The Emergence of Antibody Fragments and Derivatives

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Antibody fragments have distinct advantages over antibodies, and with both commercially viable fragments in the marketplace and numerous candidates in various stages of development, they look set to offer a credible therapeutic alternative to full-length mAbs.

The groundbreaking work of Köhler and Milstein, first published in 1975 (1), introduced methodologies for the production of monoclonal antibodies (mAbs) of precise and consistent specificity, and has for this class of proteins being able to deliver their therapeutic promise.

Currently five mAbs – Herceptin® (trastuzumab, Genentech/Roche), Avastin® (bevacizumab, Genentech/Roche), Rituxan® (rituximab, Biogen Idec/Genentech/Roche), Humira® (adalimumab, Cambridge Antibody Technology/Abbott) and Remicade® (infliximab, Centocor/J&J) – respectively account for close to three-quarters of the revenue in this biopharmaceutical sector, with the total market being made up of just over 20 mAbs. The dominance of the five above-named mAbs is due to a number of factors, including: being first to market, market expansion across indications and the inherent accumulation of clinical data, ensuring that newer therapies are used only as a second line. However, there are more than 200 mAbs in various phases of clinical trials. Generally, the outlook is very promising for first-generation mAbs, as their exposure to generic competition is limited. In the main, this can be ascribed to a mixture of technical, intellectual property and regulatory barriers.

What is clear is that this sector is commercially attractive, but also a very competitive marketplace – both now and for the foreseeable future. These factors go some way to explaining both the emergence and growth of derivatised antibody fragment technologies. Antibody fragments also represent part of the natural evolution of the sector, by being able to utilise the wealth of information already accumulated for mAbs, such as humanisation and affinity maturation. For reference, it is beyond the scope of this review to cover alternative scaffolds such as ankyrin repeats, anticalins, adnectins, affibodies and kunitz domain proteins.

ANTIBODY FRAGMENTS

Antibody fragments – by their inherent nature – can have distinct advantages over full-length mAbs. One example is their ability to penetrate tissues or organs more comprehensively, thereby providing scope for enhanced therapeutic effects (for example, the delivery of cytotoxic radioisotopes), and improved diagnostic techniques (for example, rapid clearance of radio-labelled imaging agents). The smaller size of antibody fragments may also allow interactions with cryptic epitopes, such as enzyme active sites, which mAbs may not be able to access. Similarly, removal of the constant (Fc) region can decrease non-specific effector function and lower potential antigenicity. Such fragments can be viewed as the smallest functional building block from which it is possible to make more complex molecules. Common to all antibody fragments is a conserved framework region with three complementarity-determining regions (CDRs), which can specifically interact with an antigen.

Of the many antibody fragments now described, two dominant fragments in commercial development are antigen binding fragments (Fabs) and single chain variable fragments (scFvs). Of the 54 antibody fragments that have entered clinical studies, 56 per cent are Fabs and 35 per cent are scFvs, with the remaining nine per cent being made up of other derivates (2). It should be noted that this distribution can be attributed in part to the phases of development within the field of antibody fragment technologies. With regard to size, scFvs are ~30kDa (approximately 85 per cent smaller than whole mAbs at ~150kDa), and Fabs are ~70kDa, (approximately 65 per cent smaller). More recently, an even smaller functional unit – called domain antibodies (dAbs) – have been shown to have therapeutic promise; these are either equivalent variable heavy or variable light chains from scFvs. Other similar domain antibodies, but with less well-known formats, include variable novel antigen receptors (V_{NAR}), derived from cartilaginous fish, and variable heavy chains (V_HH) from camelids (*Camelidae*). Both V_{NAR} and V_HH formats possess smaller domain sizes than scFvs (12kDa and 15kDa, respectively), and are similar in size to dAbs. They appear to have diverged from typical murine or human immunoglobulins and, as whole antibodies, only have one variable-like domain. Similarly, unlike conventional murine or human variable domains, the antigen-specific binding loops (CDRs) are typically longer, allowing

them to access antigen cavities that typical CDR binding loops would be unable to penetrate.

