The Perfect Host

Microbial hosts can be used to express a range of antibodies and antibody fragments. The different types of fragments need to be understood, including the features that are critical to successful expression, along with the potential advantages and disadvantages.

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The worldwide market for antibodies in 2009 was estimated at $42.8 billion sales (1), and the markets for antibody-based therapeutics have continued to grow since. To date, full-length IgG antibodies have dominated the market. They are stable, have long half lives *in vivo*, and the full biological properties of an antibody. However, they also have a number of potential disadvantages including high cost-of-goods (due to mammalian cell manufacture), poor tissue penetration (due to their size), and limitations in targets that can be bound effectively.

The successful commercial launches of a number of microbial expressed antibody derived proteins, including Cimzia® (UCB) and Lucentis® (Roche), has demonstrated the therapeutic value of antibodies produced in microbial systems. Microbial expression of antibodies can have clear benefits in terms of cost of goods, and faster process development, leading to quicker time to market. In addition, for certain indications, the absence of glycosylation and/or the Fc region can have key benefits.

Antibody Structure

The structure of a full-length IgG (or mAb) is shown in Figure 1. They have a molecular weight of approximately 150 kDa, comprising 12 domains of roughly 12.5 kDa each. They are made up of four separate peptide chains, two identical heavy (H) chain polypeptides and two identical light (L) chain polypeptides (see Figure 1). The H and L chains are composed of four and two domains respectively, with each domain having a similar β-barrel structure and containing one disulphide bridge. The H and L chains are linked together by a combination of disulphide and non-covalent bonds. Each chain contains both variable (V) and constant (C) domains, with the variable domains of the heavy and light chain (VH and VL) containing the highly variable complementarity determining regions (CDR). These regions are located at the N-terminal part of the antibody molecule. Together VH and VL form the unique antigen-recognition site. The amino acid sequences of the remaining C-terminal domains are much less variable.

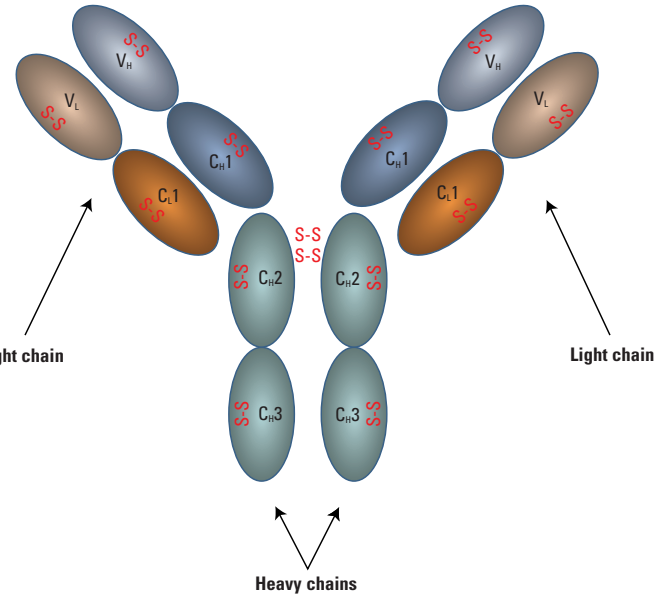

Figure 1: Schematic representations of antibody (IgG) structure

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The non-antigen binding part of an antibody molecule, the constant domain (Fc), mediates several immunological functions, such as binding to receptors on target cells and triggering effector functions that eliminate the antigen. There are applications where the Fc mediated effects are not required or even undesirable. The Fc fragment is also the site of glycosylation in IgGs. Glycosylation, which is the post-translational attachment of carbohydrate groups, is a key feature of certain eukaryotic proteins. In the case of antibodies the glycosylation can play a key part in the effector functions of an antibody. In addition, the nature of glycosylation needs to be similar to that found in native antibodies. Different organisms have different glycosylation patterns (the type and structure of the carbohydrate groups). If this is recognised as being 'foreign', then this will itself be recognised by antibodies and an immune response to this protein will be formed. Fortunately mammalian glycosylation is broadly similar across most species so that glycoproteins from most mammalian cells will not be recognised as foreign by humans. However, glycoproteins from fungi and plants are less similar to human, and may provoke an immune reaction.

Antibody Fragments

A large variety of different types of antibody fragments have been designed and tested. A selection of these antibodies is shown in Figure 2 (see page 62). Note that they all retain at least one

of the variable domains, as this is the key part of the molecule in antigen binding.

