Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Structural Biology

# Engineering antibody therapeutics

Mark L Chiu and Gary L Gilliland

The successful introduction of antibody-based protein therapeutics into the arsenal of treatments for patients has within a few decades fostered intense innovation in the production and engineering of antibodies. Reviewed here are the methods currently used to produce antibodies along with how our knowledge of the structural and functional characterization of immunoglobulins has resulted in the engineering of antibodies to produce protein therapeutics with unique properties, both biological and biophysical, that are leading to novel therapeutic approaches. Antibody engineering includes the introduction of the antibody combining site (variable regions) into a host of architectures including bi and multi-specific formats that further impact the therapeutic properties leading to further advantages and successes in patient treatment.

**Address**
Janssen Research & Development LLC, 1400 McKean Road, Spring House, PA 19477, USA

Corresponding authors: Chiu, Mark L (mchiu@its.jnj.com) and Gilliland, Gary L (ggillila@its.jnj.com)

**Current Opinion in Structural Biology** 2016, **38**:163–173

This review comes from a themed issue on **New constructs and expression of proteins**

Edited by **Rob Meijers** and **Anastassis Perrakis**

For a complete overview see the Issue and the Editorial

Available online 12th August 2016

**http://dx.doi.org/10.1016/j.sbi.2016.07.012**



## Introduction

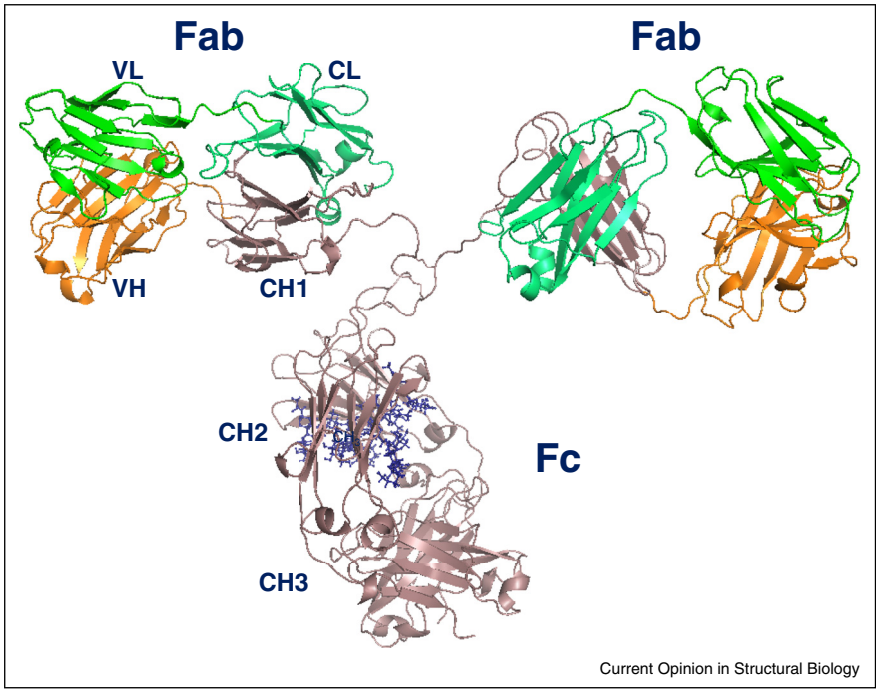
Monoclonal antibodies (mAbs) and proteins derived in part from mAbs have established themselves as one of the largest groups of biologics (biotherapeutic proteins) that are now being applied to a wide variety of therapeutic applications [1]. The discovery and development of hybridoma technology [2] created the foundation for modern day mAb discovery and development. This has been augmented over the years by the application of phage [3] and other display systems [4], transgenic animals [5•], and other approaches for mAb discovery. The successful development of a therapeutic mAb often requires generation of a selective and potent molecule, humanization of sequences, affinity maturation, Fc engineering to modulate effector functions, and engineering to deal with biophysical liabilities that would negatively impact manufacturability. In recent years, the production of bispecific mAbs and Ab-derived therapeutics in a variety of alternative formats are making their way into the clinic [6••]. Because of the vast number of publications on the discovery, application, and engineering of mAbs, this review will focus on updates of the generation of the target, or antigen binding region, of the antibodies and their incorporation into therapeutics.

Natural antibodies (Figure 1), or immunoglobulins, play active roles in the immune system by recognizing and defending against pathogens. The binding regions of the mAb are made up of the heavy chain (HC) and light chain (LC) variable domains that determine the specificity and affinity of the antibody. The stem of the Y-shaped Ab is called the Fc region, and it is composed of two pairs of constant glycosylated HC domains. The Fc is responsible for effector functions and contributes to the extended half-life of the molecule in blood plasma. The Fc effector functions include involvement in a number of cell-killing mechanisms through its protein and carbohydrate interactions with Fcγ receptors and components of the complement system [7•] as well as agonism activity mediated through crosslinking [8••]. The Fc half-life functionality is carried out mostly through interactions with the FcRn [9].

Antibody variable (V)-regions of the Fab are one of the focus areas of therapeutic engineering because of the role they play in antigen or target binding [10•]. The antigen combining site is formed by the combination of six hypervariable or complementarity-determining regions (CDRs), three from both the HC and LC (Figure 2). The amino acid residues of the V-regions that are in direct contact with the antigen are referred to as the *paratope*. The surface region of the antigen in direct contact with the Ab paratope is referred to as the *epitope*.

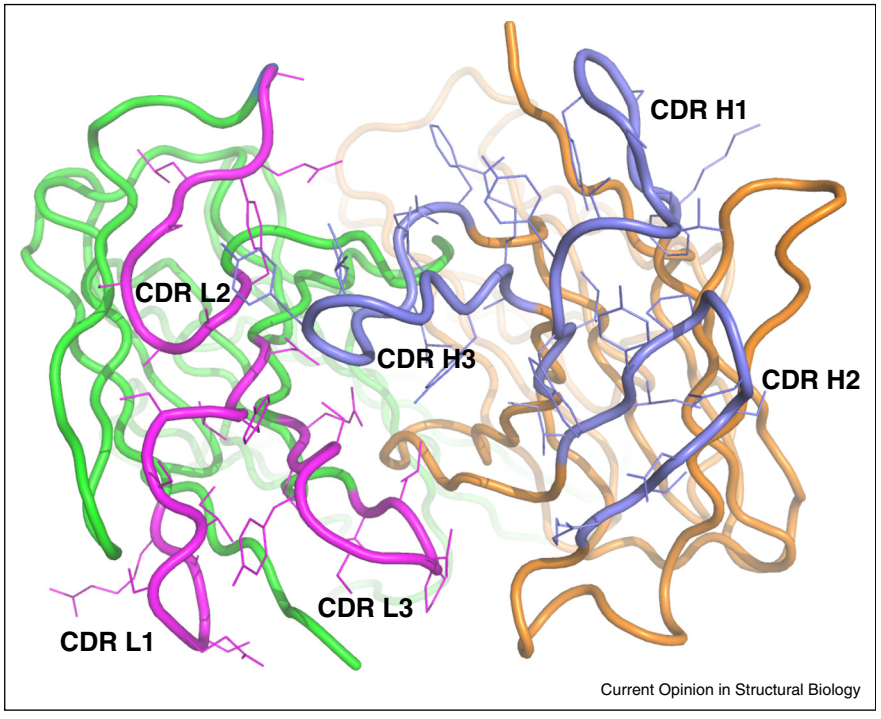
As the first structures of antibody Fab fragments became available, it was discovered that of the six CDRs, all but CDR-H3, have a limited number of main-chain conformations, often referred to as canonical structures [11]. Recently, the early analyses have been expanded to include the V-regions of all available antibodies and antibody constructs [12,13]. This recent work has also identified a small set of canonical structures for short CDR H3 loops. This knowledge is being used to guide V-region modeling/engineering studies, and is also being used to detect residues in the CDRs that could influence the biophysical properties (see Engineering for manufacturability section).

