Drugs of the Future - Bispecific Antibodies

An investigation of future development needs

Thomas Andersson, Anisha Khan, Therese Koivula, Terese Larsson, Fabian Svahn, Amanda Wahlsten

Client: GE Healthcare Bio-Sciences AB
Client representative: Daniel Larsson
Supervisor: Karin Stensjö

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Biology Education Centre, Uppsala University
Abstract

This report reviews the field of bispecific antibodies, artificially engineered antibodies that have the ability to bind two or more different antigen simultaneously. Historical as well as recently developed techniques are demonstrated, together with formats in preclinical and clinical development. We studied the field with the future needs of the developers in mind, when it comes to the processes and tools that can be offered by GE Healthcare Biosciences AB.

The development of bispecific antibodies gave rise to new challenges and product-related impurities, which are handled by various methods. We argue for, based on the formats in clinical and preclinical development, that the methods already used to purify monospecific antibodies remain the most successful methods for the purification of bispecific antibodies. This, together with the design strategies that resolve the initial bottle-necks, ensures that the needs of the developers are met to the same extent as for monoclonal antibodies. The methods and formats demonstrated here do not represent all that are available or under trial.
Abbreviations

BEAT - bispecific engagement by antibodies based on the T cell receptor
BiTE - bispecific T cell engager
bsAb - bispecific antibody
CH - constant heavy chain
CHO cell - chinese hamster ovary cell
CL - constant light chain
DVD-Ig - dual-variable domain immunoglobulin
Fab - antigen-binding fragment
Fc - crystallizable fragment/constant region
FcR - Fc receptor
Fv - variable fragment
HC - heavy chain
HEK cell - human embryonic kidney cell
Ig - immunoglobulin
IgG/IgA/IgD/IgE/IgM - different classes of immunoglobulins present in humans
KIH - knob-into-hole
LC - light chain
mAb - monoclonal antibody
MAT-Fab - monovalent asymmetric tandem Fab bispecific antibodies
scFv - single chain variable fragment
SEEDBody - strand-exchange engineered domain
SEC - size exclusion chromatography
VH - variable heavy chain
VL - variable light chain
**Definitions**

**Asymmetric design** - antibody design that has no plane of reflection.

**Bispecific** - an antibody with the ability to bind two different antigens.

**Bivalent** - when an antibody binds only two antigens.

**Design strategy** - a way to design antibodies to circumvent product-related impurities.

**Downstream** - the processes involved in the harvesting and purification of a certain product.

**Epitope** - the part of the antigen that is recognized by the antibody.

**Format** - the structure of an antibody, unrelated to the choice of antigen binding sites.

**Heterodimer** - an antibody formed by association of two different polypeptides.

**Homodimer** - an antibody formed by association of two of the same polypeptides.

**Monospecific** - an antibody able to bind only type of antigen.

**Monovalent** - an antibody able to bind only one antigen at a time.

**Immunogenicity** - the ability of a substance such as an antigen or epitope to induce an immune response.

**Parental antibodies** - antibodies used to produce e.g. a bispecific antibody.

**Platform** - a method to obtain a certain antibody format.

**Single-gene constructs** - a product produced by transcription from a single gene.

**Symmetric design** - antibody design made up of identical parts facing each other.

**Upstream** - the processes involved in the design and expression of a certain product.

**How we refer**

If a reference is placed at the end of a sentence before full stop, it refers only to the sentence itself. If a reference is placed after a full stop, it refers to all the text in the paragraph between this reference and any previous reference.
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1 Introduction

The market for recombinant proteins has in the later years been dominated by monoclonal antibodies (mAbs). Monoclonal antibodies are monospecific, meaning they target one type of antigen. These artificial antibodies have been successful as drugs in e.g. oncology.

Today, a new type of molecule with great therapeutic potential is on the rise. Bispecific antibodies (bsAbs) are artificially engineered antibodies that have the ability to bind two or more antigen simultaneously. An increasing number of bispecific antibodies enter clinical trials and there are already over 100 different formats of bsAbs (Brinkmann & Kontermann 2017). Bispecific antibodies are thereby opening up for entirely new therapeutic applications, as well as improved old ones.

GE Healthcare Biosciences AB (GE Healthcare) suggested this project because they are involved in the purification of monoclonal antibodies and therefore want to know the future needs of their clients. The project gives an overview of the field of bispecific antibodies where we have explored problems and their solutions, and listed formats in clinical and preclinical development. When looking at the different formats we have studied their structure, therapeutic effect and upstream and downstream processes. We have also listed advantages and disadvantages. We consider these topics to be the most important for understanding the field. The formats in clinical development demonstrate the varying structures and design strategies currently used, while the formats in preclinical development demonstrate what will become relevant for GE Healthcare in the future. An overview of the formats and strategies demonstrated in this report can be seen in figure 1.

![Figure 1. A schematic figure of the clinical and preclinical bispecific antibody formats and design strategies described in this report. For further details about strategies see section 3.1 and 3.2. For further details about clinical and preclinical formats see chapter 4 and 5, respectively.](image-url)
Based on our investigation of the formats in clinical and preclinical development, one of our conclusions is that the methods already used to purify monospecific antibodies remain the most successful methods for the purification of bispecific antibodies. The production of bispecific antibodies initially suffered from a low yield and product-related impurities. However, many different innovative strategies combat this issue. Taken together, we conclude that the downstream production of bispecific antibodies no longer differs significantly from monoclonal antibodies, and that the needs of GE Healthcare’s potential clients are met. A “business-as-usual” approach is therefore viable. This conclusion is described in more detail in chapter 6.

2 Understanding the structure and production of antibodies

This chapter explains the structure and production of antibodies, both the naturally occurring and their engineered bispecific relatives. The development of bispecific antibodies is based upon the existing field of mAbs. It is therefore important to demonstrate these mAbs, before moving on to bispecific antibodies.

2.1 Monoclonal antibodies

Native antibodies or immunoglobulins (Ig) are glycoproteins produced by one type of immune cells called B-lymphocytes, as a part of the adaptive immune system. The mAbs are natural or recombinant antibodies that are produced by hybridoma cells. Hybridoma cells are formed by fusion of B cells and cancerous plasma cells called myeloma cells. All monoclonal antibodies produced by one hybridoma cell line are thus identical. (Wood 2011) Monoclonal antibodies are usually bivalent i.e. can bind two epitopes. An epitope is the part of the antigen which an antibody targets. The mAbs are monospecific since it can only bind to one type of antigen, i.e. the two antigen binding sites are identical. (Lodish 2016)

The most common isotype of antibodies produced by the immune system in humans is immunoglobulin G (IgG). It consists of four different polypeptide chains, see figure 2. Two of these are identical heavy chains (HC). The heavy chains both consist of one variable domain denoted VH and three constant domains denoted CH1, CH2 and CH3 respectively. The C-terminal of the heavy chains is at the end of the CH3 domain. (Lodish 2016) The heavy chains are held together at the hinge region which is responsible for a covalent linkage between the two chains. It is moreover a flexible region which enables the distance between the Fabs to vary in length. (Brinkmann & Kontermann 2017) There are also two identical light chains (LC) which are covalently attached to the heavy chains by disulfide bonds. These consist of one variable domain denoted VL and one constant domain denoted CL. The domains of bsAbs are denoted in the same way. The light chains together with the VH and CH1 forms the antigen binding fragments (Fabs) that can bind a single antigen. (Lodish 2016)

The "spine" of the antibody that covalently links the two heavy chains is called the crystallizable fragment (Fc). It consists of the CH3 and CH2 domains of both heavy chains
and can bind various receptors. These Fc receptors (FcRs) give the Fc region the ability to enable different effector functions in the immune system. Antibodies with Fc regions can bind to Fc receptors of certain immune cells that mediate cytotoxicity i.e. the killing of cells. This cytotoxic activity can cause high immunogenicity. (Wood 2011) We have seen that, in an attempt to reduce this, some formats in development utilize modified IgGs with reduced effector functionality. The Fc region also binds the neonatal Fc receptor which significantly extends the half-life of IgG antibodies (Kuo & Aveson 2011). A longer half-life of a therapeutic antibody is associated with many benefits with some being improved treatment efficiency, fewer treatments and reduced costs.

Figure 2. A schematic figure of an IgG mAb. The IgG mAb consists of two pairs of heavy (HC) and light chains (LC) bound together by disulfide bridges shown as black bars. The two heavy chains are displayed in blue while the two light chains are shown in green. One HC consists of the domains VH, CH1, CH2 and CH3. One LC consists of the domains VL and CL. The two Fab regions constitute the two binding regions of the antibody. The crystallizable fragment (Fc), also known as the constant region, enables the antibody to bind different receptors.

Besides the IgG isotype, there are four other isotypes of Fc regions in mammals; IgM, IgA, IgD and IgE. The Ig gets its name and class based on its Fc isotype. These have different functions in the immune response. The light chain also has two isotypes; kappa (κ) and lambda (λ). These are characterized based on the amino acid sequence in the constant region. A mAb will contain two heavy chain associated with either two κ chains or two λ chains. (Lodish 2016)

2.2.1 Today’s purification methods of monoclonal antibodies

According to GE Healthcare’s handbook “Affinity chromatography, Vol.1: Antibodies”, the purification of mAbs is most often done using affinity chromatography. If high purity is required, a further polishing step can be performed. For this, size exclusion chromatography
(SEC) is frequently used. The chromatography resins, *i.e.* the stationary phase, contains an immobilized ligand which has affinity to the mAb of interest. This gives affinity chromatography its high specificity. There are, according to the handbook, three commonly used affinity ligands: protein A, protein G and protein L. Protein A and G both bind to the Fc region of the antibody while protein L binds to the VL domain of the κ isotype. The interaction with protein L does not require any Fc region which gives it a wider range of possible target antibodies. For more details on available purification methods, see appendix 1.

### 2.3 Bispecific antibodies

The main difference between mAbs and bispecific antibodies is that bsAbs can bind two different epitopes, either on the same or another antigen, see figure 3. They are, excluding IgG4, artificial proteins not found in nature. Currently, bispecific antibodies are commonly produced via fusion in mammalian cell lines. The most prevalent cell lines are CHO and HEK cells. For more information about these cell lines and how they are used to produce antibodies, see appendix 2.

**Figure 3. A schematic figure of a bispecific antibody.** The chains displayed in pink and purple are the heavy and light chains from one monospecific antibody. The heavy and light chain of the other monospecific antibody are displayed in blue and green, respectively.

There are nowadays more than 100 different formats of bsAbs. Today, an increasing number of bsAb enter clinical trials and some are already on the market. (Brinkmann & Kontermann 2017) As it will be apparent in this report, the structure of different formats varies a lot. From small formats with only variable regions, *i.e.* fragment-based/non-IgG-like formats, to larger formats with constant regions, *i.e.* Fc-based/IgG-like formats (Fan *et al.* 2015). There is no single “best format”. Different formats come with varying advantages and disadvantages and can be successful for different therapies (Brinkmann & Kontermann 2017).

There are various applications of bsAbs including diagnosis, prophylaxis and therapy. A common approach is the retargeting of effector cells, such as T cells, to a tumor cell for
cancer therapy. This is possible by designing the bsAbs to bind one epitope on the T cell and one epitope on the tumor cell and in this way directing the immune system to the diseased cell. (Duell et al. 2019) Bispecific antibodies are also potential pharmaceuticals for treatment of other diseases such as autoimmunity, bleeding disorders and infections. (Brinkmann & Kontermann 2017)

2.3.1 Fragments and linkers are common structural elements

As mentioned, there are many formats of bsAbs which may vary a lot in structure. Different fragments are occurring as parts of bsAbs. Some frequently used fragments can be seen in figure 4. As can be seen in the figure, some of the fragments are connected by linkers. We have seen that the most often used linker is the G4S-linker. Read more about linkers in appendix 3.

![Figure 4. Some frequently used fragments in bsAbs as presented in “Antibody Production in Microbial Hosts” by Rathore & Batra (2016). scFv: single chain variable fragment. A VH and a VL domain connected by a linker. Fab: antigen binding fragment. Consists of HC constant and variable region connected with LC constant and variable region. scFab: single chain antigen binding fragment. A Fab with a linker between the HC and LC. Diabody: consists of two VH/VL domains connected by linkers. sVD: single variable domain comprised of a VH, often derived from camelid or shark antibodies.](image)

2.3.2 The quadroma method pioneered the construction of bispecific antibodies but suffers from low yield

The quadroma method was one of the first developed methods for producing bispecific antibodies. The method requires two hybridomas that are combined by somatic fusion generating a hybrid hybridoma, also called a quadroma. The hybrid cell line in a quadroma co-expresses the genes encoding two heavy chains and two light chains. Each pair of heavy chain and light chain are from respective parental hybridomas. (Schaefer et al. 2015) This allows the combination of heavy and light chains of two different monoclonal antibodies in one single cell. The constant region of these chains can be of the same or different isotypes
and even retrieved from different species. (Brinkmann & Kontermann 2017) The resulting bsAbs generated from quadroma cell lines have the binding properties of their parental antibodies and resemble conventional IgG-like antibodies (Fan et al. 2015). Quadroma continues to be used in the development of novel bispecific antibody formats. One of the only two bispecific antibodies that have received market approval is catumaxomab (Creative Biolabs, 2019). Catumaxomab is a trivalent Triomab produced by the quadroma technology where cell lines from rat and mouse are utilized (Duell et al. 2019).

However, there are issues with the quadroma technology. One of the main problems is the formation of unwanted antibodies along with the target bsAbs, see figure 5. There can be up to nine variants of unwanted antibodies that are either non-functional or monospecific. These variants may also be referred to as product-related impurities. The excess formations are due to homodimerization instead of heterodimerization of the heavy chains as well as random binding of light chains with heavy chains. The former is referred to as the heavy chain problem while the latter is referred to as light chain mispairing. (Sedykh et al. 2018) Homodimerization refers to when two heavy chains with the same binding properties are associated meanwhile heterodimerization is the opposite, hence resulting in a bispecific antibody. Light chain mispairing, on the other hand, is the incorrect association of a certain heavy and light chain pair. This results in non-functional binding domains for the specific antigen the bsAb is being constructed for. (Brinkmann & Kontermann 2017) This normally leads to a low yield of target bsAbs which is a significant disadvantage with the quadroma technology (Sedykh et al. 2018).