The simplest of these are single antibody variable domains (VH or VL). However, single domains from humans and rodents are unstable, and have low antigen binding affinity compared to IgG. However, it has been found that camelids (including camels and llamas) possess V domains that are both stable and can have high affinity. These camelid domains have been exploited by a number of companies as a technology platform for antibody fragments (such as Ablynx and Domantis).

A single chain Fv fragment (scFv) consists of a VL and a VH domain linked together covalently using a peptide. The peptide linker sequences are designed to ensure that the correct binding site is formed, and also that the molecule is stable.

Fab fragments (fragment antigen binding) consist of the full-length light chain (VL and CL), and a half-length heavy chain (VH and CH1). A disulphide bond between the CH1 and the CL links the two domains together. Fab fragments retain the antigen binding part of the molecule, but lack the Fc portion. Lucentis and Cimzia are both Fab fragments.

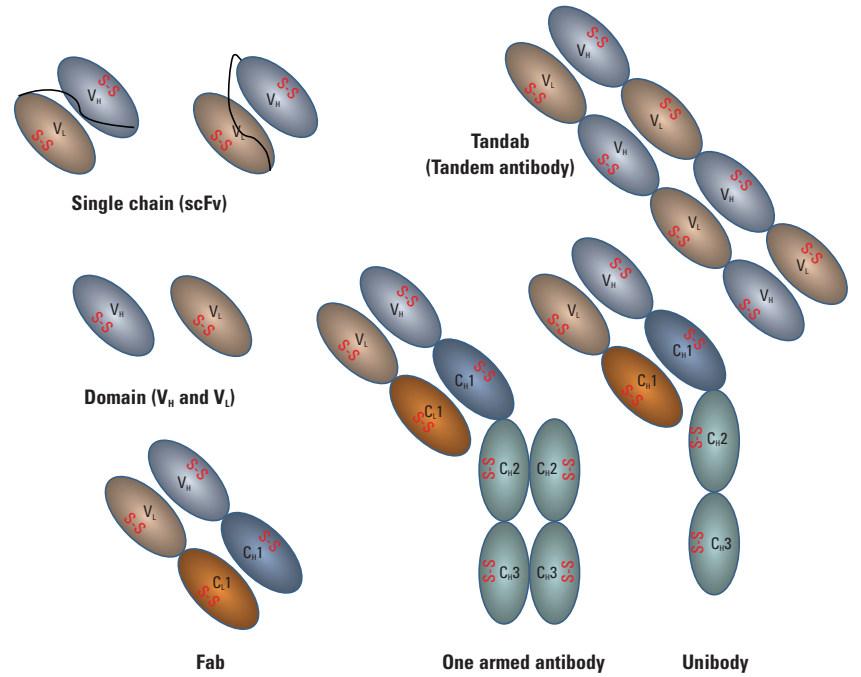
In addition, there is now a wide range of different formats based on the above fragment types, with a range of novel features, such as two different antigen binding sites.

All antibody fragments contain at least one disulphide bond. In fact each antibody domain contains a highly conserved disulphide, which is integral to the correct folding and function of the domain. Therefore any expression system has to be capable of the formation of the disulphide bonds as well as the correct folding of the molecule.

Expression Systems

The majority of commercial antibodies are full-length IgGs that have been produced in mammalian cells. The advantages of mammalian cells is that they can readily form correctly folded proteins, with intact disulphide bonds, as well as the synthesis and attachment of mammalian glycosylation units. In addition to the internal domain disulphides, IgGs also require disulphide bonds to join all the four peptide chains together. Although there have been reports of the production of IgGs in microbial systems, most companies continue to use mammalian cells for IgG production. This is because of the mammalian type glycosylation, as well as the fact that IgGs are generally produced at higher levels in mammalian cells. However, many antibody fragments can be produced at high levels in microbial systems, and lack the glycosylation site in the Fc portion, so that microbial expression becomes a preferable expression route.

Figure 2: Schematic representations of various antibody fragment types



When comparing different expressions systems, it is important to recognise the impact of both product titre, as well as production time, on the ultimate cost of goods. For example, 1g/L is considered a reasonable titre for mammalian cells, but this can take three to four weeks of bioreactor time to achieve. Microbial systems, because they grow much quicker, have a shorter manufacturing time, with bacteria typically taking two to four days and fungi/yeast seven to 10 days. As bioreactor occupancy is a major cost, this needs to be taken together with the titre in order to determine the total cost of production.

It has been found that expression levels are very specific to individual antibody sequences. For example antibody fragments that differ in a few amino acid changes can show widely varying expression levels in the same expression system.

There is a wide range of potential microbial hosts that have been used for antibody expression, but to date the only system that has been used for launched antibody-based products is *E coli*.