**Figure 1**




Structural model of a human IgG1. The Fab region is composed of the interaction between the variable and CH1 domains of the heavy and light chains. The light chain is colored in green with the variable region colored in bright green. The heavy chain is colored brown with the corresponding variable region colored in orange. The CH2 domain has glycosylation sites that orient between the two heavy chains in the Fc region.

**Figure 2**




Representative arrangement of the complementarity determining regions (CDR) of the Fab region. The CDRs are composed of three respective loops of the variable chains of the heavy and light chains. The six CDRs can interact with antigens to mediate the specificity and potency of an antibody.

Described in the following sections are the methods used to generate and engineer therapeutic antibodies. These representative methods and how they relate to the therapeutic antibody discovery process are shown in Figure 3. It should be realized that the development of every therapeutic antibody follows a unique path that incorporates many, but not all, of those described in the sections below.

## Animal immunization and hybridoma generation

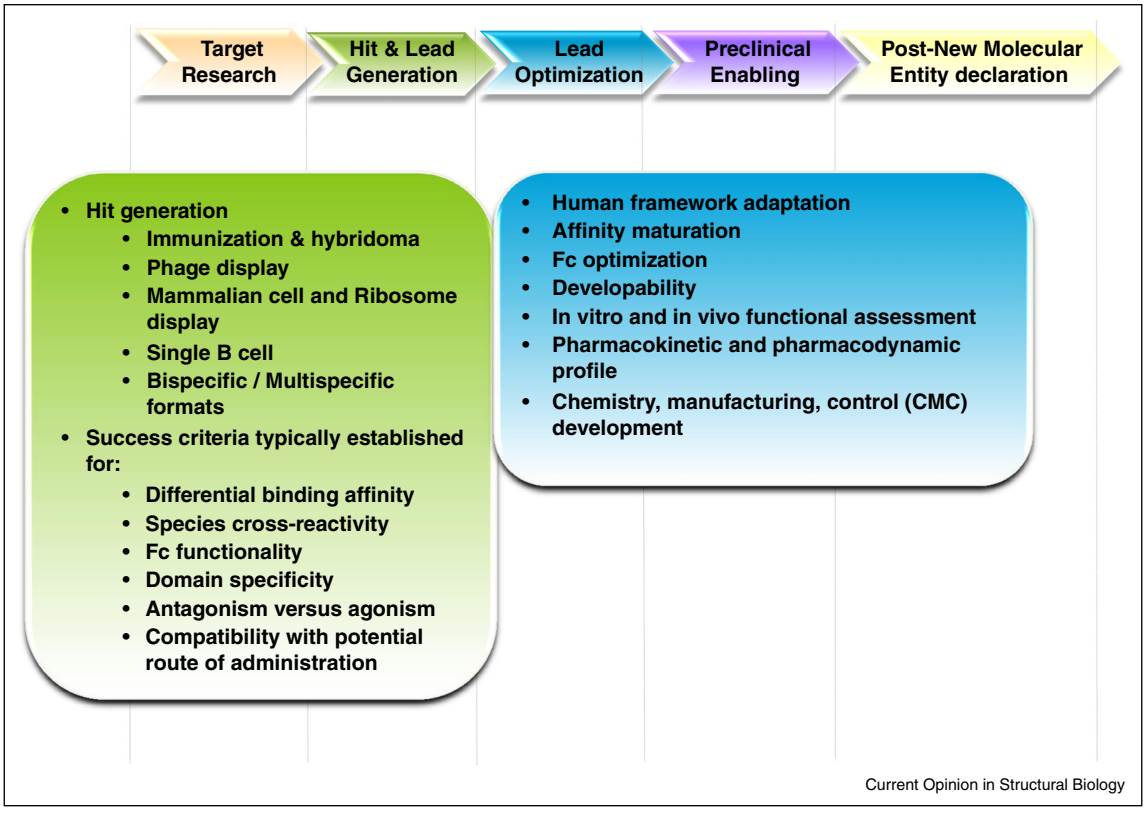
The field of therapeutic Abs began with the generation of mAbs via the fusion of murine B cells and murine myeloma cells to produce single fusion cell lines (hybridomas) which produce monoclonal Abs (mAbs) with a single unique specificity [2]. In this way, mAbs against almost any antigen are created, but the direct therapeutic use of murine mAbs in humans is limited by both the high incidence of harmful immune responses against the murine mAb and the lack of sufficient effector function for murine mAbs. Today murine mAbs destined for therapeutics, after their generation by immunization of animals (mice or rats), are then engineered to prevent an immune response in patients against non-human sequences. Initially this was partially achieved through chimerization, replacement of the constant regions of the rodent Ab with those of a human Ab [14]. Today V region humanization often uses CDR grafting in which the CDRs are combined with human V region framework sequences [15]. This process often reduces the mAb affinity, and hence, it is usually followed by affinity maturation (see Affinity Maturation section).

In order to eliminate the efforts required for humanization of mAb therapeutics, transgenic mice and rat strains have been developed to generate human mAbs directly. In these animals, the murine immunoglobulin loci have been replaced with human sequences. A number of different approaches are now being used to generate mAbs directly by using transgenic animals [5•,16].

## Microbial display

Because the antibody paratope is well understood, as mentioned in the Introduction section, several approaches have been developed to produce synthetic combinatorial libraries for the identification of human

**Figure 3**




Applications of molecular biology during the engineering of antibody therapeutics. In a typical flow chart for the process of generating a therapeutic antibody, engineering is required at hit & lead generation and lead optimization. During hit and lead generation, the focus is being able to generate the desired specific and potent activity. In lead optimization, the binding arm sequences are changed to have a more human framework sequences to minimize potential immunogenicity. Additional changes in the Fab and Fc sequences are made to tune the functional activity and to minimize manufacturability risks.

mAbs for specific targets. This is especially useful in developing mAbs against antigens that would be difficult to obtain using animal based technologies, including toxic, non-immunogenic, or self-antigens.

The display technology was originally described by Smith in the form of phage display of peptides through their incorporation as a fusion into the sequence of phage gene III protein [17]. Modified versions of this phage display approach are widely used as mAb discovery platforms. Among the selection technologies currently available, the display of antibody libraries on bacteriophage M13 by genetic fusion of antibody fragments to the phage minor coat protein pIII or its C-terminal domain is most frequently used. The challenge is to link the DNA sequence within the phage particle to the protein or peptide displayed on the surface of the phage particle. This linkage facilitates the selection of the specific binding property. Phage display is often used with Ab fragments that include single chain variable region fragments and Fabs. Screening involves the selection of molecules with the desired properties including antigen binding, cross reactivity in binding between defined species, increased stability, *etc.* [18].