The general development of phage display technologies has allowed the generation of large libraries of fragments and derivatives thereof. These libraries have been successfully used to screen for the isolation of antibody fragments specific for different types of ligands – ranging from haptens and carbohydrates, through to proteins – utilising common high-throughput screening methodologies. Interestingly, many of these fragments are amenable to protein engineering that is either not possible or difficult to achieve with whole mAbs. For example, the linking of two identical or different scFvs to form a diabody can give rise to either bivalency or bispecificity – thereby potentially enhancing avidity to antigens or mediating cross-linking, such as the recruitment of cytotoxic T-cells to mediate the termination of a target cell. Similarly, multimeric forms of other antibody fragments have been described in the literature, such as Fab_2, Fab_3, Triabodies and Tetrabodies (3).

Currently, only three FDA-approved antibody fragments are commercially available: ReoPro® (abciximab, Centocor/Lilly), a platelet aggregation inhibitor Fab; Lucentis® (ranibizumab, Genentech/Novartis), an affinity matured Fab fragment derived from Genentech's anti-VEGF mAb Avastin® for the treatment of 'wet' age-related macular degeneration (vision loss); and more recently, Cimzia® (certolizumab pegol, UCB) an anti-inflammatory PEGylated Fab for the treatment of Crohn's disease and rheumatoid arthritis.

PRODUCTION ISSUES

Taking the mAb Remicade® as an example, it is possible to highlight the need to develop cheaper and more efficient methods for the commercial production of mAbs and, potentially, via antibody fragments. Typically, Remicade® is administered bi-monthly with a dosing regimen of 5mg/kg; thus, with a conservative estimate of at least half a million patients worldwide being treated, the annual demand would exceed 1,000kg. This can be compared with other therapeutic proteins, where the demand can be 10-fold less (4). Therefore, production represents one of the main cost-drivers for commercial manufacture of mAbs and, in the future, antibody fragments. Mammalian Chinese hamster ovary (CHO) cells are the predominant cell lines used in the manufacture of mAbs; however, generally, mammalian cell lines are difficult to culture and grow, and are expensive to upscale. Hence, there is a growing interest in microbial cell culture, where such obstacles have already been addressed.

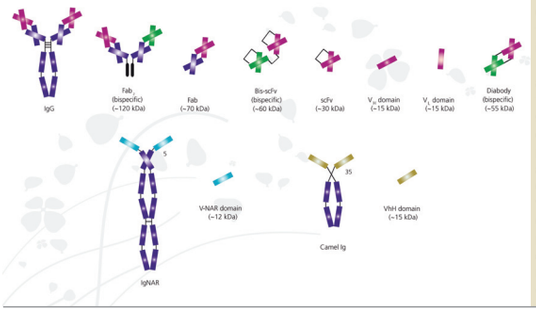
At Novozymes, we have developed proprietary yeast expression systems to help address some of these issues. *Saccharomyces cerevisiae* is a predominant yeast used in the production of antibody fragments, both as discrete entities and as fusions, where the antibody fragment is genetically fused to another protein such as albumin. Improvements in yeast expression have resulted in higher yields and decreased or no proteolysis. Similarly, the solubility and stability issues associated with antibody fragment production have been addressed (5); hence, multigram expression and secretion of functional antibody fragments and fusions is common. This, together with the requirement for inexpensive media and ease of scale up to industrial-scale fermenters, bodes well for yeast production to be a viable and cost-effective alternative to mammalian cell culture production.

Attempts have been made similarly to express antibody fragments using prokaryotic expression systems, such as *Escherichia coli* (6); however, they can have inherent limitations. Secretion of antibody fragments into the culture media can lead to cell lysis and associated product loss. Also, prokaryotes do not always have the machinery for correct folding or necessary post-translational modifications associated with eukaryotic protein expression. These limitations can mean that insoluble aggregates are produced. Attempts to re-fold such products *in vitro* can place cost-prohibitive demands on a manufacturing process, as well as the robustness of a production process at scale. Similarly, the cellular complexities associated with fusion protein expression make prokaryotic expression difficult (7).