Figure 5. A schematic figure of the possible heavy and light chain formations in a quadroma cell line. The green and blue chain represent the light and heavy chain from one of the parental antibodies. The purple and pink chains represent the light and heavy chain from the other parental antibody. The first two formations seen in the “incorrect” group are biologically functional but not bispecific. The rest of the incorrect formations are not functional.
2.3.3 The transition from the quadroma method to recombinant fusion constructs expressed in mammalian cell lines

All the formats studied and demonstrated in this report were the result of genetic fusion and expressed in mammalian cell lines. We interpret this to be due to the product-related impurities when using the quadroma method. Expressing the different chains in a mammalian cell does not solve the problem with product-related impurities. However, it allows for the expression of genetically modified chains. This, in turn, enables the use of different design strategies that combat the problem with incorrect chain pairing. Different design strategies are demonstrated in chapter 3, together with formats in clinical development. For information about the cell lines used in the production, see appendix 2.

3 An overview of design strategies

This chapter describes different strategies and approaches that solves issues regarding the manufacturing of bispecific antibodies. We have defined a strategy as a manufacturing approach that is applicable in the design of bsAbs where the format in development is not uniquely defined by the method used. A format will in this report refer to a structurally distinct antibody, not restricted to having a certain set of binding domains. In other words, two different formats can use the same strategy to solve a certain problem, while still being structurally different. The strategies described are presented in table 1, where whether they solve the heavy chain problem and/or light chain mispairing is indicated.

Table 1: Table showing the different strategies discussed in the report. It also includes what type of chain problem each strategy solves i.e. heavy chain (HC) problem and/or light chain (LC) mispairing.

<table>
<thead>
<tr>
<th>Strategy</th>
<th>Solves HC problem</th>
<th>Solves LC mispairing</th>
</tr>
</thead>
<tbody>
<tr>
<td>KIH</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>SEEDBody</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>BEAT</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>CrossMab</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Orthogonal Fab</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>DuetMab</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>CH1-CL interface mutations</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

3.1 Strategies for solving the heavy chain problem

One way of diminishing the amount of product-related impurities is by solving the heavy chain problem. That is, promoting heterodimerization by making homodimerization unfavorable. Three strategies that solve this problem, the pioneering knob-into-hole as well as SEEDBody and BEAT, are discussed below.
3.1.1 Knob-into-hole (KIH)

The KIH method is described in the patent “Knobs and holes heteromeric polypeptides” (US8216805B2, 2012). The method utilizes the introduction of protuberances; smaller protruding elements, or “knobs”, at one or multiple interfaces of an initial polypeptide (US8216805B2, 2012; Ridgway et al. 1996). These protuberances are introduced together with corresponding cavities, or “holes”, at corresponding interfaces of an additional polypeptide, see figure 6 (US8216805B2, 2012; Ridgway et al. 1996). Both the protuberances and cavities is introduced into two separate constructs of choice at their respective corresponding interfaces, and is done by altering the nucleic acid encoding the polypeptide in question (Klein et al. 2016; US8216805B2, 2012). By replacing smaller amino acids side chains, *e.g.* alanine or threonine, with larger ones, *e.g.* tyrosine or tryptophan, it is possible to introduce protuberances at predetermined locations of interference (US8216805B2, 2012). Corresponding compensatory cavities is subsequently possible to introduce into a polypeptide by means of replacing larger amino acid side chains with smaller ones.

The knobs and holes established are positioned in the CH3 region of antibodies in such a fashion as to hinder the formation of homodimers by making such formations sterically unfavorable. Subsequently, this promotes the formation of heterodimers, and does so by offering an effective and sterically favorably “docking system”, as can be seen in figure 6.

![Figure 6. A schematic illustration of heterodimerization induced by the knob-into-hole method. The purple bar represents an engineered “knob” designed on one, here seen in red, antibody in such a way and place as to fit into a sterically complementary “hole” located on a, here blue, separate antibody monomer.](image)

The knob- and hole-dimers may be expressed in a single cell line or in separate expression systems. The host cell can be transformed with a single vector or independent vectors containing DNA encoding all polypeptides. After expression, the polypeptides are combined to form a fully assembled antibody. This allows the formation of desirable heterodimers with high precision and limited mispairing. However, this method is not limited by a set number of protuberance and cavities. Sterical unfavourability or favorability between heterodimers can
be increased or decreased by introducing multiple corresponding knobs and holes. (US8216805B2, 2012)

3.1.2 SEEDBody
SEEDBody (Sb) is another approach to induce correct heavy chain pairing developed by Davis et al. (2010). SEED stands for strand-exchange engineered domain. Much like the KIH strategy, it manipulates the interface in the CH3 region to promote heterodimerization. The approach “interlocks” beta strand regions of the human IgG and IgA CH3 domains without introducing non-native disulfide bonds. Both SEED CH3 domains are built out of alternating CH3 beta strand sequences of human IgA and IgG. They are complementary, thus promoting correct heterodimerization, see figure 7. (Davis et al. 2010)

The yield of correctly heterodimerized Sb in general ranged from 85% to 95% of total protein purified from transfected human embryonic kidney (HEK) cells in a study by Davis et al. (2010). The yield was also equivalent to Fc-based fusion proteins (Davis et al. 2010), known for their high expression in mammalian cell lines (Lo et al. 1998). For more information about mammalian cell lines, see appendix 2.

![Figure 7. A schematic figure of the SEEDBody method.](image)

A: the two heavy chains with their engineered SEED CH3 domains depicted with red and blue bars. B: The two heavy chains associated as heterodimers. C: A schematic figure of the interlocking IgG and IgA beta strands at the CH3 domains.

The overlapping IgA and IgG sequences in the Fc region could potentially result in loss of important Fc functions, such as protein A or G affinity. Moreover, if binding to the neonatal Fc receptor is lost, the Sb would have significantly shorter half-life, since the neonatal Fc receptor prevents proteins from degradation. However, protein A binding was shown to be retained and the Fc region was also shown to confer longer half-life with the total half-life being equal to the control IgG antibody (Davis et al. 2010). They consider this to be an “indirect but strong evidence that the Sb binds to the neonatal Fc receptor (FcRn) and is also
consistent with the observed retention of protein A association by the overlapping region of the Sb structure.”

This is good news for the SEEDBody approach, as the loss of FcRn binding would be a significant disadvantage compared with less disruptive methods. Davis et al. (2010) further suggest that fusing the Sb with a scFv to the N-terminal would be a good way of producing bsAbs since it evades the problem with light chain mispairing. We think that other methods to combat light chain mispairing described in this report could also be applied together with SEEDBody.

3.1.3 BEAT – Bispecific Engagement by Antibodies based on the T cell receptor

BEAT was developed by the company Glenmark. Although the name BEAT technically only applies when a T cell receptor is used as binding site, we consider this a general strategy to solve the heavy chain problem. The second binding site has affinity to a generic disease associated antigen. In the leading BEAT format, the second target is the HER2 receptor on breast tumor cells. (Moretti et al. 2013; WO2015063339A1, 2015) This antibody is further described under clinical formats.

The BEAT strategy provides a way of producing full and correctly assembled heterodimeric immunoglobulins similar to native forms. The BEAT heavy chains each contain a modified CH3 domains to promote the formation of an interface in the Fc region. The engineered modifications are made by exchanging residues in the Ig constant domain of the antibody with residues from two domains in the T cell surface receptor. The T-cell receptor is partly formed by an interaction that creates a protein-protein interface between two domains called alpha and beta. This interaction is used in the BEAT technology to design new heterodimeric domains in the antibodies. Heterodimerization of the heavy chains therefore mimics the natural association of T cell receptors alpha and beta domain. This modification creates a stable interface which already occurs naturally and is applicable to all BEAT antibodies. (Skegro et al. 2017)

The standard format of BEAT antibodies is a scFv x Fab format, i.e. one of the antigens binding Fab arms is replaced by a scFv, see figure 8. This design avoids any possible light chain mispairing that otherwise could occur. It also allows versatile combinations because it is not restricted to the use of common light or heavy chains. (Moretti et al. 2013; WO2015063339A1, 2015)
To solve impurities related to homodimerization, the BEAT antibody is engineered with an asymmetric binding to protein A. The heterodimer has reduced or eliminated binding to protein A, which allows for efficient one-step purification via protein A chromatography. This can be created using Fc heavy chains from IgG isotypes with weak or no ability to bind protein A, e.g. IgG3, or by substitutions in the Fc binding region. (US9683053B2, 2017; WO2015063339A1, 2015)

Protein A can also bind the VH chain from the human subclass called VH3. VH3 chains have demonstrated improved expression and stability over other heavy chain subclasses. Therefore, VH3 antibodies have been widely developed in the biotechnology industry. (WO2015063339A1, 2015) When using antigen binding sites from a VH3 origin, the protein A binding sequence has to be substituted or located on the heavy chain that does bind to protein A in the Fc region. This way, the purification problem is solved by the significant difference in affinity for protein A between the homo- and heterodimers. Two homodimers are formed, one with no protein A affinity and one with a much stronger protein A affinity. (WO2015063339A1, 2015)

This is a unique way of designing bispecific antibodies to avoid the bottle-neck relating to formation of product-related impurities. Similar approaches have not been seen in our investigation, where other design strategies primarily focus on promoting correct association of heavy and light chain. The BEAT strategy not only promotes correct heterodimerization but also enables efficient separation of possible formation of homodimers from the product.

As mentioned before, a problem with bsAbs containing an Fc region is the interaction with receptor expressing immune cells inducing an unwanted cytokine release response. This is solved in the BEAT technology by substitutions in the Fc binding region. It can be done by introducing suggested substitutions in the constant region of the first and/or second

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**Figure 8. A schematic figure of BEAT** as presented in the patent WO2015063339A1, 2015. The antibody includes one scFv represented in purple and one Fab arm.
12

polypeptide. This produces a constant region without Fc receptor binding ability.
(WO2015063339A1, 2015)

3.1.4 The strategies to solve heavy chain pairing are not optimal for large scale manufacturing and can be improved

KIH, SEEDBody and BEAT suffer from the necessity of delivering at least two gene constructs that need to be expressed proportionally in the same or different cell lines. Manufacturing could be more easily scaled up if the formats could be expressed using single gene constructs. We might see more formats developed via single gene constructs in the future. However, the design strategies that require co-delivering of at least two gene constructs are still powerful tools to ensure correct heterodimerization of the heavy chains. When constructing the BEAT formats, they also engineered varying protein A affinity into the heavy chain constant regions, facilitating the purification process of the impurities that are still formed. We argue for that this is not an option that is exclusive to the use of BEAT and could be applied to the KIH and SEEDBody strategies as well. There is however a pending patent application (WO2015063339A1, 2015) for this strategy.

3.2 Strategies for solving the light chain problem

Since applying the technologies discussed above only solves the problem with homodimerization of the heavy chains, further methods have to be applied to solve light chain mispairing. That is, two different LC can give rise to four different antibodies were only one is bispecific, see figure 9.

![Figure 9. A schematic figure of the four possible antibodies which can be generated after solving the HC problem. The encircled antibody is bispecific whereas the others are non-functional.](image)

Solving this problem is more difficult than the heavy chain pairing issue because of the complex interactions within the Fabs (WO2018035084A1, 2018). One easy approach to avoid this problem is to use common light chains, though it would drastically reduce the possible antigen binding sites. Below, the strategies CrossMab, orthogonal Fab, DuetMab and LC-mutations that solve the light chain problem are discussed. Usually, a light chain pairing
method is combined with an approach for the HC problem to ensure correct chain pairing overall. The strategies described in this section cause the format in question to deviate from the native IgG structure in varying degrees and may require a lot of engineering.

### 3.2.1 CrossMab

CrossMab is one of the first technologies developed to solve the light chain mispairing problem. It has evolved to become one of the most used and validated techniques. The advantages of CrossMab is that it is compatible with standard upstream techniques and purification processes used during the production of conventional IgGs. Furthermore, it conserves the properties of normal IgGs in terms of, for instance, stability and glycosylation. CrossMab enables the making of bivalent antibodies but also trivalent and tetravalent antibodies can be made. (Klein et al. 2019)

The CrossMab technology produces correctly formed antibodies by making crossovers, see figure 10. This promotes correct light chain formation. Together with the use of KIH for correct heavy chain pairing, both the light chain problem and the heavy chain problem are solved. The crossover is done by exchanging domains within one Fab arm of the antibody. The exchange can occur between different domains within the antigen binding fragment. A CrossMab\textsuperscript{Fab} is constructed by, on one side, designing a new heavy chain consisting of a Fc and the Fab of the original light chain and using the original VH and CH1 domains as the new modified light chain. The crossover can also be applied on only a part of the Fab arm by exchanging the variable domains producing CrossMab\textsuperscript{VH-VL} or CrossMab\textsuperscript{CH-CL} by exchanging constant domains, see figure 10. The domain interchange makes the two binding arms significantly different and light chain mispairing can no longer occur due to not being able to form heterodimerization interfaces between light and heavy chain regions. Furthermore, this crossover can be done without affecting the binding affinities to the antigens. (Brinkmann & Kontermann 2017, Schaefer et al. 2011)
A schematic figure of different CrossMab crossovers as inspired by figure 1 in an article by Schaefer et al. (2011). A commercial mAb and three different CrossMab versions resulting from different crossovers in the same Fab arm are depicted. With the CrossMab technology, the light chain mispairing problem is solved. For CrossMab\textsuperscript{Fab} the VH and VL domains are swapped as well as the CL and CH1 domains. For CrossMab\textsuperscript{VH-VL} the VL and VH domains are swapped and for CrossMab\textsuperscript{CH1-CL} the CL and CH1 domains are swapped. These swaps are represented by arrows. All three versions have the knob-into-hole modification represented as the purple bar.