Alternatively, more focused approaches can vary the sequences at specific positions in the CDR regions by the wobbling gene synthesis. A variety of methods can be used to screen the relevant residues [19] such as phage display to generate human Abs directly from naïve human libraries [20]. The sequences have also been optimized for expression in *E. coli* in a monovalent Fab format thereby minimizing the potential of aggregation [21]. The intention of this work was to create a monovalent Fab display that was optimal for the selection of high-affinity antibodies, yet without losing antibodies with medium affinity. It was shown that sufficient numbers of Fab–pIII complexes are incorporated into the phage particle to achieve this. The Fab format is stable and does not form dimers or multimers, as is often the case for scFv (a single chain version of the VH and VL domains of an Ab). This format allows the selection for affinity and not avidity and rapid off-rate screening in crude periplasmic extracts. One drawback, however, is that they usually rely on random combination and, thus most likely, unnatural VH and VL antibody pairings.

## Eukaryotic ribosome display

We extend upon the excellent reviews of synthetic Ab technologies [22•]. Poor expression and toxicity to the host bacteria are often issues with Abs isolated from naive libraries. Likewise tolerance of an antigen that is highly homologous to host proteins will result in poor response in getting antibodies. This problem can be bypassed by using an *in vitro* protein evolution to create Abs that can bind to a desired ligand. The libraries of DNA sequences that code for Abs for selection can come from synthetic Ab repertoire libraries [22•], yeast display [23], mammalian cell display [24], and *in vitro* vaccinia virus display [25]. In these processes, each Ab sequence is transcribed and translated *in vitro*. The DNA library are typically fused to a spacer sequence lacking a stop codon so as to prevent release factors from binding and triggering the disassembly of the translational complex. Thus the Ab can protrude out of the ribosome and fold thus being a part of a complex of mRNA, ribosome, and protein which can bind to the surface-bound ligand. The complexes that bind well are immobilized and after subsequent elution that allow for dissociation, the corresponding mRNA can then be reverse transcribed back into cDNA for identification. Repetition of this process with greater selective pressure can be used to isolate even better binders. Additional factors present in eukaryotic ribosome display can enhance protein translation and folding especially with a variety of post-translational modifications better than the prokaryotic counterpart. Ribosome display can select rare sequences and high-affinity combining sites. Moreover with PCR, further diversity can be introduced into the DNA pools after selection to provide an efficient route for protein evolution. In addition, cell-free systems can be used to find binders to toxic and proteolytically sensitive or unstable proteins which can be intractable for selection using microbial or animal methods. Nonetheless, these ribosome display screenings depend on the quality of the antigen used for selection.

## Single B cell

One drawback of the Ig transgenic murine systems is that they cannot precisely imitate a human immune response because of the effects of the murine genetic background on antigen processing and B cell regulation. As a result, the recovered antibodies might not display the precise specificities of naturally occurring antibodies in humans. Although this technique has produced numerous useful Abs, its applicability is limited by differences in binding properties between antibodies expressed in bacterial and eukaryotic cells. In addition, phage display may result in heavy-chain and light-chain combinations that do not occur in the same B cell *in vivo*. As it is thought to be important in antibody development to maintain the original VH and VL pairing as exists in human B cells, efficient strategies have been designed which are based on the direct amplification of VH and VL region encoding genes from single human B cells and their subsequent expression in cell culture systems. It is possible to exploit human immune reaction in the discovery of monoclonal antibodies. Simply put, human immune response works in the same way as that in a mouse or other non-human mammal. Therefore, persons experiencing a challenge to their immune system, such as an infectious disease, cancer or a vaccination are a potential source of monoclonal antibodies directed at that challenge [26,27]. Human B cells have also been immortalized by electrofusion

or Epstein-Barr virus (EBV) transformation, with or without the use of toll-like receptor ligands. However, these techniques can be inefficient in some patients, such as those with HIV infection, and transformed clones can be lost because of instability.

Recently, cell-based microarray and microengraving techniques are being applied to large populations of primary B cells to facilitate the rapid screening and detection of single cells secreting antibodies with the desired reactivity profiles [28,29].

The single B cell antibody approaches harbor the potential to isolate functional mAbs reactive against conformational determinants that are presented predominantly *in vivo* and difficult to emulate *in vitro*. Because of the strategy straightforwardness, the requirement of relatively few cell numbers and the high efficiency in obtaining specific mAbs in a rapid way are balanced by the downsides: The need of adequate human donors and the limitation to certain target molecules. B lymphocytes expressing affinity matured Abs can be selected and expanded [30,31•]. Furthermore, valuable mAbs, have been isolated from vaccinated [32] naturally immunized donors [33] and patients with autoimmune disease [34].

Future advancements on microfluidic platforms, including PCR cycling combined with next generation sequencing methods, are likely to exceed the current systems and allow for high-throughput Ig gene sequence analyses of individual antibody repertoires. By increasing the availability of such repertoires, single B cell antibody technologies are likely to take the lead in the development of novel mAb therapeutics.

## Affinity maturation

Abs generated either from phage, eukaryotic ribosome display, or the transgenic route are often subjected to further engineering that includes replacing residues with liabilities in the binding region to improve its developability and 'germ lining' to replace unusual residues in the framework residues with those present in the pre-mutated germ line genes to reduce the potential for immunogenicity. Immunogenicity can have multiple causes and is influenced by: dose, route of delivery, presence of protein aggregates, use of concomitant medication, dosing strategy, genetic background as well as amino acid sequence. In addition to reducing the non-human primary sequences, there can be efforts to deimmunize protein sequence by removing T cell epitopes identified either *in silico* or using T cell stimulation assays [35]. When such studies are necessary, they often lead to a reduction in binding affinity and specificity. This is usually remedied by an *in vitro* affinity maturation process [10•,36].

Protein engineering can modulate the affinity of an Ab to its Ag. This is relevant for Abs generated by phage display or using transgenic mice with human IgG as these technologies tend not to generate Abs with the same affinity as immunization based techniques with wild-type animals. Methods can be in 2 categories — display based methods and structure based methods.

Display based methods rely on building libraries of variants with changes that affect Ag interaction. Panning under high stringency binding conditions can select for improved affinity. Diversity can be introduced randomly across the V gene or can be targeted to specific locations. Because of the size limitations of library construction, it can be difficult to randomize an entire binding site. Thus, a mitigating approach is to focus on CDR-H3 that often forms the majority of Ag contacts or to proceed sequentially through the CDRs, optimizing one at a time and then using the modulating sequences as the basis for subsequent library generation [37].

Analysis of the antigen–antibody complex structures deposited in the PDB database had shown that the shape of the antigen binding site differs for protein-, hapten-, and peptide-binding antibodies and that the number and the location of the antibody-antigen contact sites differ for the various types of antigen [38]. Structure based methods rely on precise structural information of the Ab–Ag interactions from structures or homology models from which a small number of highly specific mutations in the Ab binding site can be constructed [39]. Thus structural modeling can focus a more directed effort to optimize antibody V-regions to deliver the desired potency and selectivity.

