The mosaic puzzle of the therapeutic monoclonal antibodies and antibody fragments - A modular transition from full-length immunoglobulins to antibody mimetics

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A B S T R A C T
The use of monoclonal antibodies represents an important and efficient diagnostic and therapeutic tool in disease management and modern science but remains limited by several factors including the uneven distribution in diseased tissues as well as undesired activation of side immune reactions. Major scientific advancements including Recombinant DNA Technology, Hybridoma Technology, and Polymerase Chain Reaction have considerably impacted the use of monoclonal antibodies providing technical and effective solutions to overcome the shortcomings encountered with conventional antibodies. Initially, the introduction of antibody fragments allowed a more uniform and deeper penetration of the targeted tissue and reduced unwanted activation of Fc-mediated immune reactions. On another level, the immunogenicity of murine-derived antibodies was overcome by humanizing their encoding genes with specific sequences of human origin and transgenic mice able to synthesize fully human antibodies were successfully created. Moreover, the advancement of genetic engineering techniques supported by the modular structure of antibody coding genes paved the way for the development of a new generation of antibody fragments with a wide spectrum of monospecific and bispecific agents. These later could be monovalent, bivalent, or multivalent, and either expressed as a single chain, assembled in multimeric forms or stringed in tandem. This has conferred improved affinity, stability, and solubility to antibody targetting. Lately, a new array of monoclonal antibody fragments was introduced with the engineering of nanobody and antibody mimetics as non-immunoglobulin-derived fragments with promising diagnostic and therapeutic applications. In this review, we decipher the molecular basis of monoclonal antibody engineering with a detailed screening of the antibody derivatives that provides new perspectives to expand the use of monoclonal fragments into previously unexplored fields.

1. Introduction

The very first report denoting the term «antibodies» emanated from Emil von Behring and Shibasabura Kitasato’s work in 1890[1]. In their milestone publication, they established the concept of passive immunization, describing the use of serum drained from diphtheria-inoculated horses that provides a back then novel treatment modality for infected animals. It was an early insight in the potential use of antibody-rich serum as neutralizing agents (antitoxins) in clinical applications. One decade later von Behring was awarded the Nobel Prize in Physiology and Medicine in 1901 for his scientific contributions[1–3]. Despite a long century of focused research, the chemical nature and the mode of action of the so-called «antibodies» (Fig. 1) remained ambiguous for many years to come after that. In 1959, Edelman demonstrated that antibodies are composed of separate chains of proteins joined together by sulfur bonds forming crosslinks. Porter showed that when these chains were treated enzymatically with papain, they could be separated into three fragments. Two of these fragments maintain their capacity to bind the antigens (weighing between 50 and 55 KDa each), while the third (about 80 KDa) lacks this ability[4–6]. In 1963, Porter’s team projected a new feature of the multi-subunit polypeptide forming a Y-shaped complex assembled through five inter-chain disulphide bridges maintaining the tetrameric protein[7]. Another significant milestone in our understanding and use of...
antibodies comes from Dreyer and Bennett. They described the molecular concept of the variable (V) and constant (C) domains production as a result of the genetic expression of two similar but not identical genes assembled together by the previously identified disulphide bonds.[8] Further details were provided by Hood and Talmage who established the famous statement “two genes, one polypeptide chain”, demonstrating that the immunoglobulin Lambda light chain is encrypted by two distinct genes whose expression results in the synthesis of a single polypeptide chain.[9] Based on the comparison of aminoacid termini of about 64 light chains, Hood and Talmage suggested that much of the antibodies’ diversity could be rooted back the germ line variability in addition to the genetic recombination at the level of the gene families.[10] The complete sequence of a gamma-globulin was first reported by Edelman showing the presence of the variable (V) and constant (C) regions in both structures of the Heavy (H) and Light (L) antibody chains.[5] In order to acquire the functional sequence of the immunoglobulin gene, Hozumi and Tonegawa demonstrated that both the variable and constant gene segments, were permissive to undergo a process of somatic hypermutation leading to the rearrangements of the complete functional gene.[11,12].