Depending on the crossover domain, different side products can still be formed. This occurs especially when producing CrossMab\textsuperscript{VH-VL} or CrossMab\textsuperscript{Fab}. The side products are usually removed by applying different chromatography techniques. This can however be problematic and further solutions have been developed. For instance, to avoid formation of impurities when producing VH-VL CrossMabs, charged amino acid pairs can be introduced to promote correct assembly of the antibody by creating electrostatic attraction. (Brinkmann & Kontermann 2017, Klein et al. 2019)

3.2.2 Orthogonal Fab
Orthogonal Fab solves the light chain problem by the genetic engineering of the interface between the heavy and light chains to get so called orthogonal Fab interfaces. This achieves higher affinity for matching light and heavy chains. When simultaneously expressing parental monoclonal antibodies incorporating these interfaces, heavy and light chain pairing in the obtained bispecific IgG antibody is improved with an average of 93% correctly assembled antibodies. (Lewis et al. 2014)

When engineering an orthogonal Fab it is important to take the number of mutations into account as it affects the expression of the antibody. In the study by Steven M Lewis et al. (2014) a general trend was that “the designed proteins that were well expressed had fewer mutations, and all proteins with more than nine mutations were not expressed”. This was the
result from mutations in the CH1-CL and VH-VL interfaces.

This method has been successful when applied to antibodies with various specificities but does not give 100% correct assembly of the Fabs. It is worth mentioning that obtaining the correct assembly of Fabs was specific for the antibodies in the study and it is therefore unclear whether this strategy works for all antibodies. (Lewis et al. 2014)

3.2.3 DuetMab
DuetMab replaces the native disulfide bond in the CH1-CL interface with an engineered disulfide bond, see figure 11. (Mazor et al. 2015) This enhances cognate light chain pairing but needs to be combined with another design strategy that ensures correct heavy chain heterodimerization.

![Figure 11. A schematic figure of the DuetMab approach. The orange line represents the engineered disulfide bond which replaces one of the native disulfide bonds.](image)

Three different positions in the CH1-CL interface are possible candidates for favouring the formation of a novel disulfide bond according to the DuetMab approach. An amino acid on the HC and one on the LC is replaced with cysteine in one of the Fab regions. The native disulfide bond on the other Fab region is left intact. This approach was experimentally validated by Mazor et al. (2015) and applied together with KIH. Combined, they resulted in nearly 100% formation of bispecific antibodies.

It is advantageous that the modifications are in the CH1-CL interface and not in the variable domain, as this could have detrimental effects on antigen binding (Mazor et al. 2015). Although, engineering in the CH1-CL interface could mean that κ and λ constant light chains would somehow affect the usefulness of this approach. However, it was shown to be compatible to both isotypes (Mazor et al. 2015).

It could be that DuetMab cannot be applied to all Fab interfaces with such successful results. Nonetheless, 32 other DuetMab antibodies were successfully developed by Mazor et al. (2015), which points to the fact that the approach is generally applicable. Mazor et al. (2015)
concluded that DuetMab could be generically applied to bispecific antibodies in development since the approach: (i) does not contain variable domain engineering, (ii) is compatible with both kappa and lambda isotypes and (iii) was able to induce correct heterodimerization.

### 3.2.4 CH1-CL interface mutations

This design strategy is similar to the orthogonal Fab and DuetMab strategies in that it introduces modifications in the CH1-CL interface. The modifications disrupt pairing with wild type antibody chains and promote pairing with cognate engineered chains. (Bönisch et al. 2017)

This design strategy was developed by Bönisch et al. (2017). Their selected mutations were based on previously published repulsive CH3-CH3 interface mutations (Ying et al. 2012) due to structural similarity between the CH3-CH3 and CH1-CL interfaces. Modifications of the residues in the CH1-CL interface that were homologous to the repulsive CH3-CH3 were therefore explored and their impact on structure examined. When suitable candidates were found, complementary mutations to restore the CH1-CL interaction were examined to restore the association between the modified chains. The number of modifications negatively correlated with expression which was also the case for Lewis et al. (2014) developing the orthogonal Fab approach.

Four different engineered interfaces survived experimental validation. The interfaces were able to promote cognate pairing with all interfaces by introducing mutations that repulsed the wild type chain together with mutations that restored the interaction with the modified chain. Increases in yield were cell line dependent but still significant. A negative correlation between correct chain pairing and total yield was observed. The authors speculate that this could be due to that cell lines with high expression “overwhelm inherent quality control mechanisms”. (Bönisch et al. 2017)

### 3.2.5 Excessive engineering in the light chain interfaces leads to lower yield and can have implications for antigen binding affinity

The orthogonal Fab, CH1-CL interface mutations and the DuetMab approach involve varying degrees of engineering Fab in the interface. As was demonstrated by developers of the orthogonal Fab and the CH1-CL interface mutations approach, the yield was negatively correlated to the amount of performed mutations (Mazor et al. 2015, Lewis et al. 2014). Mutations in the variable region can interfere with the antigen binding affinity of the format, but not necessarily. The applicability of these strategies should vary depending on whether mutations are introduced in the variable region or not. The mutations introduced in CH1-CL interface mutations by Bönisch et al. (2017) might therefore be an approach that can be applied to a wider range of formats and targets than the orthogonal Fab strategy.
4 An overview of clinical formats

There are several bispecific antibodies in clinical trials today. Many of them use different formats, where each format has different properties. To get an insight of the formats that currently are being used in the field, a study of clinical formats was performed. For each respective format, the structures are described. Their therapeutic effects are also presented in order to understand where these molecules have potential as drugs. The upstream and downstream process are described as well as the advantages and disadvantages of the formats. A presentation of the bispecific antibodies in clinical trial described in this report can be seen in table 2.

Table 2. Overview of the clinical formats described including whether they contain a Fc region, possible purification strategies, advantages and disadvantages of the format. The possible purification strategies, and their respective abbreviations, are; PAC, protein A chromatography; PGC, protein G chromatography; PLC, protein L chromatography; SEC, size exclusion chromatography; IEC, ion exchange chromatography.

<table>
<thead>
<tr>
<th>Name</th>
<th>Fc region</th>
<th>Possible purification strategies</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>DVD-Ig</td>
<td>Yes</td>
<td>PAC/PGC</td>
<td>Avoids HC/LC mispairing</td>
<td>Lower binding affinities</td>
</tr>
<tr>
<td>scFv fusions</td>
<td>Yes</td>
<td>Standard processes, depending on conjugated protein</td>
<td>Longer half-life, avoids LC mispairing</td>
<td>Low stability because of linkers</td>
</tr>
<tr>
<td>BEAT</td>
<td>Yes</td>
<td>PAC/PGC</td>
<td>No HC/LC mispairing, avoids effects that might cause immunogenicity</td>
<td>Disallows the use of VH3 variable domains without further engineering steps</td>
</tr>
<tr>
<td>XmAb</td>
<td>Yes</td>
<td>PAC, IEC</td>
<td>Extended half-life, purification advantages</td>
<td>Possible risk of immunogenicity</td>
</tr>
<tr>
<td>BiTE</td>
<td>No</td>
<td>His-tag, PLC</td>
<td>High specificity</td>
<td>Short half-life</td>
</tr>
<tr>
<td>TandAb</td>
<td>No</td>
<td>His-tag, PLC</td>
<td>High specificity, improved half-life over BiTE</td>
<td>Short half-life</td>
</tr>
</tbody>
</table>
4.1 Fc-based formats

As previously mentioned, formats of bispecific antibodies can primarily be grouped into Fc-based, *i.e.* IgG-like, and fragment-based, *i.e.* non-IgG-like. All formats described in this section belong to the IgG-like group, since they are based on the “native” form of an IgG antibody.

The Fc-based formats outperform the fragment-based in terms of half-life. However, the Fc-based formats have their own problems regarding immunogenicity and the formation of product-related impurities. Applying strategies to solve problems associated with chain pairing produces formats that in varying degrees differ from the native structure. Extensive alteration of the antibody structure, or the existence of remaining impurities, can lead to toxicity effects. (Wood 2011)

4.1.1 Dual-variable domains Ig (DVD-Ig)

Dual-variable domain immunoglobulin (DVD-Ig) is an established format. The company AbbVie have developed several DVD-Ig like bispecific antibodies that currently are in clinical trials. One product in clinical trial is ABT165. (Sedykh et al. 2018)

AbbVie have filed a patent application titled “Isolation And Purification Of DVD-Igs” (US20160272673A1, 2016). The information described in the section “Production methods” is based on the assumption that the methods and protocols presented in the application is what they apply to their DVD-Ig products. AbbVie have a granted patent “Anti-dll4/vegf Dual Variable Domain Immunoglobulin And Uses Thereof” (US9045551B2, 2015) for a DVD-Ig with the same bindings domains as ABT165. “Production methods” is also based on the assumption that the information in the patent can be applied to the DVD-Ig ABT165. The upstream and purification processes of this particular format have been supplemented from aforementioned patent application.

**Structure**

DVD-Ig is a format with an Fc region and bispecific characteristics on each Fab arm, making it a tetravalent bispecific antibody. This is achieved by additional fusions of a second variable domain to the variable domains of a monospecific antibody with a linker, see figure 12. (Fan *et al.* 2015)
Figure 12. A schematic picture of DVD-Ig as presented in figure A in the patent US9045551B2, 2015. The pink represents the additional binding domains fused to the monospecific antibody in blue and green.

**Therapeutics effects**

ABT165 targets solid cancer tumors by binding to the delta-like ligand 4 (DLL4) and VEGF receptor. (Sedykh *et al.* 2018) Both DLL4 and VEGF are angiogenic factors, they contribute to angiogenesis which is the process where new blood vessels is formed (Li *et al.* 2018). When binding to these factors, the growth of the tumor is inhibited due to conformational changes in the ligand-receptor system, affecting the signaling to initiate angiogenesis. This is because the cells cannot survive without blood vessels. (Li *et al.* 2018; US9045551B2, 2015)

**Production methods**

In the example in patent US9045551B2 (2015) the heavy and light chains that compose the variable domain for binding to DLL4 and VEGF was synthesized with two-step PCR. Known domain sequences from humans were used to design the chains. Primers were designed with flanking regions to the cloning vector as well as a linker region between each variable domain. These were inserted into a vector and positive cloning vectors were identified through bacterial transformation. After being harvested and purified, the vector encoding genes for the recombinant bispecific binding protein were expressed via mammalian host cells. (US9045551B2, 2015) Host cells may include CHO cells, NSO myeloma cells, COS cells and SP2 cells. (US20160272673A1, 2016)

Affinity chromatography is used for purification, preferably protein A chromatography. Here, the Fc region is utilized to capture the DVD-Ig. The patent (US20160272673A1, 2016) suggests that suitable resin for this matter is MabSelect or MabSelect SuRe from GE Healthcare or ProSep Ultra Plus from EMD Millipore. The purification of a DVD-Ig may include further steps of ion exchange chromatography and/or hydrophobic interaction chromatography as well. Such steps may be anion exchange chromatography, mixed mode chromatography of either cation exchange or anion exchange type, hydrophobic interaction chromatography and viral filtration. (US20160272673A1, 2016)
Advantages and disadvantages

One of the advantages with DVD-Ig is that it avoids the heavy chain problem as well as light chain mispairing since the Fab arms are identical. Therefore, no mispairing can occur which improves yield and the incorporation of an Fc region improves stability. (Fan et al. 2015) A possible disadvantage with the format is lower binding affinities to target molecules due to steric hindrance. This especially applies to the inner variable domain. The structure and length of the linker connecting two variable regions within one Fab arm also affect the binding affinity on the inner variable domain. To avoid this, the linker should preferably be longer, about 15 amino acids, and be adopting a helical structure. (Brinkmann & Kontermann 2017)

4.1.2 scFv fusions

This class of bispecific antibodies are generated by fusing fragments, or so-called binding motifs, to other protein domains. A review by Brinkmann & Kontermann displayed many applied formats of this kind with large diversity in their structure. For example, have a variety of binding moieties been applied, for example scFvs, Fabs, single chain Fabs (scFabs) or even single variable domains (sVD) derived from camelid and sharks, see figure 4. However, scFv are most commonly used among these. The protein domain can be a whole IgG, only a Fc region or alternatively other scaffold proteins. (Sedykh et al. 2018)

It is also possible to generate asymmetric bispecific antibodies using this approach for example by fusing a Fab binding arm to a scFv-Fc moiety. This have been applied to formats that are further described later in this report. (Brinkmann & Kontermann 2017)

4.1.2.1 scFv - IgG fusions

One way of designing symmetric bispecific antibodies is by fusion of scFvs to an IgG (scFv-IgG). (Brinkmann & Kontermann 2017)

Structure

The fusion of the scFvs to the IgG can be done to the C-terminus of the heavy chain, or to the N-terminus of the heavy/light chain. The antibodies generated are therefore symmetric and usually tetravalent with two binding sites for each antigen. Thus, no heavy/light chain mispairing can occur in the production process. The first developed format of this kind was the Morrison format, see figure 13. Since then, many of the scFv-IgGs developed have been based on this format. The Morrison format was constructed by fusion of two scFvs targeting one antigen to the C-terminus of an IgG. (Brinkmann & Kontermann 2017, Sedykh et al. 2018)
Figure 13. A schematic picture of the Morrison format as presented in figure 3 in an article by Dengl et al. (2016). The pink represents the scFv that has been attached to monospecific antibody.

**Therapeutic effect**
One IgG-scFv that have reached phase I in clinical trials is generated by the company Eli Lilly. This antibody is developed to target HER1 + cMET in solid tumors. (Brinkmann & Kontermann 2017)

**Production method**
The production of these formats is, because of their symmetric design, easy and standard processes used in the production of antibody fragments and IgG-like antibodies can be applied. Because they contain both Fc region and scFvs, it is assumed that protein A, G and protein L can be used.