## Bispecific antibodies

Since most diseases involve several parallel signaling pathways, multiple inhibitions of receptors and ligands can cause better interferences of various ligand–receptor signaling pathways; simultaneous targeting can limit the development of disease cell resistance from single or combination therapy. Polypharmacology to engage multiple targets simultaneously can improve the therapeutic index of drug molecules so as to better treat a disease. Although such effort using combination therapy has been used in small-molecule drug discovery, this is being expanded for therapeutic Abs [40]. There are advantageous mechanisms of action that can be developed using Abs that possess two binding specificities, bispecific antibodies (BsAbs) [41]. An extensive effort to build platforms for the redirection of immune cells to deliver cytotoxicity of target cells can only be achieved by using bispecific agents. The challenge is to identify a specific set of Abs and determine the effective dose window to generate the desired effect when targeting different specificities. There are excellent reviews of the wide range of protein engineering of bispecific Abs: Antibody fragments without Fc domains; Antibody fragments with Fc domains; Fc domains that can also be binders, and full

IgG BsAbs [42••]. Alternatively, the BsAb platforms can generate monovalent monospecific Abs to obtain more specific biology [43,44].

One of the first applications of bispecific agents were T cell redirection in which one Fv arm targeted CD3 domain in the T cell receptor complex and the other Fv arm bound to an epitope on a target cell thereby bringing cytotoxic T cells into proximity with a target cell to promote lysis and elimination of the target cell. The first sets of bispecific antibodies had several drawbacks: they were difficult to generate large scale batches of homogenous protein; their design were chimeric Abs that resulted in having an undesirable immune response (human anti-drug Abs) decreased drug efficacy, safety, and possibility of multiple administrations (Removab). The second generation bispecific agents utilized antibody fragments, such as a scFv, that were linked with peptide linkers to create bispecific antibody-based molecules. Introduction of a stability-engineered scFv as part of an IgG-like BsAb enabled scalable production and purification of BsAbs, but such constructs required optimization to produce the favorable biophysical properties required for a successful therapeutic [45]. Thus, there was a need to generate BsAbs without the need for custom optimization of each fragment based design. Recombinant bispecific antibody fragments without the Fc domains have rapid *in vivo* clearance and require optimization of protein engineering to generate large scale amounts for manufacturability [46]. Although methods to increase half-lives such as PEGylation, N-glycosylation, HSA or other carrier protein fusions are possible, such fusions can introduce immunogenicity. Bispecific agents can also be generated using scaffolds which may not be based on Abs [6••]. However the use of bispecific agents using alternative scaffolds and Ab fusions has been hindered in some cases by difficulties in the level of purity coming from large-scale production of the constructs. These formats in some cases can have low solubility, contain non-native linkers, lack stability, and have rapid clearance *in vivo*.

Here, we focus on human IgG like bispecific Abs that have been put together using several innovative technologies including knob in hole, strand exchange engineered domain, chemical linked BsAbs, immunoglobulin domain crossover, controlled Fab arm exchange, dual variable domain. Having the BsAb in a format that is more like a human Ab decreases the risk of generating a molecule for a successful clinical profile [47].

MAbs can be selected and occasionally engineered to have Fab arms with multiple specificities [48,49]. However, generating a Fab arm with this activity is still not yet routine. Instead, it is preferable to have a platform that can take a wide array of mAbs that could be processed to generate a matrix of BsAbs. The variable regions of different Abs can be concatenated to generate a dual variable domain Ab [50]. However optimization of the sequential order and the choice of linkers are required to find the ideal candidate molecules. Alternatively, a native BsAb can be obtained by conducting Ab selections using either common light or heavy chain strategies [51]. Such constructs can require a concerted selection scheme using either display or transgenic animals to obtain human Abs with such properties [5•]. These forms of BsAbs can be prepared by co-transfection of the genes of two parental Abs that have Fc mutations that stabilize the heterodimer: knobs and holes [52]; electrostatic steering [53]; immunoglobulin cross-over [54]; and strand exchange engineered domain [55]. Because such constructs require three or four cistron gene transfections, there can be permutations of possible domain interactions. Thus, there can be challenges to maximize the BsAb yields during cell growth and from downstream purification. To improve the separation of the desired BsAb from other potential combinations of LC/HC pairings, different BsAb engineering has been employed such as using differential protein A binding [56], lambda/kappa light chain [57] based affinity chromatography. An extensive maturation process may have to be established for the optimization of BsAb yield and stability [58].

To broaden the screening process of a wide array of mAbs in a BsAb format, there are methods to generate BsAb using purified parental mAbs with the respective CH3 domain point mutations [59•,60•]. This controlled Fab arm exchange process is promising because of biophysical, activity, and pharmacokinetic similarity as well as compatibility of manufacturability to human Ab [61]. In addition, these BsAb technologies allow for incorporation of Fc engineering to enhance the potential of therapeutic efficacy.

## Lead optimization

After identification of therapeutic Abs for different mechanisms of action such as neutralizing soluble mediators, binding and killing of cells, and regulating cell function, the Abs will require optimization of the affinity, selectivity, epitope engagement, Fc functionality for the target biology efficacy. The Fab arms can be selected to modulate antigen specificity and binding affinity using affinity maturation of the variable domain; humanization to decrease immunogenicity; engineering of the variable domain to lower the isoelectric point to lower elimination of IgG [62,63].

In addition to the Fab arm activity, the Fc domain can be modified to increase the therapeutic potency of the Abs. The choice of the constant (Fc) region centers on whether specific effector functions (stimulatory functions, tumor cytotoxicity, pathogen phagocytosis, immune cell pathogenicity, hypersensitivity, toxin/virus neutralization, B cell activation) are required and the need for a suitable

*in vivo* half-life [64]. The choice of IgG1, IgG2 and IgG4 isotypes can affect engagement with Fc receptors that act as activators, inhibitors and regulators of immunity [8••,65••]. Each isotype has a different hinge and CH1 structure which can affect overall Ab activity. Likewise, there are point mutations to the Fc domains that can enhance or abrogate Fc receptor engagement [66]. Cell depleting activity can also be mediated by complement activation and ADCC through Fc engineering of IgG hexamers assembled at the cell surface [67]. It is critical to tune selection of the Fab and Fc domain activity since there can be interactions between the Fab and Fc domains with targets with different epitopic density [68••].

## Pharmacokinetic modulation

IgG antibodies have a half-life that binds to the neonatal receptor, FcRn, in a pH dependent manner which contributes to their long half-life [69]. Fc engineering can increase Ab half-life though stronger binding to FcRn which could lead to less frequent dosing [70,71]. Likewise there are mutations which can shorten the half-life [72]. Notwithstanding, there are other factors besides FcRn binding such as overall charge of the variable region which can affect pharmacokinetics [73].

Human heavy chain genes also exhibit extensive structural polymorphism(s) and, being closely linked, are inherited as a haplotype. Polymorphisms (allotypes) within the IgG isotype were originally discovered and described using serological reagents derived from humans; demonstrating that allotypic variants can be immunogenic and provoke antibody responses as a result of alloimmunization [74]. Different IgG1 allotypes have different influence with FcRn binding which affect pharmacokinetics [75]. Thus, if anti-therapeutic antibody responses are encountered in chronic diseases when patients are dosed on a continuing basis, the mechanism of action should be determined if the cause is due to variable region or allotype recognition.

## Engineering for manufacturability

Although Abs from phage, hybridomas, or display technologies can be selected for affinity, potency, Fc functionality, and biological activities, such lead molecules can have poor biophysical properties that can result in problematic developability issues such as poor expression, solubility, promiscuous cross-reactivity, and poor pharmacokinetic profiles. Establishing early stage developability screening assays capable of predicting late stage behavior is therefore of high value to minimize development risks.

