describes a technology platform for development and production of Fab-based antibody therapeutics using an *E. coli* expression system. The technology covers all stages of therapeutic development from design of the therapeutic entity and generation of a high-affinity antibody through to production of clinical-grade material. When the manufacture of clinical material is outsourced, the processes must be robust, transferable, and compatible with operation at a range of scales. Herein is presented an approach to ensuring the successful development, transfer, and scale-up of production processes while minimizing development times to ensure speed to the clinic. Finally, some examples of common problems associated with process transfer and scale-up are discussed.
entities, they do not necessarily provide the optimal structure for all such mechanisms of action. Hence a diverse range of antibody formats has been designed to incorporate the specific properties required for each intended application.

For many applications (such as recruitment of effector cells or the complement system for cell killing), the effector functions of the antibody Fc region are not required for therapeutic activity. Furthermore, in some circumstances the presence of the Fc region is undesirable: e.g., when trying to suppress an immune response. In such cases, a Fab fragment containing a single antigen-binding domain can be formatted to provide the required binding activity and optimal pharmacokinetic properties.

The monovalent Fab provides a suitable alternative structure to the full-length IgG when functioning as an antagonist. For example, it has been demonstrated that monovalent Fabs can neutralize cytokines with equivalent potency to whole IgG (1). If valency influences efficacy, such as in the selective delivery of a drug or radioisotope to a tumor cell, multivalent species can be built up from a univalent Fab fragment. Trivalent Fab-maleimide constructs (TFMs) show prolonged retention at a tumor site and increased rates of internalization compared with whole IgG (1). In addition, TFMs show reduced rates of toxicity and more rapid clearance from the blood because they lack the Fc antibody domain.

**The PEG Solution**: Rate of clearance is a key issue that must be addressed for antibody fragments to be used therapeutically, where a long circulating half-life is beneficial for efficacy. Whereas full-length IgG have relatively long serum residence times in humans, Fab and di-Fab fragments are cleared rapidly from the blood. Their half-life can be improved through the covalent attachment of polymers such as polyethylene glycol (PEG). PEG can be conjugated to antibodies by random attachment, typically through lysine residues. However, this technique produces a heterogeneous product and generally leads to a loss in binding activity, probably due to the attachment of PEG within or near the antigen-binding sites of some fragments.

Fab fragments can be engineered to contain a single unpaired cysteine residue in the hinges (the Fab’ fragment), which provides a free thiol group for site-specific PEG attachment (2). Because the PEG is thus attached at a specific point away from the antigen-binding site, the fragment’s antigen-binding activity is maintained. When divalent binding is important for antibody function, a PEG polymer terminating in two thiol-reactive groups can be synthesized and reacted with Fab’ to form a site-specific PEGylated di-Fab’. Conjugation of Fab’ or di-Fab’ to PEG molecules of progressively increasing molecular weight results in increasingly longer serum residence times, with 40-kDa PEG conjugates showing pharmacokinetic profiles similar to whole IgG (Figure 1). PEGylation of proteins has also been shown to reduce immunogenicity and proteolysis (3).

**GENERATING HIGH-AFFINITY ANTIBODIES**

Monoclonal antibodies (MAbs) of defined specificity have traditionally been generated using hybridoma technology. An antibody-secreting hybridoma cell line is derived by fusing an immune B-cell expressing a specific antibody gene with an immortal myeloma cell. Low fusion efficiency, however, leads to immortalization of only a small fraction of the specific antibody-secreting cells available in the immunized animal. In addition, the screening processes used to isolate a clonal population that secretes an antibody of interest are often labor-intensive and time consuming; thus this technology is generally restricted to the generation of rodent MAbs.