**Advantages and disadvantages**
As noted, the advantage of using fragments like scFv, instead of the original Fab arms, is that mispairing between light chains and/or heavy chains can more easily be avoided. However, many critical issues relating to the stability of the linker between the IgG and the appended scFv fragment have been experienced in antibodies of this kind. Though, existing formats have been developed to improve the structure of the appended IgGs to solve the problems, for example using different linkers. (Brinkmann & Kontermann 2017, Sedykh et al. 2018)

4.1.2.2 scFv–Fc fusions
The fusion idea was extended to create IgG-like antibodies by fusion of different binding modules to an Fc region, generating a scFv-Fc. (Brinkmann & Kontermann 2017, Sedykh et al. 2018)

**Structure**
The Dual-Affinity Re-Targeting (DART) is one fragment format that have been used to create bispecific antibodies by fragment-Fc fusions. DART fragments are based on the diabody.
format. A diabody consists of two separate polypeptide chains, each with a variable heavy and light chain domain with the binding specificity of an antibody, see figure 4. DART is further developed with additional stabilization by covalently linking the polypeptides through a C-terminal disulfide bridge, see figure 14. Studies have shown large increase in stability of the DART molecules in comparison to other diabodies e.g. BiTEs. (Brinkmann & Kontermann 2017, Rader 2011)

**Figure 14. A schematic figure of a DART fragment** as presented in figure 2, box 9 in the review by Brinkmann & Kontermann (2017). Fusion of one DART fragment represented in pink and purple to an Fc region.

**Therapeutic effect**
DART-Fc fusion proteins have primarily been developed for T cell retargeting by containing a CD3 binding site. By combining this with the equipment of different antibody binding sites DART-Fc have proven to successfully target and counteract an array of multiple variants of commonly occurring diseases. These range from colorectal cancer and acute myeloid leukemia (AML) to solid tumors. These applications are currently in clinical phase I and II. (Brinkmann & Kontermann 2017)

**Production method**
A bispecific antibody of this format can be produced by fusion of two different binding domains, each to a separate Fc chain. Any problems with impurities can then be avoided by applying one of the existing strategies that drives heterodimerization. For example, DART-Fc antibodies can be generated by fusing one or more DART fragments to one Fc that contains the KIH substitutions. This design enables purification with protein A and G. (Brinkmann & Kontermann 2017)

**Advantages and disadvantages**
Though being smaller in size compared to native IgG it is possible to retain any desired Fc effector functions and many of the other properties relating to IgGs. Furthermore, fusing fragments to Fc region prolongs the short half-life of Fc-less antibody fragments.
4.1.3 BEAT bispecific antibodies

The biotech company Glenmark have currently developed three formats based on the BEAT platform described in section 3.2.3. The first bispecific antibody generated from the proprietary BEAT platform is called GBR 1302. The two other formats are described in appendix 4.

Structure

The structure of the antibody is consistent with the BEAT format and has one CD3 binding scFv portion fused to a BEAT heavy chain and one Fab targeting the HER2 receptor. (WO2015063339A1, 2015; Chen et al. 2019)

Therapeutic effect

GBR 1302 is a bispecific antibody that targets the HER2 and CD3 antigen simultaneously. This enables treatment of different HER2 positive cancers, such as breast and gastric cancer, by binding the CD3 T cell antigen and the HER2 receptor which is overexpressed in many tumor cells. (WO2015063339A1, 2015)

Production methods

In the patent describing the BEAT antibodies (WO2015063339A1, 2015), all antibodies were produced by using following production steps. Preparing DNA expression vectors using standard molecular technology. This can be made by preparing three expression constructs, one for the scFv-Fc fusion, one for the heavy chain and one expressing the corresponding light chain. Following, the DNA vector(s) are transfected or co-transfected into a mammalian cell line. In the patent, HEK cells were used but also CHO cells can be used according to Glenmark. The BEAT antibodies were purified using a two-step process consisting of a capture-elution mode chromatography step using protein G. This step is followed by gradient mode chromatography using protein A chromatography and pH elution. Examples of possible protein A resins that can be used in the purification is MabSelect SuRe or Mabselect protein A column but are not limited to these two. (WO2015063339A1, 2015)

Advantages and disadvantages

In a study by Skegro et al. (2017) several BEAT antibodies of different formats were constructed and resulted in up to 95% purity after purification with protein A. According to Glenmark the BEAT antibodies can be produced by using conventional production methods. One study produced BEAT antibodies using a platform approach consisting of expression in CHO-cells and one-step purification with protein A. This resulted in purification levels of 97% which supports Glenmark's suggestion (Moretti et al. 2013). Heterodimerization also showed higher efficiency when compared to using the KIH and CrossMab approach. (Skegro et al. 2017)

Either stepwise or gradient pH elution can be applied in the elution process. This efficiently dissociate most interaction and the homo and heterodimers usually separate with one pH unit in the range between 3-4. It is important to consider that some antibodies could be damaged
by low pH and immediate neutralization and minimizing the time in low-pH are necessary. (WO2016071355A1, 2016)

4.1.4 XmAb
The biotech company Xencor has developed antibodies with their platform called XmAb. The platform enables alterations with desirable effects to the Fc domain of the antibodies. The following section describes the XmAb formats, with focus on the bispecific antibody XmAb22841.

Structure
One of the XmAb technologies are the Xtend technology that modifies the Fc domain with two amino acid changes. The modification increases affinity to the neonatal Fc receptor which prevents the antibody from degradation. Hence, this interaction extends the antibody’s half-life, making it a more desirable therapeutic drug. (Xencor, 2019)

The bispecific antibody, XmAb22841, with the Xtend modification recently reached phase I. Other XmAb bispecific antibodies in clinical phase I are of the same format as XmAb22841. This format is similar in structure to an IgG molecule, but with one Fab arm replaced by a scFv, see figure 15 (Xencor, 2019).

![Figure 15. A schematic figure of an XmAb format](image)

Figure 15. A schematic figure of an XmAb format as presented in “Simultaneous checkpoint-checkpoint or checkpoint-costimulatory receptor targeting with bispecific antibodies promotes enhanced human T cell activation” by Hedvat et al. (2018). The format is composed of an IgG molecule, but with one Fab arm replaced by a scFv represented. The Xtend modification marked in orange on the Fc region extends the antibody's half-life.

Therapeutic effect
The main therapeutic field of bispecific XmAb formats in preclinical and clinical development is oncology according to Xencor’s pipeline. In the case of XmAb22841, the Fab arm targets LAG-3, a lymphocyte activation gene that suppresses T cell activation and
cytokine secretion (Long et al. 2018). The scFv targets the antigen CTLA-4, which inhibits immune responses (Buchbinder & Desai 2016).

**Production methods**

In a study by Moore et al. (2019), the XmAb platform was engineered and tested in the process of producing a bispecific XmAb antibody. It was described that the platform enables antibodies with a heterodimeric Fc domain, i.e. an Fc domain with two different heavy chains. The upstream and downstream process of the XmAb antibody was described based on proteins containing a heterodimeric Fc. Due to overall high heterodimer yield of 95% and similar biophysical properties, they claimed that other bispecific XmAbs can be manufactured with the same approach as in the study. The approach described below is based on the same format as XmAb22841.

In order to construct the XmAb format an antibody heavy and light chain and a scFv Fc-fusion were subcloned into vectors. The scFv and Fc region were connected with a GS-linker. The Fc region was altered with substitutions in order to increase the differences in $pI$ between the two heavy chains. This would increase the $pI$ differences between homodimers and heterodimers, which would then facilitate the purification of heterodimers. They sought to minimize the risk of immunogenicity by utilizing buried substitutions, but the exact risk has to be further investigated in clinical studies. For the production of the proteins, plasmids encoding all chains were co-transfected into HEK cells. The antibody was purified using protein A chromatography and ion exchange chromatography. (Moore et al. 2019)

**Advantages and disadvantages**

As mentioned, the Xencor’s Xtend technology extends the antibodies half-lives. This is advantageous since less frequent medication is needed. Due to alterations in the Fc domain, $pI$ differences can be used in the purification. Furthermore, as for BEAT described in section 4.1.3, the use of one scFv in the design of XmAb22841 circumvents the light chain mispairing problem. The risk of immunogenicity for the XmAb formats was sought to be minimized in the study by Moore et al. (2019). However, since this was not determined it is still unclear whether the formats are immunogenic or not.

**4.2 Fragment-based formats**

Several formats that heavily deviate from the native Ig-like structure are in development, as will be seen in this section. The bispecific antibodies that were the first to survive clinical trials and reach the open market are such. These fragment-based formats both have advantages and disadvantages compared to the Ig-like formats. Since they lack the Fc region, they are not as efficiently purified by protein A and protein G chromatography as the Ig-like antibodies. Antibody fragments are instead usually purified by using a His-tag or protein L chromatography. There are numerous of Fc lacking formats, such as BiTEs, BiKEs, TriKEs and DARTs (Duell et al. 2019). These fragments consist of scFvs linked in different ways.
4.2.1 BiTE

The following section describes the fragment-based format BiTE, which stands for bispecific T cell engager as well as the drug blinatumomab developed by Amgen. Blinatumomab is the first representative of the BiTE class that has been approved for use. (Sedykh et al. 2018)

Structure

BiTE is a class of small molecules comprised of two scFvs with different specificities, which are connected via a flexible GS-linker, see figure 16 (Huehls et al. 2015).

**Figure 16. A schematic figure of a Bispecific T cell Engager (BiTE)** as presented in figure 2, box 3 in a review by Brinkmann & Kontermann (2017). The fragment is composed of two scFvs represented in green and blue. The scFvs have different specificities and are connected with a GS-linker.

Therapeutic effect

BiTE simultaneously targets CD3, a receptor that activates cytotoxic T lymphocytes, and a surface antigen of tumor cells (Sedykh et al. 2018). The cytotoxic activity is only activated when both specificities are targeted, which in turn circumvent the issue of undesired T cell activation (Huehls et al. 2015). Currently, there are only two bsAbs approved for use in the US and Europe; blinatumomab and catumaxomab. blinatumomab belongs to the BiTE class and this therapeutic drug targets the proteins CD3 and CD19, the latter of which is a surface protein of B lymphocytes. Blinatumomab is used for the treatment of acute lymphoblastic leukemia. (Sedykh et al. 2018)

Production methods

In a study by Naddafi et al. (2018), the upstream and downstream process of blinatumomab was described. Both CHO cells and *Escherichia coli* (*E. coli*) strains where tested as expression hosts for the upstream process. The gene coding for blinatumomab was cloned into expression vectors, 6xHis-tagged and purified on a Ni-NTA chromatography column. The Ni-NTA column is used for purification of 6xHis-tagged recombinant proteins (Thermo Fisher, 2019). The result showed that the purified antibody from the CHO cell expression system had higher binding activity compared with the purified antibody from the *E. coli* expression system. This is due to a more properly folding of proteins in mammalian cells compared to *E. coli* cells.
**Advantages and disadvantages**

An advantage of the BiTE format is the very high and specific antitumor activity (Sedykh et al. 2018). However, due to its small size, BiTEs tend to have a short half-life. Treatment with blinatumomab, for instance, requires a constant intravenous infusion in a repeated four-week cycle. (Duell et al. 2019)

### 4.2.2 TandAb

The TandAb format, see figure 17, was developed to overcome BiTE’s issues of short half-life while still retaining good tissue penetration (Zhang et al. 2017).

**Structure**

The TandAb format is a homodimer made up of four scFvs connected by GS-linkers, see figure 17. Unlike the BiTE format, a TandAb is tetravalent and therefore maintains the avidity of larger formats.

![Figure 17. A schematic figure of the TandAb format](image)

*Figure 17. A schematic figure of the TandAb format, as represented in fig 3 by Sedykh et al. (2018). The two scFv displayed in pink are from one parental antibody, while the two scFv displayed in blue are from the other parental antibody. A: the TandAb format without bound antigens. B: the TandAb format with bound antigens.*

**Therapeutic effect**

An example of the TandAb format being applied in the development of a bispecific antibody is the AFM11 bsAb being developed by Affimed (Affimed 2019). It is an anti-CD3 and anti-CD19 antibody, just like blinatumomab. When the performance of blinatumomab was compared to AFM11, the latter demonstrated improved antitumor potency, especially at low ratios of effector to target. It is currently in phase I clinical trial.

**Production methods**

Purification by protein A or G chromatography is not possible due to the lack of an Fc region. Purification via His-tag as a capture step is a possibility (Ellwanger et al. 2017) and seems to be the most common capture method. Purification via protein L chromatography is an
alternative as well. A bimodal polishing strategy, *i.e.* based on two orthogonal properties, can be performed based on purity needs. This can include size exclusion chromatography together with protein A or G chromatography, where fragment-based antibody formats can be collected as flow-through. (Spiesberger *et al.* 2015)

**Advantages and disadvantages**

Much like BiTE and other smaller formats, TandAb suffers from a short half-life. However, it is slightly larger than BiTE. The molecular weight, >100kDa, exceeds the first pass renal clearance threshold which confers the TandAb format with better half-life than BiTE. (Ellwanger *et al.* 2017) At the same time, the TandAb format can still penetrate tumors due to its small size (Duell *et al.* 2019). Also, it is expressed as a single gene product which is beneficial when scaling up the production process.

5 Preclinical formats

The following chapter present preclinical bispecific antibodies. We have defined a preclinical format as one not represented in clinical studies, regardless of the specific antigens it binds. This is because the factors affecting the downstream processing of the format does not vary when different antigen binding sites are used. In other words, the application of an established format with new targets does not make it a preclinical format.

To get an insight of the formats that currently are in preclinical development, a study of preclinical formats was performed. For each format, the structures are described. Their therapeutic effects are also presented in order to understand where these molecules have potential as drugs. The upstream and downstream process is described as well as the advantages and disadvantages of the formats. A presentation of bispecific antibodies in preclinical trial can be seen in table 3.
Table 3. Overview of the preclinical formats described in this chapter including what purification strategies and cell lines were used, what targets the formats have and their therapeutic use. PAC, protein A chromatography; SEC, size exclusion chromatography; PGC, protein G chromatography; IEC, ion exchange chromatography.