The complexity of an antibody mAb can be subject to multiple mechanisms of degradation [76]. Therapeutic antibodies need to possess the chemical and physical stability to withstand degradation that can occur during the manufacturing process, packaging, storage, and delivery [77•]. Poor stability can result from amino acid modifications such as deamidation, oxidation, isomerization, cyclization, *etc.* [78], glycation [79,80], and Ab disulfide shuffling [81]. The process to generate recombinant Abs can result in aggregation, lack of solubility at concentrations required for the therapeutic product profile, loss of structural integrity due to fragmentation or clipping [82,83] and chemical instability of the amino acid residues [84]. Partial unfolding of the tertiary structure of Abs can result in exposure of normally buried hydrophobic faces that they can interact to result in aggregation [85]. While Abs possess glycans attached to N297 of the CH2 domain of the Fc, glycosylation of the variable regions is not preferred because of variability of the therapeutic product was well as potential interference on antigen binding [86]. Better understanding of the aforementioned heterogeneity of therapeutic molecules can permit development of strategies to better identify the potential modifications to mitigate the risks [87•].

Different methods that include oxidation, glycation, high and low pH holds have been introduced to characterize the relationship between protein stability and antibody structure [88••]. Relative stability of therapeutic antibody candidates can be evaluated primarily through mAb response to thermal degradation, yet this technique is not always predictive of stability in manufacture, shipping, and storage. Stress studies can be conducted to select leads as well as guide sequence optimization [77•,88••].

Computational tools can predict potential manufacturing risks that can occur within the CDR regions through analysis of the CDR interactions with framework residues to permit more rational approaches for stability improvement and for the elimination of hydrophobic patches that can affect solubility [89•,90,91]. If such mutagenesis significantly affects activity, then protein formulation will be required to maintain the Ab integrity [92•,93•]. Ideally, *in silico* assessment of manufacturing risks of hits coming from immunization, microbial display, eukaryotic ribosome display, and single B cell can help expedite the therapeutic Ab discovery efforts by focusing efforts on more viable leads.

For antibody therapeutics that rely on Fc-mediated effector function for their clinical activity, the terminal sugars of Fc glycans have been shown to be critical for safety or efficacy [94,95]. The production of Abs in different hosts can alter the IgG1 Fc glycoforms and also affect the chemical degradation profiles [96,97]. However, differences in glycosylation coming from different cell types is of less importance compared with process related parameters such as cell growth conditions [98]. Different glycosylation variants have also been shown to influence the pharmacodynamic and pharmacokinetic behavior

while other Fc glycan structural elements may be involved in adverse immune reactions [99].

## Conclusions and future directions

Since the arrival of the first therapeutic mAb in 1986, there are more than 294 mAbs which are approved or investigational drugs in the clinic (36 chimeric mAbs, 258 humanized mAbs). The large surface area of the CDRs optimize the potential of obtaining the potency and specificity to achieve clinical proof of concept target activation, inhibition, of blocking of ligand interactions. Most mAbs have targeted soluble and membrane proteins, glycoproteins, glycolipids, and carbohydrates. Since these targets may have dynamic structures, the selection of the relevant mAb will require screening a wide array of epitopes. To obtain mAb diversity, multiple approaches to generate V regions may be required. Each screening method has complementary advantages and disadvantages to obtain diverse molecules. Upon discovery of mAbs that target different epitopes, the next effort would be to trigger different mechanisms of action using Fc engineering. The properties of eliciting Fc effector function, control of pharmacokinetics and tissue specificity can tune the mAb to different pathogenic disorders. To increase the potential of differentiation with other mAbs, increase of functionality and differentiation can be obtained with combining different mAbs with distinct properties into a bispecific format. In addition, there is already great effort to link multispecificity using coupling with small molecule drugs as well as other protein domains to elicit new biological activity. Likewise the coupling of Fab and Fc activity provides greater potential for innovation. Concomitant to the process of mAb functional activity optimization, it is critical to couple evaluation of manufacturability to the discovery of the lead molecule. Engineering stability, homogeneity by removal of aggregation hotspots, and developability can minimize chemistry, manufacturability, deviceability, and control liabilities. The approaches that have been described will continue to evolve leading to faster development of therapeutics with better biological and biophysical properties that will address unmet patient needs.

## References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest


1. Reichert JM: **Antibodies to watch in 2016**. *MAbs* 2016, **8**:197-204.
2. Kohler G, Milstein C: **Continuous cultures of fused cells secreting antibody of predefined specificity**. *Nature* 1975, **256**:495-497.
3. Omidfar K, Daneshpour M: **Advances in phage display technology for drug discovery**. *Expert Opin Drug Discov* 2015, **10**:651-669.
4. McCafferty J, Schofield D: **Identification of optimal protein binders through the use of large genetically encoded display libraries**. *Curr Opin Chem Biol* 2015, **26**:16-24.
5. • Bruggemann M, Osborn MJ, Ma B, Hayre J, Avis S, Lundstrom B, Buelow R: **Human antibody production in transgenic animals**. *Arch Immunol Ther Exp (Warsz)* 2015, **63**:101-108.
Progress on generating human antibodies from transgenic mammals.
6. •• Spiess C, Zhai Q, Carter PJ: **Alternative molecular formats and therapeutic applications for bispecific antibodies**. *Mol Immunol* 2015, **67**:95-106.
Review of bispecific and multispecific molecules using antibody fusions and alternative scaffolds.
7. • Bournazos S, Ravetch JV: **Fcgamma receptor pathways during active and passive immunization**. *Immunol Rev* 2015, **268**:88-103.
Excellent review of the different biological activities arising from Fc receptor engagement.
8. •• Beers SA, Glennie MJ, White AL: **Influence of immunoglobulin isotype on therapeutic antibody function**. *Blood* 2016, **127**:1097-1101.
Critical review of isotype selection for therapeutic antibodies targeting different mechanisms of action.
9. Stapleton NM, Einarsdottir HK, Stemerding AM, Vidarsson G: **The multiple facets of FcRn in immunity**. *Immunol Rev* 2015, **268**:253-268.
10. • Gilliland GL, Luo J, Vafa O, Almagro JC: **Leveraging SBDD in protein therapeutic development: antibody engineering**. *Methods Mol Biol* 2012, **841**:321-349.
Review of structure based therapeutic antibody engineering to guide affinity, Fc effector function, and biophysical properties.
11. Chothia C, Lesk AM: **Canonical structures for the hypervariable regions of immunoglobulins**. *J Mol Biol* 1987, **196**:901-917.
12. Adolf-Bryfogle J, Xu Q, North B, Lehmann A, Dunbrack RL Jr: **PyIgClassify: a database of antibody CDR structural classifications**. *Nucleic Acids Res* 2015, **43**:D432-D438.
13. North B, Lehmann A, Dunbrack RL Jr: **A new clustering of antibody CDR loop conformations**. *J Mol Biol* 2011, **406**:228-256.
14. Morrison SL, Johnson MJ, Herzenberg LA, Oi VT: **Chimeric human antibody molecules: mouse antigen-binding domains with human constant region domains**. *Proc Natl Acad Sci U S A* 1984, **81**:6851-6855.
15. Jones PT, Dear PH, Foote J, Neuberger MS, Winter G: **Replacing the complementarity-determining regions in a human antibody with those from a mouse**. *Nature* 1986, **321**:522-525.
16. Laffleur B, Pascal V, Sirac C, Cogne M: **Production of human or humanized antibodies in mice**. *Methods Mol Biol* 2012, **901**:149-159.
17. Smith GP: **Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface**. *Science* 1985, **228**:1315-1317.
18. Hoogenboom HR: **Selecting and screening recombinant antibody libraries**. *Nat Biotechnol* 2005, **23**:1105-1116.
19. Osbourn J, Groves M, Vaughan T: **From rodent reagents to human therapeutics using antibody guided selection**. *Methods* 2005, **36**:61-68.
20. Marks JD, Hoogenboom HR, Bonnert TP, McCafferty J, Griffiths AD, Winter G: **By-passing immunization. Human antibodies from V-gene libraries displayed on phage**. *J Mol Biol* 1991, **222**:581-597.
21. Rothe C, Urlinger S, Lohning C, Prassler J, Stark Y, Jager U, Hubner B, Bardroff M, Pradel I, Boss M *et al.*: **The human combinatorial antibody library HuCAL GOLD combines diversification of all six CDRs according to the natural immune system with a novel display method for efficient selection of high-affinity antibodies**. *J Mol Biol* 2008, **376**:1182-1200.
22. • Adams JJ, Sidhu SS: **Synthetic antibody technologies**. *Curr Opin Struct Biol* 2014, **24**:1-9.
Review of design strategies to obtain diverse high affinity antibodies.