An alternative technology that can be used to generate high-affinity antibodies from any species — including humans — is the selected lymphocyte antibody method (SLAM) (4). This technique permits the screening of large cell populations without the need to generate a hybridoma, so a much larger proportion of the immune cell repertoire is analyzed. B-cell lymphocytes isolated from the blood or spleen are cultured in vitro and then screened directly using methods allowing identification of single cells that produce an antibody of interest in very large populations of antibody-secreting cells. Those

![Figure 1: Pharmacokinetics of 125I-labeled IgG, di-Fab’, and di-Fab’-PEG conjugates in rat. Di-Fab’ is conjugated to PEG molecules of differing molecular weights, ranging 5–40 kDa. The graph shows percentage of injected dose (%ID) per mL of blood over time.](image)
individual cells can then be isolated and their heavy- and light-chain variable region genes recovered. Because SLAM improves sampling efficiency of the B-cell repertoire compared with hybridoma techniques, the probability of recovering very rare antibodies with specific functional characteristics and affinities below the pico-Molar level greatly increases.

**E. coli As an Expression Host**

Mammalian cell culture is currently the system of choice for producing therapeutic whole antibodies. However, the long culture times and scale limitations associated with this expression system, combined with an apparent constraint in worldwide manufacturing capacity and relatively high cost of goods, make cell culture production for large-market indications problematic. For most antibody fragments, the host organism of choice is *E. coli*. Antibody fragments can be expressed to high titers of a soluble form within the bacterial periplasm and purified by simple, efficient means.

As an expression host, *E. coli* offers a number of advantages over mammalian cell systems. Its faster growth rate provides shorter culture times, and fermentation processes can be operated at up to five times the scale of mammalian cell cultures. In addition, *E. coli* can be grown in a simple, chemically defined medium free from serum components, without the requirement of expensive viral clearance steps. These factors combine to make *E. coli* an attractive host organism for biopharmaceutical production supplying large markets. Microbial cell lines can also be generated rapidly with no screening for high expressers, no gene amplification, and no adaptation to serum-free medium. These facts considerably reduce process development times to just 8–10 months from selection of the high-affinity antibody to GMP manufacture.

Conventionally soluble Fab’ fragments are produced in *E. coli* by secretion to the periplasmic space, where oxidizing conditions and disulphide-bond–forming machinery facilitate correct inter- and intrachain disulphide bond formation (5, 6). Final fermentation yields of soluble Fab’ in the range of 1–2 g/L have been reported (7). However, different Fab’ fragments are tolerated by *E. coli* to different degrees, with poorly tolerated Fabs tending to misfold or aggregate within the cells. Soluble yields are thought to be influenced principally by the primary sequence of a given protein.

The solubility of low-yielding Fabs can be improved by substituting specific amino acids that increase solubility but do not affect binding affinity (8) or by grafting antigen-binding domains onto the framework of fragments known to express well in *E. coli* (9). Yields can be influenced by the relative rates of expression of the heavy and light chains, with optimal yields obtained when functional expression of the two chains are closely matched (10). Optimal balance can be achieved at the level of transcription or translation, e.g., using different promoter–inducer systems or through modifications to the translation initiation region.

**Manufacturing PEGylated Fabs**

This manufacturing platform was developed for the production of PEGylated Fab’ fragments using proprietary humanization, *E. coli* expression, and PEGylation technology. Once a therapeutic candidate is selected and the appropriate format for its intended mode of action identified, an emphasis is placed on speed to the clinic. Short process-development times are favored, but the processes developed must be robust and transferable to facilitate scale-up for the clinic. Strategies employed to minimize development times include the use of generic manufacturing processes (optimized for each new product) and the parallel development of fermentation and purification steps.

Following selection as a development candidate, an antibody is humanized and its primary sequences optimized for expression as a Fab’ fragment in *E. coli*. To ensure that high yields of soluble Fab’ are consistently achieved, an expression vector system has been developed that enables optimization of the relative rates of light- and heavy-chain expression. Humanized Fab’ fragments are cloned into a suite of expression vectors that differ exclusively in the short sequence between their Fab’ light chain (LC) and heavy chain (HC) genes (the intergenic sequence or