<table>
<thead>
<tr>
<th>Name</th>
<th>Purification strategy</th>
<th>Cell line</th>
<th>Target</th>
<th>Therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>biAbFabL</td>
<td>PAC + SEC</td>
<td>HEK</td>
<td>IL-17A/F + IL-23</td>
<td>Autoimmune diseases</td>
</tr>
<tr>
<td>taFab</td>
<td>PAC + SEC</td>
<td>HEK</td>
<td>IL-17A/F + IL-23</td>
<td>Autoimmune diseases</td>
</tr>
<tr>
<td>VCVFc</td>
<td>PAC+SEC</td>
<td>HEK</td>
<td>IL-17A/F + IL-23</td>
<td>Autoimmune diseases</td>
</tr>
<tr>
<td>VCDFc</td>
<td>PAC + SEC</td>
<td>HEK</td>
<td>IL-17A/F + IL-23</td>
<td>Autoimmune diseases</td>
</tr>
<tr>
<td>MAT-Fab</td>
<td>PAC</td>
<td>HEK</td>
<td>CD3/CD20</td>
<td>Oncology</td>
</tr>
<tr>
<td>iBiBody</td>
<td>PAC or PGC</td>
<td>HEK</td>
<td>CD3/FLT3</td>
<td>Oncology (AML)</td>
</tr>
<tr>
<td>Tandem forms</td>
<td>PAC or PGC</td>
<td>CHO/HEK</td>
<td>TLR4/TLR2</td>
<td>Unspecified</td>
</tr>
<tr>
<td>κλ antibody</td>
<td>PAC + ligand for κ/λ LC</td>
<td>CHO</td>
<td>CD47/mesothelin or CD47/CD19</td>
<td>Oncology</td>
</tr>
<tr>
<td>ADAPTIR</td>
<td>PAC + SEC</td>
<td>CHO/HEK</td>
<td>4-1BB/5T4</td>
<td>Oncology</td>
</tr>
<tr>
<td>BiIA-SG</td>
<td>PGC</td>
<td>CHO/HEK</td>
<td>CD4/HIV-1 gp160</td>
<td>HIV-1</td>
</tr>
</tbody>
</table>

5.1 biAbFabL, taFab, VCVFc and VCDFc

The bispecific antibody formats biAbFabL, taFab, VCVFc and VCDFc are in preclinical development by Bristol Myers Squibb with a pending patent application named “Bispecific antibodies and methods of using the same” (US20150086552A1, 2015).

Structure

The structure of biAbFabL can be seen in figure 18a. It is IgG-based with two Fab-domains attached via linker to the C-terminus of the constant region. It utilizes a common light chain, i.e. the two binding entities share the same light chain. The antibody is tetravalent.

The structure of taFab can be seen in figure 18b. It is similar to the DVD-Ig that is already well established, see section 4.1.1. The difference here, however, is that the taFab format also contains the constant light and constant heavy chain attached to the N-terminus of the
“native” Fabs. In other words, it contains entire Fab-regions attached via linkers between the hinge region and the C-terminus of the native Fab. DVD-Ig on the other hand, contains only the variable fragments added to the N-terminus. The linker used was (Gly4Ser1)n, wherein n is 1, 2 or 3. The format is tetravalent.

The structure of VCVFc can be seen in figure 18c. It can also be compared to the established DVD-Ig format. In this format, a variable domain is added between the Fab region and the “hinge” of the bsAb. The variable light domain is linked to the light chain constant region of the Fab, and the variable heavy domain is linked to the CH1 region of the Fab. Unlike in biAbFabL, the two different binding entities do not have to share a common light chain. The linker used is the same for all formats, i.e. a n-mer of G4S. The format is tetravalent.

The structure of VCDFc can be seen in figure 18d. It is similar to both VCVFc and a regular antibody where a single-domain antibody (sdAb) is inserted via linkers between the Fab region at CH1 and the hinge of the bsAb. This format is therefore tetravalent.

**Figure 18. A schematic figure of the biAbFabL, taFab, VCVFc and VCDFc formats**, as presented in figures 2, 3, 5 and 6, respectively, in the patent US20150086552A1, 2015. A: the structure of biAbFabL, 1: the native Fab region of a parental antibody. 2: the linked Fab region of the other parental antibody. B: the structure of taFab, 2: the native Fab region of the parental antibody. B: the structure of taFab, 2: the native Fab region of the parental antibody. 1: the Fab region from the other parental antibody inserted between the hinge and the CH1 of the native Fab region. C: the structure of VCVFc. The inserted variable domains are shown in pink and purple. D: the structure of VCDFc. The inserted sdAb is shown in orange.

**Therapeutic effect**

Anti-IL-17A/F and anti-IL-23 binding domains were selected for the antibody formats. IL-17A, IL-17F and IL-23 are cytokines, small signal proteins, that are involved in the body’s own inflammatory response. They are demonstrated to be mediating and promoting autoimmune diseases. For example, increased amounts of IL-23 have been associated with several autoimmune diseases such as IBD, Chron’s disease, psoriasis and MS. IL-23 knockout mice were resistant to some autoimmune disease inductions.
In short, the therapeutic function of these bispecific antibodies would be to combat autoimmune disease by antagonizing these cytokines, reducing or inhibiting their cell-signaling abilities.

**Production methods**

Hybridoma cells were used to produce parental antibodies. To design a humanized antibody, murine complementarity-determining regions were grafted onto human germline framework sequences for both heavy and light chains. Humanizing the antibody is key to reduce the immunogenicity of the antibody, which is a factor to be wary of when developing biological therapeutics. They also used a modified version of IgG called IgG1.1 with reduced FcR binding ability to avoid the binding of phagocytosis-mediating receptors. This would also reduce the immunogenicity of the format.

The bispecific antibodies were constructed via genetic fusion of the parental antibody sequences by using a HEK transient expression vector, with inserted genes for the chains using restriction enzymes. The vectors were transfected into *E. coli* for amplification. After harvesting and purification, HEK suspension cells were transfected with expression constructs using polyethylenimine and then cultivated. Media was then harvested. Cells were lysed using ultrafiltration. Protein was purified using a bimodal strategy consisting of a capture step using MabSelect SuRe (GE Healthcare) and then a polishing step using Superdex SEC (GE Healthcare).

Since all of the formats contain the Fc region, using protein A affinity chromatography as a capture seems natural. The appended binding entities deviating the structure from native IgG-like form does not appear to hinder this strategy in any way.

**Advantages and disadvantages**

When investigating these formats, the following things came to mind. In this invention, a common LC was used for the biAbFabL format. The use of a common light chain is generally a significant disadvantage. It can be avoided by applying any of the LC-pairing strategies mentioned in this report. Unintuitively, in the study presented in the patent application, the ability of the antibody to inhibit the IL-17A homodimer actually increased when using a common anti IL-23 LC. Generally, this is probably not the case. Design strategies such as CrossMab or DuetMab could then be applied to increase the yield of correctly paired constructs, allowing for the use of different light chains.

taFab, VCVFc and VCDFc do not require the use of common light chains or LC pairing design strategies. However, there is a risk that the binding capacity of the inner binding region, whether it is a Fab region, a scFv or an sdAb, might be sterically hindered by the outer Fab region.
5.2 MAT-Fab

In the patent application “Monovalent Asymmetric Tandem Fab Bispecific Antibodies” from Epimab Biotherapeutics Inc (WO2018035084A1, 2018), a format of a bispecific antibody is presented. It is called MAT-Fab which stands for Monovalent Asymmetric Tandem antigen binding fragment. This format will be described based on the mentioned patent application.

Structure

MAT-Fab is a tetrameric protein i.e. it consists of four polypeptide chains which is shown in figure 19 below.

![Figure 19. A schematic figure of the MAT-Fab format](image)

All nearby domains in MAT-Fab are directly connected to each other without any kind of linkers. The first light chain together with VLA-CL forms the outer Fab and the second light chain together with VHB-CH1 forms the second, inner Fab, see figure 19. To induce correct light chain pairing, a type of “cross-Fab” has been used which work similar to CrossMab, see section 3.2.1. The outer Fab has VL-CL on the heavy chain and VH-CH1 on the light chain whereas the inner Fab on the contrary has VH-CH1 on the heavy chain and VL-CL on the light chain. This arrangement of inner and outer Fab favors the proper association of VL-CL with correct VH-CH1 giving functional Fab units.

Modifications has also been done on the CH3 domains to diminish the amount of product-related impurities. This happens tentatively using the KIH strategy but the patent also mentions other options. Some examples are creating salt bridges or electrostatic interactions between the domains. The constant region may also contain modifications to prevent or impair the Fc effector functions.
Therapeutic effect
The target cells of one Fab region are effector cells from the immune system such as T cells, natural killer cells or macrophages. The target cells of the other Fab region are detrimental cells, such as cancer cells or virus infected cells. According to the patent, there are many potential target antigens on these cells. One example is antigen CD3 on a T cell and CD20 on a cancer cell.

Production methods
The design of a MAT-Fab antibody is based on parental monoclonal antibodies which have at least one property of the desired MAT-Fab antibody. Examples of properties are affinity to the wanted antigen, stability or solubility. A parent antibody may thus not have affinity to the wanted antigen if the derived part from it e.g. is a constant domain. The constant domain must in that case be linked to a variable domain from a different parental antibody with correct affinity. The parental antibodies could be naturally occurring or genetically constructed antibodies. Production of each subunit was made by introducing vector(s) with one to four of the polypeptide chains that form the MAT-Fab into host cells.

Protein A affinity chromatography was used to purify the MAT-Fab. In one example mentioned in the patent, two MAT-Fab antibodies only varying in KIH mutations were created. In this example the host cells were HEK. The monomeric fraction of these after one purification step with protein A was determined using SEC. The result was that 98.19% respective 95.7% of the MAT-Fab was present in each monomeric fraction.

Advantages and disadvantages
The fact that all nearby domains are directly connected to each other without any kind of linkers is mentioned as an advantage in the patent application. This eliminates the risk of immunogenicity due to linkers, since they can be seen as foreign to the immune system. Another advantage is that the structural organization of the polypeptide chains allows the correct intracellular assembly of the MAT-Fab antibody using the natural protein expression, folding, and secretion mechanisms. No post production processing techniques are needed to obtain the functional MAT-Fab antibody. No disadvantages were mentioned in the patent application.

5.3 iBiBody
The company Smet Pharmaceutical Inc have presented an antibody format called iBiBody in the patent application “Asymmetric Bispecific Antibodies And Their Use” (WO2019023097A1, 2019). The discussion about this format will be based on this patent application.

Structure
The “i-shaped” bispecific antibody, iBiBody, consists of a binding protein, a Fab and one or more mFc (monomeric crystallizable fragment). These parts can be linked together in different ways, see examples in figure 20.
Figure 20. A schematic design of the format iBiBody as presented in figure 5 in patent WO2019023097A1, 2019. This is three examples of how the different elements can be connected. These elements are binding protein in green, Fab in blue and mFc in pink which are connected by linkers. The star symbols represent substitutions of amino acids.

The mFc domain in the figure has two star symbols which represents substitutions of amino acids. This has been done in order to diminish the risk of homodimerization. The substitution also makes the proper chains bind stronger to each other. The binding protein can in principle consist of any protein with the ability to bind another unit. One example is an antibody fragment like Fab or diabody and a receptor or a ligand. The linkers between the different parts most often consist of repetitions of G4S or full-length or partial IgG hinge sequences.

**Therapeutic effect**
The iBiBody format is predicted to be used as immunotherapy against acute myeloid leukemia (AML), one of the most common types of leukemia. iBiBody is directed towards a cancer cell and a T cell where the epitope on the cancer cell is FLT3 and on the T cell is CD3.

**Production methods**
To produce an iBiBody antibody, DNA vectors encoding one or more of the polypeptide chains the iBiBody consists of were made. This was done with multiple overlapping PCR. These vectors were then expressed in HEK. Purification was done with protein A affinity chromatography or protein G affinity chromatography. In example two mentioned in the patent, where they designed a FLT3xCD3 iBiBody, the protein A Sepharose 4 Fast Flow column chromatography from GE Healthcare was used. In order to maintain affinity to protein A or G, two residues of the Fc portion of the iBiBody may be replaced.

**Advantages and disadvantages**
A hypothesis is that the use of IgG hinge sequences as the link between Fab and mFc give rise to decreased binding to Fc receptors due to steric obstacles. This diminish FcR binding-mediated toxicity, such as lymphopenia. Though, binding to the neonatal Fc receptor remains.
It also gives rise to increased stability by removing cleavage sites for proteases. No disadvantages were mentioned in the patent application.

### 5.4 Tandem forms

In the patent application “Novel Bispecific Antibody Format” published by Astellas Pharma Inc 2017, a total of four bispecific antibody formats are presented. They are named “tandem form A”, “B”, “C”, and “D” respectively. The structure of this format closely coincides with that of the taFab format described in 4.1, and only deviate from one another in the aspects of linkers and antigen binding sites. According to our own definition, they are technically the same format. We choose, however, to include these different takes on the format since we find that it might be useful to demonstrate different applications of this structure. (US20170306053A1, 2017)

#### Structure

These “Y-shaped” bispecific antibodies formats closely resemble that of standardized IgG antibodies, and, while being equipped with an Fc region and Fab regions, distinguished itself by having two sets of two Fab regions of different specificity linked in tandem, see figure 21. This enabled each form to retain moderately high to high binding affinity to both antigens. They are hence functional homodimeric tetravalent bispecific antibodies.

![Figure 21. The tandem format](image)

These tandem IgG formats were structured as per the illustration in figure 21, with two innermost “primary” Fabs, as well as two outermost “secondary” Fabs. The first antigen binding site in question is either anti-human TLR2, as in the case of tandem forms B and D, or anti-human TLR4, as in the case of tandem forms A or C. The primary Fab region is linked
by its N-terminus using two peptide linkers to the C-terminus of the secondary Fab region. These two linkers connect HC and LC regions together separately, of which the LC linker were cleavable for forms A and B. This resulted in a second pair of bound HC and LC whose combined variable regions comprises the second antigen binding site, either Anti-human TLR4, as in the case of tandem forms B and D, or anti-human TLR2, as in the case of tandem forms A or C. For a summation of variable regions of interest for each format variant, see table 4 below.