23. Gai SA, Wittrup KD: **Yeast surface display for protein engineering and characterization**. *Curr Opin Struct Biol* 2007, **17**:467-473.

24. Harvey BR, Georgiou G, Hayhurst A, Jeong KJ, Iverson BL, Rogers GK: **Anchored periplasmic expression, a versatile technology for the isolation of high-affinity antibodies from *Escherichia coli*-expressed libraries**. *Proc Natl Acad Sci U S A* 2004, **101**:9193-9198.

25. Smith ES, Zauderer M: **Antibody library display on a mammalian virus vector: combining the advantages of both phage and yeast display into one technology**. *Curr Drug Discov Technol* 2014, **11**:48-55.

26. Smith K, Garman L, Wrammert J, Zheng NY, Capra JD, Ahmed R, Wilson PC: **Rapid generation of fully human monoclonal antibodies specific to a vaccinating antigen**. *Nat Protoc* 2009, **4**:372-384.

27. Kwakkenbos MJ, Bakker AQ, van Helden PM, Wagner K, Yasuda E, Spits H, Beaumont T: **Genetic manipulation of B cells for the isolation of rare therapeutic antibodies from the human repertoire**. *Methods* 2014, **65**:38-43.

28. Jin A, Ozawa T, Tajiri K, Obata T, Kondo S, Kinoshita K, Kadowaki S, Takahashi K, Sugiyama T, Kishi H *et al.*: **A rapid and efficient single-cell manipulation method for screening antigen-specific antibody-secreting cells from human peripheral blood**. *Nat Med* 2009, **15**:1088-1092.

29. Ogunniyi AO, Story CM, Papa E, Guillen E, Love JC: **Screening individual hybridomas by microengraving to discover monoclonal antibodies**. *Nat Protoc* 2009, **4**:767-782.

30. Tiller T: **Single B cell antibody technologies**. *N Biotechnol* 2011, **28**:453-457.

31. • Duvall MR, Fiorini RN: **Different approaches for obtaining antibodies from human B cells**. *Curr Drug Discov Technol* 2014, **11**:41-47.
Review on methods to obtain antibodies directly from human B cells.

32. Wrammert J, Smith K, Miller J, Langley WA, Kokko K, Larsen C, Zheng NY, Mays I, Garman L, Helms C *et al.*: **Rapid cloning of high-affinity human monoclonal antibodies against influenza virus**. *Nature* 2008, **453**:667-671.

33. Scheid JF, Mouquet H, Feldhahn N, Seaman MS, Velinzon K, Pietzsch J, Ott RG, Anthony RM, Zebroski H, Hurley A *et al.*: **Broad diversity of neutralizing antibodies isolated from memory B cells in HIV-infected individuals**. *Nature* 2009, **458**:636-640.

34. Mietzner B, Tsuiji M, Scheid J, Velinzon K, Tiller T, Abraham K, Gonzalez JB, Pascual V, Stichweh D, Wardemann H *et al.*: **Autoreactive IgG memory antibodies in patients with systemic lupus erythematosus arise from nonreactive and polyreactive precursors**. *Proc Natl Acad Sci U S A* 2008, **105**:9727-9732.

35. Buttel IC, Chamberlain P, Chowers Y, Ehmann F, Greinacher A, Jefferis R, Kramer D, Kropshofer H, Lloyd P, Lubiniecki A *et al.*: **Taking immunogenicity assessment of therapeutic proteins to the next level**. *Biologicals* 2011, **39**:100-109.

36. Wu S-J, Gilliland GL, Feng F: **Solubility and early assessment of stability**. In *Biophysical Methods for Biotherapeutics: Discovery and Development Applications.* Edited by Das TK. John Wiley & Sons; 2014:65-91.

37. Yang WP, Green K, Pinz-Sweeney S, Briones AT, Burton DR, Barbas CF 3rd: **CDR walking mutagenesis for the affinity maturation of a potent human anti-HIV-1 antibody into the picomolar range**. *J Mol Biol* 1995, **254**:392-403.

38. Raghunathan G, Smart J, Williams J, Almagro JC: **Antigen-binding site anatomy and somatic mutations in antibodies that recognize different types of antigens**. *J Mol Recognit* 2012, **25**:103-113.

39. Kiyoshi M, Caaveiro JM, Miura E, Nagatoishi S, Nakakido M, Soga S, Shirai H, Kawabata S, Tsumoto K: **Affinity improvement of a therapeutic antibody by structure-based computational design: generation of electrostatic interactions in the transition state stabilizes the antibody–antigen complex**. *PLOS ONE* 2014, **9**:e87099.

40. Takeda K, Kojima Y, Uno T, Hayakawa Y, Teng MW, Yoshizawa H, Yagita H, Gejyo F, Okumura K, Smyth MJ: **Combination therapy of established tumors by antibodies targeting immune activating and suppressing molecules**. *J Immunol* 2010, **184**:5493-5501.

41. Gu J, Ghayur T: **Rationale and development of multispecific antibody drugs**. *Expert Rev Clin Pharmacol* 2010, **3**:491-508.

42. •• Kontermann RE, Brinkmann U: **Bispecific antibodies**. *Drug Discov Today* 2015, **20**:838-847.
Description of novel therapeutic mechanism of actions for bispecific antibodies.

43. Mazor Y, Oganesyan V, Yang C, Hansen A, Wang J, Liu H, Sachsenmeier K, Carlson M, Gadre DV, Borrok MJ *et al.*: **Improving target cell specificity using a novel monovalent bispecific IgG design**. *MAbs* 2015, **7**:377-389.

44. Liu Z, Leng EC, Gunasekaran K, Pentony M, Shen M, Howard M, Stoops J, Manchulenko K, Razinkov V, Liu H *et al.*: **A novel antibody engineering strategy for making monovalent bispecific heterodimeric IgG antibodies by electrostatic steering mechanism**. *J Biol Chem* 2015, **290**:7535-7562.

45. Miller BR, Demarest SJ, Lugovskoy A, Huang F, Wu X, Snyder WB, Croner LJ, Wang N, Amatucci A, Michaelson JS *et al.*: **Stability engineering of scFvs for the development of bispecific and multivalent antibodies**. *Protein Eng Des Sel* 2010, **23**:549-557.