---

**Figure 2**: Culture growth and Fab’ production during a 1000-L *E. coli* fermentation.

Periplasmic Fab’ was measured by protein G HPLC assay. Periplasmic extracts were prepared by incubating cell pellets in a Tris-EDTA extraction buffer for 16 hours at 60 °C. Supernatant Fab’ was measured by ELISA.
IGS). The IGS affects the rate of Fab’ HC translation initiation and hence the relative rates of LC and HC expression. A short IGS gives a high rate of HC translation initiation because ribosomes remain associated with RNA molecules in between light- and heavy-chain synthesis. When the IGS sequence is longer, ribosomes dissociate from the RNA following LC synthesis and must then reassociate to initiate HC translation, which leads to a slower rate of HC accumulation. Further tuning of the HC translation initiation rate can be achieved by modifying the consensus ribosome binding-site sequence. The IGS providing an optimal balance of LC and HC expression along with the highest yield of soluble periplasmic Fab’ is determined experimentally for each new development candidate. This can be done rapidly because vectors can be constructed and tested in parallel; fermentation times are short, and the number of vectors tested can be limited from experience to a small number (generally only two or three).

Once an optimal IGS vector has been selected, fermentation and downstream purification processes are developed in parallel. The E. coli strain W3110 is used for production. The cells are grown to high biomass on a simple, chemically defined growth medium before they are induced to express the Fab’ fragment. The Fab’ is expressed from a tac promoter, inducible with isopropyl β-D-thiogalactopyranoside (IPTG, C₉H₁₇O₅S) or lactose, and OmpA signal sequences direct the Fab’ heavy and light chains to the periplasmic space. The soluble Fab’ accumulates in the periplasm over a 36-hour induction period, with very little loss of Fab’ into the supernatant (Figure 2). The induction system (expression vector and inducer) and culture conditions during induction are tuned to the rate-limiting step for Fab’ production: the rate at which folding can take place in the periplasm. Antibiotic selection is not required for production fermentors, and the expression plasmid shows near-complete structural and segregational stability throughout fermentation.

**Upstream Processing:** Soluble Fab’ is extracted from the E. coli periplasm using a simple chemical extraction procedure (11). Cells harvested by centrifugation are resuspended in a Tris(hydroxymethyl)aminomethane–ethylenediaminetetra-acetic acid (Tris–EDTA) extraction buffer at elevated temperature. The Tris and EDTA synergistically permeabilize the bacterial outer membranes, releasing Fab’ into the supernatant without lysing the cells. Complete, correctly folded Fab’ fragments are stable at the temperature used; their structure and binding affinities are not affected by heat treatment. However, many E. coli proteins and incomplete or incorrectly assembled antibody fragments are unstable at high temperatures, so they will be removed from the process stream at this stage. Hence the high-temperature extraction enriches the process stream for the product, with Fab’ constituting ~50% of total protein at the end of this step.

**Downstream Processing:** Fab’ is purified from clarified periplasmic extract by two- or three-column chromatography steps. For therapeutic antibodies, the cost of purification can represent a significant portion of total production costs. This cost is reduced by avoiding the use of expensive affinity matrices and instead using nonaffinity capture methods such as ion-exchange and hydrophobic-interaction chromatography (which employ low-cost, reusable resins) and appropriately sizing the columns to production scale. A key requirement of the purification process for an E. coli expression system is effective removal of endotoxin. An anion-exchange column will remove the bulk endotoxin present in the process stream, and the purification process effectively reduces them to well below acceptable levels.

Purified Fab’ is conjugated to PEG in a controlled, site-specific reaction. A mild reduction step reduces the free hinge thiol, and the reduced Fab’ reacts with PEG-maleimide to yield a PEGylated Fab’. The reaction produces a defined product, which is beneficial from a regulatory perspective (Figure 3) and is simple to scale up for manufacture. Unreacted Fab’ and PEG are removed from the process stream by one or two further chromatography steps.