The last three decades of the twentieth century witnessed major scientific breakthroughs that marked that start of unprecedented advancement of both science and medicine. In 1972, Stanley Cohen and Herbert Boyer were first to apply for a patent on «Recombinant DNA Technology» a discovery that paved the road to the genetic engineering of many therapeutic proteins, including antibodies[13]. Later, in 1984, Jerne, Milstein and Kohler were jointly awarded the Nobel Prize in Physiology and Medicine for their development of the so-called «Hybridoma Technology» which allowed the in vitro mass production of «Monoclonal Antibodies» in cell cultures. The leading purpose of this technology is to maintain a constant supply of pure and highly specific monoclonal antibodies[14,15]. In 1985, Karry Mullis shared the Nobel Prize in Chemistry with Michael Smith for their pioneer invention of Polymerase Chain Reaction- or PCR molecular technique. This allowed the enzymatic synthesis of a large number of DNA sequences starting from a limited number of copies using a thermocycler in the presence of free nucleotides, a pair of forward and reverse primers and a thermostable DNA polymerase[16,17].

With the establishment of the various modern molecular techniques, the need for «Monoclonal Antibodies» as a very efficient tool in the molecular diagnosis and the treatment of a large spectrum of illnesses and diseases has increased[18–21]. The first FDA approved therapeutic monoclonal antibody (mAb) was designed to function as an immuno-suppressive agent used to manage post-transplantation rejections of kidney transplants[22]. Muromonab-CD3, the so-named Orthoclone (OKT3) is an IgG-2a fully murine monoclonal antibody. It is directed against CD3 which isa cluster of differentiation of about 20 KDa, representing a part of a larger macromolecular complex expressed at the surface of mature T cells and the medullary thymic lymphocytes in the proximity of the TCR chains. However, due to its increased immunogenicity, and clinical side effects (Fever, thrombotic disorders, dermatitis, and anaphylactic shock), the commercial marketing and production of murine OKT3 has was discontinued as of July 30th, 2011[23,24].

The modular nature of the antibody gene clusters coding for the constant and variable domains of the heavy and light chains allowed molecular biotechnologists to efficiently manipulate the murine and human gene segments. This has resulted in constructing hybrid entities that maintain the ability to specifically bind antigens with a lower immunogenicity and higher therapeutic efficiency. Novel monoclonal antibody “designers” apportioned the utmost level of consideration to reduce immunogenicity, and promote the therapeutic effect at the best of specificity, safety, and optimal distribution[25]. The modern improvements in genetic engineering initiated the emergence of a spectrum of immunoglobulins conjugates, such as immunotoxins, multivalent fragments and multispecific complexes that conferred an extended efficacy as therapeutic immunoglobulins. Hundreds of newly developed therapeutic antibodies and their derivatives are currently

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Fig. 1. Schematic representation of a full length antibody composed of a pair of two identical heavy chains (H) assembled with two identical light chains (L). Heavy chains are composed of one variable domain (VH) and three constant domains (CH1, CH2, and CH3). The light chains consist of one constant domain (CL) and one variable domain (VL). CH1 and CH2 domains are separated by a flexible hinge region connected by disulphide bridges.
under clinical investigations before being approved by FDA and industrialized for global marketing[26].

The design of therapeutic monoclonal antibodies is optimized to target a large spectrum of oncological illnesses and malignancies. Besides, a number of these antibodies are currently used for the management of hematological disorders, inflammatory diseases, organ transplantation, autoimmune diseases, prevention of migraine and recently for the treatment of COVID-19 patients[27]. Since the discovery and the approval of murine Muromonab (OKT3) in 1986, scientific and industrial efforts were directed towards reducing the side effects of murine antibodies characterized by their high immunogenicity[28]. Consequently, humanized antibodies appeared for the first time in 1997 with an FDA approved anti-IL-2 Receptor antibody (Daclizumab) designed for the prevention of organ transplant rejection[29]. Moreover, fully humanized antibodies generated by transgenic mice marked a revolution in mAbs production when the first anti-Epidermal Growth Factor Receptor (EGFR) was approved by FDA (Panitumumab) to become available in the market as of 2006 and recommended for the treatment of metastatic colorectal cancer[30,31]. A fully humanized anti-TNF-α monoclonal antibody (Adalimumab, Humira) was designed to block the interaction between the pro-inflammatory cytokine and its transmembrane and/or soluble receptor. The subcutaneously administrated mAb (Humira) represents a disease modifier with its ability to block the signaling pathway reducing the TNF-mediated reactions associated with rheumatoid arthritis and other inflammatory conditions such as IBD[32]. Adalimumab was FDA approval in 2002 as an effective drug recommended, not only for RA, but also for the management of a spectrum of chronic inflammatory diseases (i.e. psoriatic arthritis, ankylosing spondylitis, Crohn’s disease, ulcerative colitis, psoriasis, hidradenitis suppurativa, uveitis, and juvenile idiopathic arthritis)[33,34]. Nivolumab and Pembrolizumab (Opdivo and Keytuda respectively) are both directed against the PD-1 receptor intended to block the interaction with its ligand underlying the immune checkpoints involved in the inhibition of tumor progression[35,36]. Monoclonal antibodies which block the Calcitonin Gene-Related Peptide (CGRP) were known to be effective in reducing migraine episodes. For that purpose, three different constructs, namely Erenumab (Aimovig), Galcanezumab (Emgality), and Fremaezumab (Ajovy) were approved to prevent migraine-related pain episodes[37].