46. Wu X, Sereno AJ, Huang F, Lewis SM, Lieu RL, Weldon C, Torres C, Fine C, Batt MA, Fitchett JR *et al.*: **Fab-based bispecific antibody formats with robust biophysical properties and biological activity**. *MAbs* 2015, **7**:470-482.

47. Grote M, Haas AK, Klein C, Schaefer W, Brinkmann U: **Bispecific antibody derivatives based on full-length IgG formats**. *Methods Mol Biol* 2012, **901**:247-263.

48. Benson JM, Sachs CW, Treacy G, Zhou H, Pendley CE, Brodmerkel CM, Shankar G, Mascelli MA: **Therapeutic targeting of the IL-12/23 pathways: generation and characterization of ustekinumab**. *Nat Biotechnol* 2011, **29**:615-624.

49. Bostrom J, Yu SF, Kan D, Appleton BA, Lee CV, Billeci K, Man W, Peale F, Ross S, Wiesmann C *et al.*: **Variants of the antibody herceptin that interact with HER2 and VEGF at the antigen binding site**. *Science* 2009, **323**:1610-1614.

50. Jakob CG, Edalji R, Judge RA, DiGiammarino E, Li Y, Gu J, Ghayur T: **Structure reveals function of the dual variable domain immunoglobulin (DVD-Ig) molecule**. *MAbs* 2013, **5**:358-363.

51. Fischer N, Elson G, Magistrelli G, Dheilly E, Fouque N, Laurendon A, Gueneau F, Ravn U, Depoisier JF, Moine V *et al.*: **Exploiting light chains for the scalable generation and platform purification of native human bispecific IgG**. *Nat Commun* 2015, **6**:6113.

52. Carter P: **Bispecific human IgG by design**. *J Immunol Methods* 2001, **248**:7-15.

53. Gunasekaran K, Pentony M, Shen M, Garrett L, Forte C, Woodward A, Ng SB, Born T, Retter M, Manchulenko K *et al.*: **Enhancing antibody Fc heterodimer formation through electrostatic steering effects: applications to bispecific molecules and monovalent IgG**. *J Biol Chem* 2010, **285**:19637-19646.

54. Schaefer W, Regula JT, Bahner M, Schanzer J, Croasdale R, Durr H, Gassner C, Georges G, Kettenberger H, Imhof-Jung S *et al.*: **Immunoglobulin domain crossover as a generic approach for the production of bispecific IgG antibodies**. *Proc Natl Acad Sci U S A* 2011, **108**:11187-11192.

55. Muda M, Gross AW, Dawson JP, He C, Kurosawa E, Schweickhardt R, Dugas M, Soloviev M, Bernhardt A, Fischer D *et al.*: **Therapeutic assessment of SEED: a new engineered antibody platform designed to generate mono- and bispecific antibodies**. *Protein Eng Des Sel* 2011, **24**:447-454.

56. Tustian AD, Endicott C, Adams B, Mattila J, Bak H: **Development of purification processes for fully human bispecific antibodies based upon modification of protein A binding avidity**. *MAbs* 2016 http://dx.doi.org/10.1080/19420862.2016.1160192:0.

57. Muller-Spath T, Ulmer N, Aumann L, Strohlein G, Bavand M, Hendriks L, De Kruif J, Throsby M, Bakker A: **Purifying common light-chain bispecific antibodies: a twin-column, countercurrent chromatography platform process**. *Bioprocess Intl.* 2013, **11**:36-45.

58. Lewis SM, Wu X, Pustilnik A, Sereno A, Huang F, Rick HL, Guntas G, Leaver-Fay A, Smith EM, Ho C *et al.*: **Generation of bispecific IgG antibodies by structure-based design of an orthogonal Fab interface**. *Nat Biotechnol* 2014, **32**:191-198.

59. • Labrijn AF, Meesters JI, de Goeij BE, van den Bremer ET, Neijssen J, van Kampen MD, Strumane K, Verploegen S, Kundu A, Gramer MJ *et al.*: **Efficient generation of stable bispecific IgG1 by controlled Fab-arm exchange**. *Proc Natl Acad Sci U S A* 2013, **110**:5145-5150.
Generation of an intact and stable human bispecific antibody with minimal number of mutations.

60. • Strop P, Ho WH, Boustany LM, Abdiche YN, Lindquist KC, Farias SE, Rickert M, Appah CT, Pascua E, Radcliffe T *et al.*: **Generating bispecific human IgG1 and IgG2 antibodies from any antibody pair**. *J Mol Biol* 2012, **420**:204-219.
Generation of human bispecific IgG1 and IgG2 antibodies.

61. Gramer MJ, van den Bremer ET, van Kampen MD, Kundu A, Kopfmann P, Etter E, Stinehelfer D, Long J, Lannom T, Noordergraaf EH *et al.*: **Production of stable bispecific IgG1 by controlled Fab-arm exchange: scalability from bench to large-scale manufacturing by application of standard approaches**. *MAbs* 2013, **5**:962-973.

62. Schoch A, Kettenberger H, Mundigl O, Winter G, Engert J, Heinrich J, Emrich T: **Charge-mediated influence of the antibody variable domain on FcRn-dependent pharmacokinetics**. *Proc Natl Acad Sci U S A* 2015, **112**:5997-6002.

63. Li B, Tesar D, Boswell CA, Cahaya HS, Wong A, Zhang J, Meng YG, Eigenbrot C, Pantua H, Diao J *et al.*: **Framework selection can influence pharmacokinetics of a humanized therapeutic antibody through differences in molecule charge**. *MAbs* 2014, **6**:1255-1264.

64. Jefferis R: **Antibody therapeutics: isotype and glycoform selection**. *Expert Opin Biol Ther* 2007, **7**:1401-1413.

65. •• Jefferis R: **Isotype and glycoform selection for antibody therapeutics**. *Arch Biochem Biophys* 2012, **526**:159-166.
Excellent review of the role of glycosylation on the activity of antibodies that can be put into different isotypes.

66. Monnet C, Jorieux S, Urbain R, Fournier N, Bouayadi K, De Romeuf C, Behrens CK, Fontayne A, Mondon P: **Selection of IgG variants with increased FcRn binding using random and directed mutagenesis: impact on effector functions**. *Front Immunol* 2015, **6**:39.

67. Diebolder CA, Beurskens FJ, de Jong RN, Koning RI, Strumane K, Lindorfer MA, Voorhorst M, Ugurlar D, Rosati S, Heck AJ *et al.*: **Complement is activated by IgG hexamers assembled at the cell surface**. *Science* 2014, **343**:1260-1263.

68. •• Teeling JL, Mackus WJ, Wiegman LJ, van den Brakel JH, Beers SA, French RR, van Meerten T, Ebeling S, Vink T, Slootstra JW *et al.*: **The biological activity of human CD20 monoclonal antibodies is linked to unique epitopes on CD20**. *J Immunol* 2006, **177**:362-371.
Excellent comparison of the role of anti-CD20 antibody epitopes drive the biological activity.

69. Martin WL, West AP Jr, Gan L, Bjorkman PJ: **Crystal structure at 2.8 A of an FcRn/heterodimeric Fc complex: mechanism of pH-dependent binding**. *Mol Cell* 2001, **7**:867-877.

70. Hinton PR, Xiong JM, Johlfs MG, Tang MT, Keller S, Tsurushita N: **An engineered human IgG1 antibody with longer serum half-life**. *J Immunol* 2006, **176**:346-356.