Fungal Expression

Fungi and yeast are eukaryotic, so might be expected to be better than bacteria at synthesis of a complex proteins such as antibodies. There have been numerous reports of production of antibodies in filamentous fungi, and yeasts. Filamentous fungi, such as *Aspergillus awamori* and *Trichoderma reesei*, which have been used commercially to produce other recombinant proteins, have been shown to be able to secrete IgGs, Fabs and scFvs (2). However, in order to produce IgG, it was necessary to produce the individual chains as fusion proteins

with glucoamylase, which is an enzyme *Aspergillus* secretes at high levels. In order to produce authentic IgG, it was then necessary to use a protease to cleave the fusion protein. Although the product titres were reasonable, the final product was heterogeneous, and the process was not suitable for commercial manufacture.

Yeasts have been used to produce a number of commercial therapeutic proteins. Baker's yeast (*Saccharomcyes cereviseae*) has been shown to be able to produce antibody fragments but the titres tend to be fairly low. There has been more success with methylotrophic yeast such as *Pichia pastoris*, with yields of up to 4.9 g/L for an scFv (3). However, expression of two chain antibodies, such as Fabs, have been less successful, with high proportions of incorrect product (for example light chain dimers) often being produced (3).

One drawback with the use of fungi and yeast for IgG production is that their native glycosylation is very different from humans, and so it is not suitable for therapeutic molecules. However, this problem can be avoided with the use of engineered strains, in which the mammalian glycosylation pathways are expressed. This has been shown to lead to mammalian type glycosylation and subsequently human-like IgGs can be produced (4,5).

Bacterial Expression

The most common approach to antibody fragment production in *E coli* (and other Gram negative bacteria) is to fuse a bacterial secretion signal sequence to the start of the gene. This enables the protein to be secreted into the periplasm, where disulphide bond formation and folding of the protein can take place. For single chain antibody fragments, such as scFvs, it is possible to obtain correctly folded product at high yields (2-3 g/L). However, there can be a large variability in the yields for similar antibody fragments of the same type, which may only differ by a few amino acids. There has been some work in identifying residues that may lead to poor secretion, but determining whether a given antibody will secrete in useful amounts is still largely empirical (7). Gram negative bacteria such as *E coli* do not secrete large quantities of proteins, and so the secretion and folding pathways have a limited capacity. One way to improve yields is to control the rate of protein expression using a system such as pAVEway™ (MSD BioManufacturing Network), so that the rate of expression matches the secretion and folding capacity of the cell (8). As *E coli* has a limited secretion capacity, using a powerful promoter can lead to blocking the secretion machinery, and it has been found that reducing the rate of expression can lead to higher yields of secreted soluble product (9).

Two chain molecules such as Fabs have extra disulphides compared with single chain antibody fragments, and require correct folding of the light and heavy chain, as well as correct pairing of the two chains to form an active molecule. For this reason, the yields tend to be lower than for equivalent single chain molecules, with 1-2 g/L being the best achieved, but the yields are also protein-sequence-dependent (10).

The secretion of full-length IgG in *E coli* has been reported with a yield of 150 mg/L, but this required significant process optimisation (11). This further strengthens the case for mammalian cells as the expression route of choice for full-length 1g/Gs, where yields of less than 1g/L are routinely achieved.

Disulphide bonds do not form readily inside bacterial cells. Expression of antibody fragments within the cell usually leads to misfolding of the protein, and subsequent inclusion body formation (insoluble protein). It is possible to express antibody fragments intracellularly as misfolded inclusion bodies, which can then be extracted, denatured and refolded to form the correct structure. While this is feasible for single chain molecules, and is a potential alternative to secretion, for antibodies with more than one chain, such as Fabs, this route usually result in very low yields.

The cost advantages of being able to produce soluble active molecules intracellularly in *E coli* has led to a number of new scaffold proteins, or antibody mimetics, being developed. These are proteins derived from natural sources that have been engineered to

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contain variable regions, which can act as antigen binding regions. Many of these scaffolds have been selected due to their the ease of expression in *E coli*; for example, DARPins (Molecular Partners) and anticalins (Pieris), which can be formed solubly within the cell.

There are reports of both Gram negative (other than *E coli*) and Gram positive bacteria being used to express antibody fragments, but most of the yields are fairly low (2). Gram positives do secrete much higher amounts of protein than Gram negatives, but to date the yields from expression of antibody fragments has not been any higher than for *E coli*.

Conclusion

Although mammalian cells remain the system of choice for full-length antibodies, microbial systems, and in particular *E coli*, are the first choice for most antibody fragments. If expression of a molecule can be achieved in *E coli*, then this will usually lead to a process with the lowest cost of goods, and fastest speed to market.

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