2. Adverse effects of full-length monoclonal antibodies

Several factors contribute to immunogenicity of monoclonal antibodies. These include the chemical nature of the relatively conserved and variable amino acids in the primary structure, the three-dimensional conformation, the animal species or the hybridoma host cells used the molecular mass of the protein complex, and the diversity of the heavy and light chains binding sites[38].

IgG antibodies are perceived as large multimeric complexes (dimers of dimers) of about 150 kDa which limits their penetration capacity into solid tissues and tumor masses. The molecular size of these immunoglobulins hinders their ability to cross the blood-brain barrier which restricts their interaction with the cellular targets of the Central Nervous System[39]. Functionally, these antibodies possess two highly specific antigen binding sites (bivalent and monospecific) carried by the uppermost variable domains of the heavy and light chains held together by a highly conserved, biologically active fragment, named Fc region. The presence of this later maintains an extended circulation time with penurny of target tissue concentration which leads to the reduction of its therapeutic effectiveness[19,39,40]. Although monoclonal antibodies are commonly tolerated by the human body, their adverse effects were reported at many instances. This mounts significant side effects after instillation and/or injection of monoclonal antibodies, some of which are summarized in Table 1. For instance, the anti-TNF-α antibodies were shown to induce an autoimmune activity leading to a lupus-like syndrome associated with anti-nuclear antibodies and anti-dsDNA antibodies[41,42]. Yet another monoclonal antibody directed towards the cytotoxic T-Lymphocyte associated antigen 4 (CTLA4) could stand behind the development of autoimmune colitis[43,44]. Cardio-toxicity has been clinically documented in patients treated with ERBB2 monoclonal antibodies[45,46]. The antithrombotic agents directed against the antiplatelet glycoprotein IIb and IIIa caused an immune thrombocytopenia in patients with high risk of developing spontaneous thrombosis[47,48]. Furthermore, an intense cytokine storm has been released following the infusion of an anti-CD28SA antibody subsequently leading to a full revision of the treatment protocols and the adoption of additional measures aiming at improving the safety of the clinically approved monoclonal antibodies[49].

The glycosylation of monoclonal antibodies which involves a multilayered system of poly-enzymatic reactions, has also proved to impact the antigen binding ability, biosafety, and biological activity of monoclonal antibodies[50]. This has shown to affect the immunogenicity of the antibodies, their vascular half-life and subsequently the purposes for which they are produced. In contrast to the hyper-variable domains of the Fab region involved in the recognition and binding of the antigenic determinants, the Fc region has a highly conserved structure with limited diversity[51]. This consensus sequence of the heavy chain encloses two conserved N-glycosylation sites located at the Asn297 on each of the paired chains. Site directed modifications of these N-glycans have been shown to hinder the conformation leading to the hampering of the IgG with molecules of the immune system and may also hamper the activation of the complement system[52]. Glycation, deamination, acetylation, and/or oxidation are among the more common post-translational biochemical mechanisms that alter the immunogenicity of monoclonal antibodies[53,54].

3. Transition from murine to humanized monoclonal antibodies

The continuous expansion of molecular manipulation, DNA sequencing and recombinant DNA technologies allowed the design of a novel system, including a combination of rodent and human antibody coding sequences and resulting in the synthesis of the so-called Chimeric Monoclonal Antibodies (Fig. 2B)[55]. Chimerization, first proposed by Morrison in 1980’s, aimed at decreasing the mAbs immunogenicity by grafting murine variable sequences of the heavy and light chains into human antibody coding sequences generating novel proteins with about 70% of human content[56]. Before being amplified by PCR, the hybridoma extracted genes, encoding for the rodent variable regions are separated. The amplification is further used to yield multiple copies of the human fraction of the heavy and light chains constant regions. Resulting PCR products, including murine and human sequences are inserted into a shuttle plasmid used to transfect a prokaryotic host strain. The chimeric proteins are collected in the form of cytoplasmic inclusions that are purified and tested in vivo following cell lysis. Limiting factors include aggregation of cytoplasmic recombiant proteins in the form of inclusion bodies as well as the limited growth rate of transformed bacteria[57]. Anti-GPIIb-IIIa antibody (abciximab), directed against integrin receptors of the platelets surface was approved by FDA as an antithrombotic agent that inhibits platelet aggregation in patients with a high risk of cardiovascular illnesses[58,59]. In oncology, Rituximab, a chimeric recombinant IgG1 antibody, targeting the CD20 at the surface of cancer T cells was FDA approved and indicated for the management of non-Hodgkin’s Lymphoma by the end of 1997[60,61]. Although chimerization is a major advancement in the optimization of monoclonal antibodies, they still hold a considerable fraction of the rodent immunoglobulins which clinically imposes further focus on reducing their immunogenicity.