**Table 4.** A table showing the different variants of the homodimeric tetravalent bsAb Tandem forms and their inclusion and placement of the different variable regions and type of linkers covered in this section.

<table>
<thead>
<tr>
<th>Format</th>
<th>Innermost Fab</th>
<th>Outermost Fab</th>
<th>LC Linker</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>TLR4</td>
<td>TLR2</td>
<td>Cleavable</td>
</tr>
<tr>
<td>B</td>
<td>TLR2</td>
<td>TLR4</td>
<td>Cleavable</td>
</tr>
<tr>
<td>C</td>
<td>TLR4</td>
<td>TLR2</td>
<td>GS</td>
</tr>
<tr>
<td>D</td>
<td>TLR2</td>
<td>TLR4</td>
<td>GS</td>
</tr>
</tbody>
</table>

**Therapeutic effect**

All four of the bsAbs format variants were equipped with both functional “anti-human Toll-Like receptor 2 (TLR2)” and “anti-human Toll-Like receptor 4 (TLR4)” binding sites. TLRs play a fundamental role in pathogen recognition (Kawasaki & Kawai 2014). Even though the antigen binding sites were well defined, no underlying therapeutic effects were claimed to be pursued motivating the choices. The construct would rather have been constructed in order to simply demonstrate the increased affinity of the innermost epitope brought forward by the cleaving of the LC linker.

**Production**

The format was produced by means of genetic fusion where the variants had HC and LC encoding sequences produced separately. The two Fabs were expressed in tandem and bound together using GS linker for the HC and either a GS linker or a polypeptide linker including a protease recognition site in order to enable LC linker cleavage. For information regarding the order of Fab regions and linker equipped for each format variant, see table 4. The HC and LC were then inserted into a mutual vector.

Vectors containing the chains of all constructs, to be cleaved and ligated using either the ligation kit “Ligation-Convenience Kit” (NIPPONGENE Co., Ltd.) or ligation reagent Ligation-high (TOYOBO Co., Ltd.) and were transfected in host cells, preferably CHO, HEK or NS0 cell lines, transfected with the vectors. The culturing was carried out using established methods. Possible LC polypeptide linkers were then cleaved by intracellular proteases as to improve the binding affinity of primary antigen binding sites were then performed.

The method of producing the bsAbs may include a recovering step preferably by means of
isolating or purifying from the host cell line in addition to a step regarding the direct culturing of a construct expressing cell line. The bsAbs can preferably be purified from the culture supernatant by means of various chromatography methods, for example protein A or protein G columns. Although these methods are preferred, several other methods can be utilized, such as gel filtration, ultrafiltration or ion exchange chromatography.

**Advantages and disadvantages**

The format have the advantage that it, since the Fab arms are identical, avoids the heavy chain problem as well as light chain mispairing. No mispairing can hence occur which improves its yield. This while still retaining affinity towards two antigens.

Affinity towards both antigens is naturally retained by all tandem forms, although the affinity towards the innermost antigen binding site is slightly weaker than that of the outermost one. The reason for this is due to the fact that the innermost one is somewhat “hidden” by the outermost one. Cleaving of LC linkers by protease in the culture cell have been demonstrated to improve the binding capabilities of the buried secondary Fab. This entails that there is possible to give Tandem forms A and B almost as strong affinity towards both antigens TLR2 and TLR4, which makes the usage of these forms preferable for therapeutic applications over forms C and D. No improvement in binding affinity is however possible for the outermost antigen binding sites of either format, since no such posttranslational modification is possible to achieve.

All tandem forms were capable of retaining high binding affinity to both antigens TLR2 and TLR4, but were also able to be produced efficiently using a commercial production process.

**5.5 κλ antibody**

This section describes the so-called kappa-lambda (κλ) antibody owned by the company Novimmune. Currently, they have two κλ formats, NI-1701 and NI-1801, in preclinical trial. Based on Novimmune’s patent applications, the up- and downstream processes of the κλ antibody are described.

**Structure**

The two possible light chain types of mammalian antibodies, κ and λ, can be utilized in the engineering of a bispecific antibody. By combining the two different light chains into one antibody with one common heavy chain, a κλ bispecific antibody is produced. For this, two different light chain variable regions are required since the variable regions of the heavy chains are identical, see figure 22. (Brinkmann & Kontermann 2017)
Figure 22. A schematic figure of a κλ bispecific antibody as presented in figure 2, box 2 in a review by Brinkmann & Kontermann (2017). It is composed of an IgG antibody, with the two light chain types κ and λ and a common HC. κ and λ light chains are two only possible light chain types of mammalian antibodies.

**Therapeutic effect**

Novimmune has developed the κλ bispecific antibody. One κλ antibody, called NI-1801, is currently in preclinical phase. According to their pipeline, the bispecific antibody targets and suppresses a “don’t eat me” signal receptor, called CD47, displayed by tumors. It also targets mesothelin, an antigen found overexpressed on cell surfaces of solid tumors. Novimmune has also developed a similar κλ antibody, NI-1701, which is currently in the application process for clinical trials. According to their pipeline, NI-1701 targets the mentioned CD47 protein and the antigen CD19. CD19 is like mesothelin a cell surface antigen, but it is expressed by tumors of B cell origin. (Novimmune, 2019)

**Production methods**

The κλ antibody format is also patented by Novimmune and is described in their patent application. The first step in the upstream process of the κλ antibody was to isolate two antibodies with different specificities and variable domains, but with the same variable heavy domain. The variable heavy chain domain was then fused with the constant region of the heavy chain. The two different light chain variable domains were fused to their respective kappa and lambda constant domain. Lastly, the three peptide chains were co-expressed in mammalian cells, e.g. in CHO cells. This generated three types of antibodies: two monospecific antibodies and one bispecific antibody.

Due to the kappa and lambda constant domains, affinity to these domains can be utilized in the purification process. There are three types of antibodies as mentioned - two comprising either kappa or lambda constant domains and one comprising both kappa and lambda constant domains. By using affinity to both kappa and lambda constant domains, only the bispecific antibody with both domains will remain after the purification process. This general approach is divided into a three-step chromatography. In the first step, affinity to the Fc domain is utilized which captures the antibodies. In the second step, affinity to the constant kappa light
chain is utilized, which captures antibodies with one or two kappa light chains. In the third and last step, affinity to the constant lambda light chain is utilized. This captures the bispecific kappa/lambda heterodimers. (Brinkmann & Kontermann 2017) The same approach was described in the patent application (US009834615B2, 2017) with the following three steps: (i) Protein A that captures both mono- and bispecific IgG antibodies, followed by (ii) KappaSelect (GE Healthcare) that captures antibodies containing one or two kappa light chains, and (iii) LambdaSelect (GE healthcare) that captures antibodies containing a lambda light chain. The antibody remaining is bispecific.

In two additional patent applications from Novimmune, two corresponding bispecific antibodies to NI-1701 (WO2014087248A2, 2014) and NI-1801 (WO2018215835A1, 2018) with the same antigen binding sites respectively were described. Therefore, we assume that these patents are indeed the patents for NI-1701 and NI-1801. The purification methods described in the patent applications had a similar three-step approach as described above. The differences were that the affinity matrices from Thermo Fisher, namely CaptureSelect™ IgG-CHI, CaptureSelect™ LC-kappa and CaptureSelect™ LC- lambda, were used instead.

The purification processes of the mentioned κλ antibodies are based on the kappa and lambda constant domains. Even if NI-1701 and NI-1801 are not the bsAbs described in the patents, we conclude that the purification method can be performed in the same way as described.

**Advantages and disadvantages**
The advantage of using a κλ antibody is a facilitated purification process when separating the desired heterodimer from undesirable homodimers (Brinkmann & Kontermann 2017). Furthermore, the approach does not require any linkers or modifications and the κλ antibody has the same structure as a native human antibody (Magistrelli et al. 2016). These features are advantageous from a therapeutic perspective. In the patent application of the κλ antibody, they describe the advantages of not using linkers are that the antibody overcomes issues regarding immunogenicity, aggregation and poor stability. Additional advantages are that the antibody can be produced at an industrial scale and purification is not dependent on the variable regions (Fan et al. 2015). The purification process is therefore generally applicable. However, compared with other approaches such as knob-into-hole and CrossMab, the yields are significantly lower. This is due to a larger fraction of homodimers produced compared with these forced heterodimerization approaches. (Brinkmann & Kontermann 2017)

**5.6 ADAPTIR**
ADAPTIR is an antibody developed by the company Aptevo Therapeutics, see figure 23. An example of a bispecific antibody of this format is the drug candidate ALG.APV-527 co-developed by Aptevo Therapeutics and Alligator Biosciences (Aptevo Therapeutics, 2019). The companies have a granted patent “Oncofetal Antigen Binding Proteins And Related Compositions And Methods” (US10239949B2, 2019) together for antigen binding proteins that bind the same antigen as ALG.APV-527. The following information is presented based on the assumption that the information in patent can be applied to ALG.APV-527.
Structure
The structure of an ADAPTIR bispecific antibody comprise of an Fc region and four binding domains with two different specificities. The four binding domains are scFvs and attached in pairs at the amino and carboxyl ends of the Fc region. Thus, the Fc region has two binding domains at each end for binding two different antigens respectively, making it a tetravalent homodimeric bispecific antibody, see figure 23.

Figure 23. A schematic figure of ADAPTIR as presented in figure 10 in the patent US10239949B2, 2019. The pink and green domains represent the two pairs of scFvs, composing two different binding domains fused to the blue Fc region.

Therapeutic effects
ALG.APV-527 has binding domains for the tumor associated antigen 5T4 and the tumor necrosis factor receptor 4-1BB. High expression levels of 5T4 is common on cells in various types of cancers such as breast, ovarian, pancreas, prostate and gastric cancers. 4-1BB occurs on different types of cells but upon activation on T cells, when binding to its corresponding ligand, induce cell death. Targeting these two molecules with a bispecific antibody will promote potent tumor-directed immune T cell activation which makes ALG.APV-527 a potential drug for treatment of cancer.

Production methods
For upstream processing of this antibody, viral and non-viral vectors are used. The nucleotide sequences encoding VH and VL for one binding domain are most commonly placed in a plasmid. The sequences are then synthesized or amplified and linked together with a polypeptide linker. The resulting scFv nucleotide sequence, particular for a certain binding domain, is fused with the sequence for an Fc region. The sequence for the second binding domain is synthesized or amplified and linked to the Fc region in the same way. The resulting bispecific antibodies are expressed in host cells via transient transfection of cultured mammalian cells. Some suitable and commonly used cell lines for this purpose are HEK and CHO cells. Aptevo Therapeutics state that the production of ADAPTIR antibodies can be
done using antibody-like processes at commercial scales (Aptevo Therapeutics, 2019).

Affinity chromatography methods are used to purify these bispecific antibodies. Due to the fact that the ADAPTIR molecule contains an Fc region, the antibodies can be purified with protein A or protein G chromatography. However, protein A was used for purification of the molecules in the examples in the patent. For further purification size exclusion chromatography is also applied.

**Advantages and disadvantages**
The ADAPTIR formats in the examples of the patent have shown, compared to the similar Morrison format (see figure 13) with the same binding domains to have significantly improved expression levels in HEK cells. This was achieved without any further mutations on the binding domains. This is an advantage in upstream processing because it makes the manufacturing more efficient and therefore leads to lower costs. The ADAPTIR formats also showed greater purification, the aggregate levels were lower, in comparison to the Morrison format. Purity levels for the ADAPTIR formats varied between 89-94% meanwhile the Morrison was at 82%. Another advantage with this format is the presence of an Fc region which gives them half-lives similar to natural antibodies unlike bispecific antibody fragments. As no engineering of the Fc region is required for the formation of ADAPTIR molecules, the risk of immunogenicity is reduced which is another advantage. No disadvantages were mentioned in the patent or on Aptevo Therapeutics or Alligator Biosciences websites.

**5.7 BiIA-SG**
In 2018, a patent on the bispecific antibody BiIA-SG, see figure 24, was awarded to the University of Hong Kong and Shenzhen Third People’s Hospital. The BiIA-SG was described in a study by Wu et al. (2018) and all information about this antibody are based on this study.

**Structure**
This format constitutes of a single gene-encoded tandem bispecific immunoadhesin bs-BnAb called BiIA-SG. BiIA is an engineered immunoadhesin, which is an antibody-like molecule. It tetravalently binds to the two antigens via four scFvs fused to an IgG Fc region. It lacks the two CH1 domains that are native to the heavy chains of the IgG structure, see figure 24.

The structure of the single gene-encoded BiIA-SG molecule is constructed using a gene tandem fusion method. This results in a structurally unique molecule with four scFv binding domains, two targeting HIV-1 gp120 receptor and 2 targeting human T cell CD4 receptor. The existing of two scFv for gp120 results in a significant higher binding affinity comparison to having only one.
Figure 24. A schematic picture of the BiIA-SG as presented in figure 1B in the article by Wu et al. (2018). Two scFv represented in green and pink respectively are attached in tandem orientation on the Fc domain represented in blue.

**Therapeutic effect**
The BiIA-SG was developed as a therapeutic in the treatment of genetically divergent HIV-1. There have been increasing efforts to design therapeutics against HIV-1 using broadly neutralizing antibodies (bnAbs). Developing an efficient vaccine is difficult due to the wide genetic variety of HIV-1 because of its tendency to easily mutate. Thus, bispecific bnAbs (bs-bnAbs), as BiIA-SG, that have affinity for two separate epitopes have been developed to improve the breadth and potency of the antibody. (Huang et al. 2016)

**Production**
BiIA-SG antibodies are produced by fusing genes encoding two scFvs with different binding affinities in tandem, connected by a linker to a human IgG-Fc domain. The scFvs are connected through fusion of the gp120 binding VL/VH to the N-terminal of the CD4 binding VL/VH. The single-gene constructs are introduced into plasmids and transfected into cells. CHO cells were used for protein production in *in vivo* studies but HEK cells were used for the *in vitro* analysis. After culturing and centrifugation, the antibodies were purified using Protein-G-Agarose (Life technologies) according to the manufacturer’s instructions. Purity was validated to 90% using SEC-HPLC and no extra polishing steps were mentioned.