EXTENDING HALF-LIFE

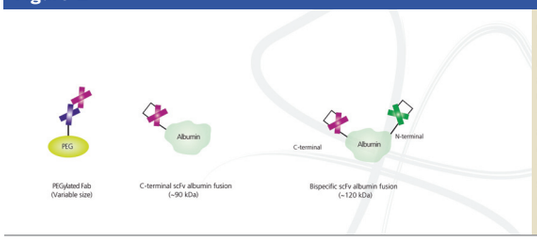
The relative ease with which antibody fragments can be produced will mean that they are credible alternatives to mAbs. However, while their short half-life may be an

Figure 1



Graphical representation of antibody fragments, as derived from a 'typical' isotype G immunoglobin (IgG), a shark Ig new antigen receptor (IgNAR) and a camelid VHH-Ig. Constant domain frameworks for all the antibodies are shown in purple, with the variable domains (V) shown in blue, brown, pink and green. Both camel and shark antibodies have a single V domain (VhH and V-NAR), and IgGs have two V domains called heavy (VH) and light (VL) respectively. A selection of the possible antibody fragments which can be derived from these full length antibodies are shown, with approximate sizes (kDa).

Figure 2



Graphical representation of a typical PEGylated Fab, a C-terminal monovalent scFv albumin fusion and a bispecific N- and C-terminally fused scFvs albumin fusion molecule. Approximate sizes are shown for the albumin fusions (kDa). The size of a PEGylated fragment is dependent upon the size and number of covalently linked polyethylene glycol (PEG) polymers.

advantage in procedures such as radio-imaging, the converse would be true for therapeutic delivery. From this viewpoint, all antibody fragments could be viewed as having a distinct disadvantage to mAbs. However, two potential means of extending antibody fragment half-life are the use of PEGylation and the expression of fusion molecules.

PEGylation has been used in the synthesis of Cimzia® for, among other things, increasing the Fab's hydrodynamic size (its size in solution), solubility and stability. In general, the process involves the covalent attachment of polyethylene glycol (PEG) polymers to the therapeutic molecule. However, although PEGylation has been used widely for improving biopharmaceutical drugs, there are potential drawbacks with antibody fragments. By virtue of its nature, PEG is polydisperse, and thus variations in both the number and positions of covalently linked PEG chains give rise to variability of the final product; this in turn has the potential to affect clinical and product characteristics. This polydispersity is more problematic the higher the molecular mass of PEG used. Methods do exist for site-specific PEGylation and these give rise to well-defined products – but they are reliant on the protein having defined positions suitable for PEG attachment. This may involve having to pre-engineer a protein and, for smaller antibody fragments, this is potentially a time-consuming exercise, along with a limited number of sites available for mutations.

An emerging viable alternative to PEGylation for potentially increasing circulation half-lives – as well as stability, solubility, proteolytic resistance, non-toxicity, non-immunogenicity and higher recombinant expression yields – is the genetic fusion of antibody fragments to long circulatory half-life proteins, such as human serum albumin.

In this context, we have developed a proprietary technology platform called albufuse® albumin fusion. As this involves the production of a single fused polypeptide molecule, it is a single-step expression solution that eliminates the need for costly post-production processing. The flexibility of such fusion technology enables antibody fragments to be bound at either or both the N- or C-terminus; thus, the opportunity to generate a fusion molecule with monovalent, bivalent or bispecific affinity is possible and has indeed been realised. We have used our experience in both yeast expression and fusion protein technologies to demonstrate the production and secretion from *S. cerevisiae* of functional antibody fragment fusions in excess of 5g/L, in defined-media high cell-density fermentations.

CONCLUSION

Although still a relatively small sector, the general interest in antibody fragment technologies is growing. The development of phage display libraries and general methods of high-throughput screening has meant that the development of potential drug candidates has been significantly enhanced. Similarly, with both commercially viable therapeutic antibody fragments in the marketplace, and numerous candidates in various stages of both pre-clinical and clinical development, these molecules are credible alternatives to full-length mAbs as therapeutics.

Note

The author would like to direct interested readers to Reference 2 for a comprehensive analysis of both the historical and current status of the clinical development of antibody fragments.

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