In summary, the processes to be optimized include extraction and primary capture, purification of bulk antibody fragments, Fab’ PEGylation, and purification of the PEGylated product. To reduce development times, these activities are carried out in parallel whenever possible. Affinity-captured Fab’ is used for initial PEGylation studies and to establish binding and elution conditions for the column steps used for Fab’ purification. Meanwhile, the extraction and primary capture steps are optimized in sequence. Naturally, certain risks are associated with using generic processes and optimizing steps in parallel. Therefore, experience gained from developing purification steps for previous development candidates is used, and the complete process is demonstrated and optimized in sequence before scale-up for clinical manufacture.
Successful scale-up and technology transfer to a contract manufacturing organization (CMO) requires careful planning and good communication. Prior knowledge of the manufacturing site and intended scale of operation allows process fit to be discussed with the CMO at an early stage. Another benefit is that equipment, scale, and other site-specific limitations are identified early. Any such limitations can be taken into consideration when designing the manufacturing process that will be transferred. Timelines for technology transfer should also be defined early, and items or activities with long lead times should be identified (e.g., cell banking and ordering chromatography resins).

A problem frequently associated with the scale-up of high–cell-density microbial fermentation is the ability to satisfy oxygen demand or cooling requirements at large scale. If it is known in advance that those factors may be problematic on scale-up, the fermentation process can be designed such that oxygen uptake and heat generation are reduced to within achievable levels using nutrient-limiting feeds.

Knowledge of the key process control parameters can facilitate successful transfer and scale-up. For example, Fab’ distribution between the periplasm and the supernatant is sensitive to temperature and the concentration of medium components such as inorganic phosphate, calcium, and magnesium ions. Small changes in one or more parameters can cause loss of Fab’ to the supernatant, reducing final product yield. Special attention to maintaining control of those parameters on scale-up will maintain control of Fab’ distribution with no loss in periplasmic yield (Figure 4).

Differences in the design and operation of bench and production scale equipment can also lead to unexpected problems at the commercial scale. One such example relates to the scale-up of centrifugation. Laboratory batch centrifuges give tight, dry pellets, whereas continuous disk-stack centrifuges produce a heavy phase “slurry,” either carrying over supernatant or losing product in the heavy phase. Neither effect is observed at the small scale. Following scale-up of one process, it was observed that a large proportion of the Fab’ (about 45%) was routinely being lost during the disk-stack centrifugation step that removed cells from the process stream. Those losses were greater than what was predicted due to removal of supernatant in the heavy phase alone. The Fab’ appeared to partition preferentially with the heavy phase, an effect that had not been observed during process development in the laboratory. The problem was resolved by removing the centrifugation step and instead capturing the Fab’ directly from unclarified cell extract using an expanded-bed adsorption (EBA) column. The number of processing steps was consequently reduced, and the yield of Fab’ from the extract increased to over 90% using EBA chromatography at the 2500-L scale.

**Small Can Be Better**

For many therapeutic applications, the properties of an antibody required to mediate a therapeutic effect reside within the Fab’ fragment. This fragment can therefore be used as a “building block” for a therapeutic entity, allowing far greater flexibility in design than can be achieved using standard IgG. Therapeutics based on the Fab’ fragment can be formatted to address specific mechanisms of action through control of valency (by linking a number of Fab’ units) and half-life (by PEGylation).

The technology platform described here uses an *E. coli* expression system for the production of PEGylated antibody fragments. Novel technologies such as SLAM provide the means for the rapid generation of antibodies of very high affinity. Humanized Fab’ fragments can be produced at high titers in soluble form within the *E. coli* periplasm and can be purified by simple, cost-effective means. Site-specific PEGylation provides a method for modifying half-life without loss of binding affinity — and creates a well-defined product. Manufacturing processes for making PEGylated antibody fragments are robust, transferable, and compatible with large-scale operations. *E. coli* production systems also allow high-volume production and offer short process development times, which enables rapid progression to the clinic.
ACKNOWLEDGMENTS
The author would like to thank the following people for their contributions to this work: Neil Weir, Andrew Chapman, Alastair Lawson, Andy Popplewell, David Humphreys, David Glover, Mari Spitali, and Pari Antoniw. Celltech currently has four PEGylated Fab’ fragments in clinical or advanced preclinical development.

REFERENCES

LC Bowering is team leader for microbial fermentation at Celltech R&D Ltd., 216 Bath Road, Slough, Berkshire, SL1 4EN, United Kingdom; 44-1753-534655; leigh.bowering@slh.celltechgroup.com.