**Advantages and disadvantages**
Other existing bn-bsAbs are designed using conventional methods, for example the KIH and CrossMab technologies. The BiIA-SG structure have been compared to an alternative format based on the KIH method called BiIA-DG (double gene). The BiIA-DG had two scFv fused to the Fc region and was therefore bivalent. The BiIA-SG format demonstrated higher performance in regards to both activity and developability, due to being tetravalent and single gene-encoded.

In a preclinical study using humanized mice, BiIA-SG displayed improved breadth and
potency compared to regular treatment methods (Wu et al. 2018). This concludes that BiIA-SG is a promising format for further clinical development.

The lack of further effort to get higher purity could be due to the fact that the development is in its early preclinical stages. Additional purification steps, such as SEC polishing, could therefore be necessary for the drug to be able to enter further clinical phases.

6 Conclusions

We argue for, based on the formats in clinical and preclinical development, that the methods already used to purify monospecific antibodies such as protein A, G, or L affinity chromatography remain the most successful methods for the purification of bispecific antibodies. This, together with the resolving of the initial bottle-necks caused by chain mispairing, ensures that the needs of the developers are met in the same grade as for monoclonal antibodies. A “business-as-usual” approach is therefore viable which is, however uninteresting, good news for companies providing solutions for the downstream processing of biological therapeutics. It must be noted that the continuous development and optimization of these products and protocols is of course still beneficial to advance the field. We regard this to fall under a “business-as-usual” approach in chapter 7. Our conclusions are based on the arguments listed below.

6.1 The Fc region is important for both design and production

When developing bispecific antibodies for therapeutic use, the Fc region plays an important role in both the downstream processing and the biological functionality. From a drug design perspective, the Fc region is highly significant to extend the half-life of the antibody and to enable various effector functions. This domain also enables purification by protein A or protein G affinity chromatography, the most common capture steps for monospecific antibodies.

The BEAT strategy innovatively engineers different protein A binding affinities into the heavy chains. This ensures that the two homodimers have either increased or decreased binding affinity compared to the heterodimer. The desired heterodimer can therefore be purified in a one-step gradient process using protein A as a ligand. The importance of this region from a drug design perspective, together with innovative designs such as BEAT, points toward Fc-based formats being common in the future. This, in turn, points towards the continuous use of protein A and protein G chromatography.
6.2 Fragment-based formats usually lack protein A and G affinity but are still readily purified using standard methods

Fragment-based bispecific antibodies that lack the Fc region have also seen clinical success and are represented on the market. They can be purified using His-tag affinity chromatography or protein L affinity chromatography, together with size exclusion chromatography. In such cases, protein A or protein G chromatography is also a possible polishing step since the desired protein can be collected as flow-through.

The VH3 variable fragment also has protein A binding affinity. As can be seen in section 3.1.3, VH3 are established chains that have demonstrated good performance when it comes to expression and stability. If fragment-based formats incorporate VH3 chains, they could therefore be purified with protein A affinity chromatography as a capture step. Since fragment-based formats can be successfully purified using the aforementioned methods, we consider the future needs of developers met.

6.3 Currently available products offer satisfactory results

The grade of purity demanded varies with the intended use of the purified product. For example, clinical studies require higher purity than preclinical in-situ experiments. It seems, however, that the bispecific antibodies in clinical and preclinical development have been successfully purified using current products offered by GE Healthcare and other manufacturers. It is therefore our meaning that the downstream processing of these new formats is not significantly different than for their monospecific counterparts.

6.4 The initial problem with low yield has been solved using different design strategies

When bispecific antibodies were initially introduced, the production suffered from a very low yield. A number of design strategies have been developed to successfully combat this significant bottle-neck. We consider this issue to be satisfyingly resolved. Future methods could build upon these to further optimize the development of bispecific antibodies, and new methods could be developed to allow for single gene expression.

It is worth mentioning one factor that could be contributing to the continual success of conventional purification methods. Developing a format that can be efficiently purified with products available today is a goal in and of itself. As the development of biological drugs progresses, there may come a time when current products are insufficient and entirely new methods must be developed.
7 Discussion

In this chapter we discuss the possibility of selection bias, *i.e.* the underrepresentation of data highlighting problems in the development of bsAbs. We also discuss our selection process when deciding which formats and design strategies to include in the report. Finally, we discuss other possible purification methods with a couple of ideas of our own.

7.1 Selection bias

In this project, we have read numerous patent applications and articles in an attempt to present both the latest and historical developments in the field of bispecific antibodies. The patents are often the only references to the various preclinical formats described in this report. The published articles and patents we have encountered make it seem that the purification protocols were performed without any hindrances. This could be due to the fact that eventual problems encountered are under-reported by the authors to favor the publishing of articles and to increase the strength of their patent applications.

Other issues that are unrelated to the purification process can also be underrepresented or reported from different angles. For example, the developers of BiTE claimed that a short half-life makes it easier to control dosage levels. However, all the other sources see a short half-life as a significant disadvantage. Furthermore, the use of linkers is reported from different perspectives by different authors. The developers of the κλ antibody and the MAT-Fab format claim that avoiding the use of linkers is beneficial since they can be immunogenic. However, this is not something that we have seen discussed in other sources. These properties may only be mentioned as positive since they occur in the respective antibody format.

In summary, although we conclude that a “business-as-usual” approach is viable based on our own findings, it would be beneficial for GE to conduct client interviews to determine whether there were indeed problems that was not published for different reasons.

7.2 The format and design strategy selection process

This report does not describe all of the formats and design strategies available. There is an abundance of published articles and reviews describing formats and strategies in clinical development. We selected formats that were often mentioned in the numerous reviews available. When choosing design strategies, KIH and CrossMab were chosen due to them being frequently occurring in the reviews. The choice of additional strategies was made based on time constraints and not due to any order of interest.

Finding formats in preclinical developments was much more difficult. We defined a preclinical format as being a structure that is not represented in clinical trials with different binding domains. This limited our search to entirely new formats found in patent applications. These were very limited and we chose to include all of those we found.
7.3 Other possible purification methods

Our investigation lead to the result that bispecific antibodies can be produced by conventional recombinant protein expression (using CHO and HEK cells) and purified by the same methods as for monoclonal antibodies. However, continual development of GE Healthcare’s products is still necessary to maintain and develop their leading role on the market.

For example, when using protein A affinity chromatography, the captured antibodies are often evaluated by lowering the pH. This increases the risk for antibodies to “lose” their structure and to aggregate. Aggregates result in lower yield/purity and higher immunogenicity. This effect is one evidence that other purification methods could be of interest (Mazzer et al. 2015).

As described in appendix 1. It is desirable to use a method which could be applied in a platform process. In this report, we saw that many of the bispecific antibodies in development are targeting the CD3 receptor on T cells. One possibility for GE Healthcare could be to develop a ligand similar to the CD3 antigen to be used in an affinity chromatography capture step. Using the CD3 antigen as a capture step ligand would circumvent the need for e.g. His-tag usage and would therefore reduce the number of steps in the process. CD3 affinity would further be a better option from an environmental perspective in replacement of using a His-tag when producing antibody fragments since the use of His-tag implies the usage of the hazardous substances imidazole and nickel.

Another possibility is to design a format with specific purification properties in mind. For example, an antibody format could be fused with an additional scFv with binding affinity towards a certain ligand used in a capture step. The binding affinity would need to be such that the bsAbs of interest can be eluted easily enough. It would also be important that the additional scFv does not exhibit activity in vivo to avoid any immunogenicity. It is also possible to use a cleavable linker in the fusion of the scFv.

8 Water consumption in life sciences – an ethical analysis

Global water consumption is a growing issue worldwide. The European Environmental Agency has published a forecast that predicts a global water demand to reach more than 40% above current supply by 2030 (EEA 2010). The need to decrease and streamline water consumption in all sectors, including life science, is therefore urgent.

In the life science industry, water is vital for many different processes. According to Biocom in “Water Conservation by Life Sciences”, it is estimated that 80 percent of the water consumption in a biotech company is for process support, i.e. manufacturing or treatment process, cooling or research and development. Many of these processes involve purification of pharmaceuticals, often affinity chromatography. In these cases, buffer is utilized in the binding, elution and regeneration steps.
Since GE Healthcare manufactures instruments for purification, we have compared buffer consumption for different chromatography alternatives used to purify antibodies, see appendix 5. There is a difference in the amount of necessary column volumes (CV) when looking at the values for the total number of rinses with binding-, elution- and regeneration buffer. The amount varies even if both products use the same ligand. It has to be taken into account that factors such as wanted purity and method of elution affect the number of column volumes needed. It would take another project to distinguish why the total column volume differs from 25 CV to 63 CV. However, it is clear that there are options which require lower amounts of water, indicating that there is room for improvement.

In 2010-2012 GE Healthcare conducted a study where they examined whether single-use or traditional products affected the environment the most when looking at different factors. One category of interest was fresh water consumption and its impact on the environment. The study showed that the impact of freshwater consumption depended the geographical location. In for example Istanbul, Turkey, the impact of freshwater consumption was much larger than in Cork, Ireland.

Should countries with high impact of water consumption produce pharmaceuticals that have high water demanding procedures? It is important to keep the impact as low as possible since fresh water is not just vital for different processes in the life science industry, but also for human life and ecosystem quality in general. Does this make companies selling products with major fresh water consumption, such as chromatography products, responsible for the impact it might cause? Even though distributors might not be responsible it is of great significance to lower the consumption of fresh water as much as possible.

The sensitive nature of drug development for human use, whether it be for clinical trials or large-scale manufacturing, confers very high demands for purity. The same applies for the production of bispecific antibodies. Optimizing the downstream procedures in favor of reducing water consumption could be a possibility. However, changing the downstream procedure needs to happen without compromising product quality and safety for it to be deemed ethically viable. Perhaps, an approach with more potential is to improve the upstream processing of these bispecific antibodies, reducing the need for complicated purification protocols. Optimized upstream processes would therefore reduce the water needed to yield an end product with a certain purity. Currently existing strategies such as knob-into-hole and CrossMab facilitate the production process and more are sure to be developed in the future. However, too much engineering could lead to increased immunogenicity and further improvements would need to keep this in mind. Successful optimizations of the upstream process would be beneficial both for the re-adjustment towards long-term sustainable water use and for economic reasons.

When comparing the water use in life science to other products with high water demand there is an interesting discussion to be had. It is estimated that the amount of water needed to produce a single t-shirt together with a pair of jeans is 20,000 L. (WWF, 2019) For the same
volume of water, you could produce enough antibodies to treat circa 2000 patients. We base this simplified estimation on the following numbers:

- At most 20,000 L buffer is required for the harvesting of a 1000 L cell culture (Channeler 2015)
- 3-8 g mAbs are produced per L of cell culture (Kelley et al. 2018)
  - We assume the lowest yield, i.e. 3 g per L of cell culture
- The total amount of Blinatumomab administered according to the dosing scheme is 1.435 g (FASS - Blincyto 2019)

1000 L cell culture results in 3 kg antibodies. 3000/1.435 = 2090 patients treated.

This calculation is very simplified and is based on several assumptions but it may yet serve a purpose to illustrate the difference in the water consumed in the two processes. These numbers assume that the water required in the production of bispecific antibodies is the same for monospecific antibodies as for bispecific antibodies. We also do not account for the water used in the creation of the actual drug products, as the antibodies are of course not administered as a solid chunk at one time but rather as continual injections.

The ethical comparison of a pair of blue jeans and the availability of potentially life-saving therapy with biological drugs such as bispecific antibodies heavily favors the latter. It is perhaps an ill-suited argument that tries to evade the issue of water consumption within life science with a redirection toward other industries. However, if water were to be respected as a diminishing and vital resource, the consumption needs to be ethically justified.

In summary, water consumption within the life science industry and within the purification of bispecific antibodies needs to be further optimized. The reduction of water consumed in the process must come without the expense of product quality and safety. Furthermore, the water needed to manufacture a single pair of jeans and a T-shirt is equivalent with the amount needed to produce enough antibodies to treat two thousand patients, roughly calculated. All things considered, the water consumption in the production of bispecific antibodies can be improved, but it is ethically justified given the benefits provided by this technology.
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Contribution statement

All project members contributed to the project plan. Amanda and Anisha did the project plan presentation. The ethical analysis was written with more contribution from Amanda, Fabian, Terese, Therese and was reviewed by all project members.

The report was written by all project members where:

- Amanda focused on the BEAT technology and formats, CrossMAb, scFv fusions, BiIA-SG, the purification methods in antibody production and CHO cells.
- Anisha focused on the quadroma technology, DVD-Ig, ADAPTIR and HEK cells.
- Fabian focused on DuetMab, SEEDBody, CH1-CL mutations, TandAb, biAbFabL, taFab, VCVFc and VCDFc.
- Terese focused on the orthogonal Fab strategy, MAT-Fab, iBiBody and creating the figures.
- Therese focused on BiTE, XmAb and κλ antibody.
- Thomas focused on knob-into-hole strategy and tandem forms.

The remaining parts of the report have been written together. Each and every member has reviewed and edited the content and all of the pictures in the report. Every member has also collected sources and shared knowledge and feedback throughout the course of the project. The poster was made by Fabian and the content and layout was thoroughly reviewed by Anisha, with feedback from all project members. The poster presentation was done by Fabian while the final project presentation was done by Terese, Therese and Thomas.
References


**Patents**

All the patents were retrieved from a patent database called The Lens. It can be found at [https://www.lens.org/](https://www.lens.org/).