71. Dall'Acqua WF, Kiener PA, Wu H: **Properties of human IgG1s engineered for enhanced binding to the neonatal Fc receptor (FcRn)**. *J Biol Chem* 2006, **281**:23514-23524.

72. Datta-Mannan A, Witcher DR, Tang Y, Watkins J, Wroblewski VJ: **Monoclonal antibody clearance. Impact of modulating the interaction of IgG with the neonatal Fc receptor**. *J Biol Chem* 2007, **282**:1709-1717.

73. Suzuki T, Ishii-Watabe A, Tada M, Kobayashi T, Kanayasu-Toyoda T, Kawanishi T, Yamaguchi T: **Importance of neonatal FcR in regulating the serum half-life of therapeutic proteins containing the Fc domain of human IgG1: a comparative study of the affinity of monoclonal antibodies and Fc-fusion proteins to human neonatal FcR**. *J Immunol* 2010, **184**:1968-1976.

74. Jefferis R, Lefranc MP: **Human immunoglobulin allotypes: possible implications for immunogenicity**. *MAbs* 2009, **1**:332-338.

75. Ternant D, Arnoult C, Pugniere M, Dhommee C, Drocourt D, Perouzel E, Passot C, Baroukh N, Mulleman D, Tiraby G *et al.*: **IgG1 allotypes influence the pharmacokinetics of therapeutic monoclonal antibodies through FcRn binding**. *J Immunol* 2016, **196**:607-613.

76. Wang W, Singh S, Zeng DL, King K, Nema S: **Antibody structure, instability, and formulation**. *J Pharm Sci* 2007, **96**:1-26.

77. • Yang X, Xu W, Dukleska S, Benchaar S, Mengisen S, Antochshuk V, Cheung J, Mann L, Babadjanova Z, Rowand J *et al.*: **Developability studies before initiation of process development: improving manufacturability of monoclonal antibodies**. *MAbs* 2013, **5**:787-794.
Review of quality by design using stress conditions and solubility to screen antibody candidates.

78. Manning MC, Chou DK, Murphy BM, Payne RW, Katayama DS: **Stability of protein pharmaceuticals: an update**. *Pharm Res* 2010, **27**:544-575.

79. Zhang B, Yang Y, Yuk I, Pai R, McKay P, Eigenbrot C, Dennis M, Katta V, Francissen KC: **Unveiling a glycation hot spot in a recombinant humanized monoclonal antibody**. *Anal Chem* 2008, **80**:2379-2390.

80. Miller AK, Hambly DM, Kerwin BA, Treuheit MJ, Gadgil HS: **Characterization of site-specific glycation during process development of a human therapeutic monoclonal antibody**. *J Pharm Sci* 2011, **100**:2543-2550.

81. Dillon TM, Ricci MS, Vezina C, Flynn GC, Liu YD, Rehder DS, Plant M, Henkle B, Li Y, Deechongkit S *et al.*: **Structural and functional characterization of disulfide isoforms of the human IgG2 subclass**. *J Biol Chem* 2008, **283**:16206-16215.

82. Vlasak J, Ionescu R: **Fragmentation of monoclonal antibodies**. *MAbs* 2011, **3**:253-263.

83. Wu H, Kroe-Barrett R, Singh S, Robinson AS, Roberts CJ: **Competing aggregation pathways for monoclonal antibodies**. *FEBS Lett* 2014, **588**:936-941.

84. Liu H, Ponniah G, Zhang HM, Nowak C, Neill A, Gonzalez-Lopez N, Patel R, Cheng G, Kita AZ, Andrien B: **In vitro and in vivo modifications of recombinant and human IgG antibodies**. *MAbs* 2014, **6**:1145-1154.

85. Roberts CJ: **Therapeutic protein aggregation: mechanisms, design, and control**. *Trends Biotechnol* 2014, **32**:372-380.

86. Jefferis R: **Glycosylation of antibody therapeutics: optimisation for purpose**. *Methods Mol Biol* 2009, **483**:223-238.

87. • Alsenaidy MA, Jain NK, Kim JH, Middaugh CR, Volkin DB: **Protein comparability assessments and potential applicability of high throughput biophysical methods and data visualization tools to compare physical stability profiles**. *Front Pharmacol* 2014, **5**:39.
The use of different biophysical and analytical tools to characterize protein physical stability for the selection of candidate biotherapeutic molecules.

88. •• Jarasch A, Koll H, Regula JT, Bader M, Papadimitriou A, Kettenberger H: **Developability assessment during the selection of novel therapeutic antibodies**. *J Pharm Sci* 2015, **104**:1885-1898.
The use of small-scale, rapid methods that can identify candidates with better biochemical and biophysical properties.

89. • Agrawal NJ, Kumar S, Wang X, Helk B, Singh SK, Trout BL: **Aggregation in protein-based biotherapeutics: computational**

**studies and tools to identify aggregation-prone regions**. *J Pharm Sci* 2011, **100**:5081-5095.
Sequence-structure-based approach to identify aggregation prone sequences in antibodies.

90. Rouet R, Lowe D, Christ D: **Stability engineering of the human antibody repertoire**. *FEBS Lett* 2014, **588**:269-277.

91. Swift J, Saing S, Rouet R, Dudgeon K, Schofield P, Sewell W, Christ D: **Identification of aggregation inhibitors of the human antibody light chain repertoire by phage display**. *Protein Eng Des Sel* 2014, **27**:405-409.

92. • Shire SJ: **Formulation and manufacturability of biologics**. *Curr Opin Biotechnol* 2009, **20**:708-714.
Review of changes in the manufacturing process and formulation development to maximize the successful manufacture of therapeutic antibodies.

93. • Lowe D, Dudgeon K, Rouet R, Schofield P, Jermutus L, Christ D: **Aggregation, stability, and formulation of human antibody therapeutics**. *Adv Protein Chem Struct Biol* 2011, **84**:41-61.
Overview of the methods to characterize antibody aggregation and the role of engineering and formulation to mitigate such risks.

94. Higel F, Seidl A, Sorgel F, Friess W: **N-glycosylation heterogeneity and the influence on structure, function and pharmacokinetics of monoclonal antibodies and Fc fusion proteins**. *Eur J Pharm Biopharm* 2016, **100**:94-100.

95. Jefferis R: **Glycosylation as a strategy to improve antibody-based therapeutics**. *Nat Rev Drug Discov* 2009, **8**:226-234.

96. Mozziconacci O, Okbazghi S, More AS, Volkin DB, Tolbert T, Schoneich C: **Comparative evaluation of the chemical stability of 4 well-defined immunoglobulin G1-Fc glycoforms**. *J Pharm Sci* 2016, **105**:575-587.

97. More AS, Toprani VM, Okbazghi SZ, Kim JH, Joshi SB, Middaugh CR, Tolbert TJ, Volkin DB: **Correlating the impact of well-defined oligosaccharide structures on physical stability profiles of IgG1-Fc glycoforms**. *J Pharm Sci* 2016, **105**:588-601.

98. Wacker C, Berger CN, Girard P, Meier R: **Glycosylation profiles of therapeutic antibody pharmaceuticals**. *Eur J Pharm Biopharm* 2011, **79**:503-507.

99. Reusch D, Tejada ML: **Fc glycans of therapeutic antibodies as critical quality attributes**. *Glycobiology* 2015, **25**:1325-1334.