Appendix 1 - Purification methods used in antibody production

When purifying antibody in large scale production, it is usually done using a platform approach where standard operations and conditions are held constant, according to GE Healthcare. Using this generic approach, it is able to apply the same purification platform when producing several different types of antibodies. Because of this, affinity chromatography using protein A, G or L are the most commonly used in platforms when producing IgG and antibody fragments such as Fabs and scFvs. Due to the diversity of the fragment structure, a platform approach is harder to achieve and further developing is needed. Alternatively, fragments can be captured using cation exchange chromatography or even multimodal resins. (Tosoh Bioscience, 2019)

Protein A affinity chromatography

Protein A chromatography is based on the specific and reversible binding of immunoglobulin antibodies to an immobilized protein A ligand. The native forms of protein A originate as a surface protein of *Staphylococcus aureus*. It has five immunoglobulin binding domains that are able to bind to protein from many mammalian species but specifically immunoglobulins through the heavy chain in the Fc region. Furthermore, it is also able to bind to the Fab region of certain IgG subclasses/isotypes. Since first generated, many different protein A resins have been developed for different purposes by modification of the ligand. One example is recombinant protein A lacking in albumin binding regions. The protein A used today are thus recombinant forms produced in *Escherichia coli* (*E. coli*). (Sino Biological, 2019)

Due to its specificity, protein A chromatography is the standard technique for capturing recombinant antibodies. Because of its robustness, it is used both at research scale and in industry. Depending on the use of the target antibody, protein A might be the only purification step needed. In other cases, it is followed by one or several of polishing steps *i.e.* ion exchange chromatography and gel filtration. (GE Healthcare, 2019)

Protein G affinity chromatography

Protein G are similar to protein A, in case of both binding to immunoglobulin antibodies. Protein G was first isolated from *Streptococcal* bacteria strains C and G. The difference between the two are usually that protein A has greater affinity to rabbit, pig, dog and cat IgG whereas Protein G has higher affinity for mouse and human IgGs. (Sepmag, 2018)

Protein L affinity chromatography

Protein L chromatography differ from A and G because of its ability to bind members of all classes of immunoglobulins and also scFvs and Fab fragments. Protein L was originally derived from the bacteria *Peptostreptococcus magnus* but just as protein A and G are now produced recombinantly in *E. coli*. The protein L ligand bind generally through kappa light chain interaction without interfering with the antigen binding site. Therefore, protein L binding is restricted to antibodies or fragments that contain kappa light chains. In humans and mice, the light chain can also be of lambda type. Furthermore, it binds most effectively to
kappa light chains of certain subtypes. In recent years, recombinant resins have also been
developed that also bind to lambda light chains. (InvivoGen, 2019, GE Healthcare, 2019)
Appendix 2 - HEK and CHO cells, the most common cell lines for producing bispecific antibodies

Chinese hamster ovary cells (CHO cells)
Chinese hamster ovary (CHO) cells are epithelial cells widely used in research and commercially in the production of therapeutic proteins. They are used in a wide variety of fields for example in studies of genetics, toxicity screening, nutrition and gene expression but in particular in the production of recombinant protein. For example, are CHO cells the most prominent choice of mammalian host for expression of monoclonal antibodies. All CHO cells used in laboratories originate from the same source, a single female Chinese hamster that lived in 1957. Since then, a lot of different CHO cell lines have been created for different purposes. CHO cell lines used in production are often opted to achieve increased expression levels and maximize production while still preserve its stability. The first method for producing monoclonal antibodies was the hybridoma technique. Since then, problems with this method have been discovered and it is now widely replaced by recombinant expression in cellular systems, usually in CHO cells production systems. (Shatz et al. 2016)

High-level recombinant expression can be done in CHO cells lacking the dihydrofolate (DHFR). This is one of the standard genetic selection methods used to achieve transfected CHO cell lines. The DHFR gene and the recombinant gene are introduced in an expression vector and transfected into cells. They are further co-amplified within the host cell which are allowed to grow under selective conditions. The DHFR gene works as a selective marker, which mean that only cells with the DHFR gene and the gene of interest will survive. Depending on modification, growth rate and level of production between cell lines varies wildly. It has also shown that stability can be affected by to high expression levels and work have to be done to obtain highly productive but still stable cell lines. The CHO cell lines can be obtained from a number of biological resources centers such as the European Collection of Cell Cultures, which is part of the Health Protection Agency Culture Collections. (Shatz et al. 2016)

CHO cell lines have also in recent years become the cell line of choice when producing bispecific antibodies. When producing bispecific antibodies, the heavy chain (HC) and the light chain (LC) plasmids for each HC-LC fragment are transfected into CHO cells and cultured separately. The antibodies are captured as a mixture of half-antibodies and homodimners. The bispecific antibodies are assembled by combining each media mixture in vitro followed by addition of reduced glutathione (GSH) to allow disulfide bond formation. This usually results in fully formed bispecific antibodies and very little homodimer impurities. Many approaches that invoke heterodimerization, such as knob-into-hole, involves separate expression and purification of each fragment as described above. Although good results, this strategy are inefficient because of the many steps needed. The parallel processes also lead to an increases risk of contamination and costs. (Shatz et al. 2016)
Co-culturing is another alternative strategy in the upstream process of producing bispecific antibodies using heterodimerization technology *e.g.* controlled Fab arm exchange, strand-exchange engineered domain (SEED), charge pair residues and HC hinge point mutation technologies. Both cell lines of the H-L fragment are then grown in the same production flask in a one to one ratio. The protein is secreted in the culture media and addition of GSH favors correct assembling of the fragments. The assembled antibodies are then captured directly from the media. This method has many advantages over conventional methods for example cost, time when reducing culturing and purification steps needed. (Shatz *et al.* 2016)

An increasing number of strategies where the four chains are co-expressed in the same cell have in recent years been developed. This is termed single-cell expression and have, as for co-culturing, many advantages because fewer steps are needed. The main asset of this strategy is that the steps are made further upstream in the production path. This method however requires a substantial amount of genetically engineering, in form of mutagenesis work, to be done and often results in lower yields. Also, some difficulties can occur for protein with different expression levels. (Shatz *et al.* 2016)

**Human embryonic kidney (HEK) cells**

Human embryonic kidney (HEK) cells are often used as a mammalian expression system to produce recombinant proteins. This is needed for applications such as biochemical assays, structural studies and protein production for therapeutics *e.g.* bispecific antibodies. HEK cells were first derived by altering the DNA of the human fetal kidney cells with Adenovirus 5 in the early 1970s. The transformation led to the immortal cell line known today as HEK 293 cells. It is also referred to as just HEK cells. (Nigi *et al.* 2017)

Several variants of the HEK 293 cells have since then been developed for different purposes. One of the widely used formats is HEK 293Freestyle (HEK 293F) cells in the production of challenging protein complexes. This cell line has high transfection efficiency (50-80%) with the inexpensive transfection agent (polyethyleneimine). The HEK 293F cells are therefore a quick and economical method for producing a large quantity of recombinant proteins. (Nigi *et al.* 2017) Furthermore, the cell line is adapted to grown in suspension. HEK 293F cells are commonly used today in the production of bispecific antibodies when expressing the proteins through genetic fusion.

Some other HEK 293 cells are HEK 293T which enable amplification of vectors containing an SV40 ori thanks to its own SV40 T antigen. HEK 293S is adapted to suspension growth and the further development HEK 293SG contain resistance against Ricin toxin. (Nigi *et al.* 2017)

Mammalian expression systems have been developed due to the lack of machinery required for post translational modifications. Especially when trying to express human proteins in corresponding bacterial expression systems. (Nigi *et al.* 2017) An example of a post translational modification dependent on host cell is glycosylation. It is important not to lack in
this machinery in the production of antibodies since glycosylation patterns can affect Fc effector function and/or affinity to the antigen. (WO2018035084A1, 2018)

Mammalian expression systems, besides HEK cells, may include CHO cells and mouse myeloma cells. The aforementioned expression systems have the ability to produce high yield of proteins that cannot be expressed in bacteria. The main advantage with HEK 293 cells, compared to others like CHO cells, is its incorporation of more native-post-translational modifications. In other words, post-translational modifications that are present on human proteins. Cell lines derived from other species than humans can introduce non-native post-translational modifications and cause immunological reactions or affect stability and function of the protein. (Nigi et al. 2017) Another advantage when compared to CHO cells is that HEK 293 cells, when transfected, can produce higher yield of antibodies in half the time of CHO cells. (Arena et al. 2019)
Appendix 3 - Linkers are short peptide sequences commonly used in the construction of bispecific antibodies

A common element in bsAbs are linkers which are sequences connecting different domains together. A linker built up of repeating elements of glycine and serine is widely used. Glycine confers flexibility, while serine confers solubility. A linker consisting of these two residues prevent the formation of secondary structures and reduces the likelihood of the linker interfering with either folding or function (van Rosmalen et al. 2017). An example is (Gly-Gly-Gly-Gly-Ser)n, sometimes abbreviated G4S or GS-linker, where n represents the number of repeats (Chen et al. 2013). These are inserted in the expression construct between the sequences of the different domains to e.g. link added domains to a native structure, or to link different fragments together in a completely novel format. Linkers are important to fuse the domains while at the same time allow for correct folding of the fusion protein. Fusion proteins that do not incorporate linkers can otherwise suffer from lower yield or potency (Tushchuk et al. 2016). Depending on the desired functionality of the fusion protein, incorporated linkers can be flexible, rigid or cleavable (Tushchuk et al. 2016). The formats described in this report primarily used flexible GS-linkers.
Appendix 4 - Further developed BEAT formats

There are more than one potential BEAT formats, as presented in the report, see section 4.1.3. Herein is information about two BEAT formats, one in clinical and another in preclinical trial, in regards to therapeutic effects, structure and upstream/downstream processes.

GBR 1342

The GBR 1342 BEAT molecule that targets components of CD3 and CD38 have reached clinical phase I studies. The CD38 molecules is a type II transmembrane glycoprotein normally found on hematopoietic cells that are stems cells that differentiate to other blood cells and solid tumors. Furthermore, CD38 has also shown to been expressed in many malignant hematological diseases, for instance multiple myeloma. It is therefore a target of great potential as a means of treating multiple myeloma and chronic lymphocytic leukemia. This is mainly because of its expression pattern and its role as both a receptor and an ectoenzyme. MAbs have in recent years been used in the treatment of these cancers by targeting the CD20 receptor. Although, many have recently demonstrated limited effect using these therapeutics. This is likely caused by B cells losing CD20 expression when differentiating to plasma cells. The development of a bispecific antibody for treatment is therefore needed. (WO2016071355, 2016)

The structure of the bispecific antibody can constitute of different combinations of fragments according to the BEAT format. One example mentioned in the patent comprises of a heterodimeric immunoglobulin with one CD3 binding scFv-Fc and one CD38 binding Fab with only ability to bind protein A on the Fc portion fused to the scFv. According to the patent the antibody was transient expressed in HEK cells and further purified with protein A or G. (WO2016071355, 2016)

GBR 1372

The latest invention generated using the BEAT format is a CD3xEGFR bispecific antibody. It is now under preclinical investigations, according to Glenmark’s pipeline. This antibody can bind the CD3 domain of the T cell receptor and EGFR expressing tumor cells simultaneously. When used for the treatment of EGFR tumors, it activates and redirects T cells against the cancer cells. EGFR (epidermal growth factor receptor) is overexpressed in many epithelial cell cancers such as colorectal, lung and pancreatic cancer. (WO2018197502A1, 2018)

One way of treating these types of cancers is by using EGFR targeting mAbs. The problems with these are that the cancer cells tend to develop a resistance against the pharmaceuticals. This could be caused by adaptive mechanisms applied by the cancer cells. Some observed effects are variability in EGFR expression, signaling in neoplastic cells and/or other mechanisms to avoid therapy. Two very problematic mechanisms are the KRAS and B-Raf mutations observed in for instance colorectal cancer cells. This led to the invention of a bispecific CD3xEGFR antibody using a BEAT format to overcome the problem. (WO2018197502A1, 2018)
The structure mentioned in the patent consists of at least one scFv antigen binding portion and at least one Fab antigen binding portion concatenated to each other. Furthermore, the antibody has the BEAT substitutions as described above to induce heterodimerization and the ability to purify with protein A. Different BEAT formats have been constructed and tested for significant effect. One that demonstrated particular good results is constructed with one CD3 binding scFv portion fused directly to a Fab arm with an Fc region. This structure was developed by the idea that closer proximity between the antigen binding sites would have increased therapeutic effect. To get the BEAT format, substitutions in the VH3 region on the CD3 binding portion and Fc portion were introduced to remove protein A binding. In the mentioned patent, the antibody was produced using transient expression in HEK cells. Furthermore, the BEAT antibody can be purified from the supernatant using protein A affinity and pH elution. (WO2018197502A1, 2018)
Appendix 5 - Table over buffer use in different purification techniques

The ethical analysis in chapter 8 describes the issues regarding water consumption in life science. Processes that require large amounts of water in this industry is purification. Therefore, it might be interesting to compare the column volume (CV) of different purification techniques used by different companies. See table 5.

Table 5. Buffer use in different purification techniques. The table presents the buffer consumption if various purification techniques, with column volumes (CV) as unit. The data was collected from the respectively company manual.

<table>
<thead>
<tr>
<th>Column</th>
<th>Ligand</th>
<th>Amount of Binding buffer, elution buffer and regeneration buffer (CV)</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>HiTrap MabSelect suRe</td>
<td>Protein A</td>
<td>53</td>
<td>GE Healthcare</td>
</tr>
<tr>
<td>HiScreen MabSelect</td>
<td>Protein A</td>
<td>48-63</td>
<td>GE Healthcare</td>
</tr>
<tr>
<td>HiTrap Protein A HP</td>
<td>Protein A</td>
<td>25</td>
<td>GE Healthcare</td>
</tr>
<tr>
<td>HiTrap Protein G HP/Protein G Sepharose 4 Fast Flow</td>
<td>Protein G</td>
<td>25</td>
<td>GE Healthcare</td>
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<tr>
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<td>Recombinant protein (Antibody Toolbox resins)</td>
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<tr>
<td>Affi-Gel Protein A</td>
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