The molecular illustration of the variable region holding within the antigen binding site demonstrated the presence of two anti-parallel β-sheets assembled together by a disulphide bridge. The comparison of the heavy and light chains sequences revealed the presence of three distinct hypervariable regions termed Complementary Determining
Table 1
Clinical side effects associated with the more commonly used monoclonal antibodies.

| Monoclonal Antibody | Trade Name | Product’s Type | Hypersensitivity | Immunogenicity | Immunosuppression | Infusion Reactions | Infections | Anemia | Cytopenia | Autoimmunity | Coagulation Disorders | Heart Failure | Malignancy | Others |
|---------------------|------------|----------------|------------------|---------------|------------------|-------------------|------------|--------|----------|--------------|-----------------------|-------------|------------|--------|
| Infliximab          | Remicade   | Chimeric       | +                | +             | +                | +                 | +          | +      | +        | +            | +                     | +           | +          | Increased Liver Enzymes |
| Basiliximab         | Simulect   | Chimeric       | +++              | +++           | +                | +                 | +          | +      | +        | +            | +                     | +           | +          | Skin Rash |
| Rituximab           | Rituxan    | Chimeric       | +                | +             | +                | +                 | +          | +      | +        | +            | +                     | +           | +          | Hypotension & Serum Sickness |
| Cetuximab           | Erbitux    | Chimeric       | +                | +             | +                | +                 | +          | +      | +        | +            | +                     | +           | +          | Urticaria & Bronchospasm |
| Abciximab           | ReoPro     | Chimeric       | +                | +             | +                | +                 | +          | +      | +        | +            | +                     | +           | +          | Bleeding |
| Adalimumab          | Humira     | Fully Human    | +                | +             | +                | +                 | +          | +      | +        | +            | +                     | +           | +          | Increased Liver Enzymes |
| Panitumumab         | Vectibix   | Fully human    | +                | +             | +                | +                 | +          | +      | +        | +            | +                     | +           | +          | Skin Rash & Diarrheae |
| Alemtuzumab         | Campath    | Humanized      | +                | +             | +                | +                 | +          | +      | +        | +            | +                     | +           | +          | Thyroid Disorders |
| Daclizumab          | Zenapax    | Humanized      | +++              | +++           | +                | +                 | +          | +      | +        | +            | +                     | +           | +          | Cytokine Release Syndrome |
| Bevacizumab         | Avastin    | Humanized      | +                | +             | +                | +                 | +          | +      | +        | +            | +                     | +           | +          | Slow Wound Healing |
| Eculizumab          | Soliris    | Humanized      | +                | +             | +                | +                 | +          | +      | +        | +            | +                     | +           | +          | Meningitis |
| Efalizumab          | Raptiva    | Humanized      | +                | +             | +                | +                 | +          | +      | +        | +            | +                     | +           | +          | Encephalitis |
| Natalizumab         | Tysabri    | Humanized      | +                | +             | +                | +                 | +          | +      | +        | +            | +                     | +           | +          | Hepatotoxicity |
| Omalizumab          | Xolair     | Humanized      | +                | +             | +                | +                 | +          | +      | +        | +            | +                     | +           | +          | Injection Site Reactions |
| Palivizumab         | Synagis    | Humanized      | +                | +             | +                | +                 | +          | +      | +        | +            | +                     | +           | +          | Apnea & Fever |
| Trastuzumab         | Herceptin  | Humanized      | +                | +             | +                | +                 | +          | +      | +        | +            | +                     | +           | +          | Skin Reactions & Cardiotoxicity |
| Tocilizumab         | Actemra    | Humanized      | +                | +             | +                | +                 | +          | +      | +        | +            | +                     | +           | +          | Anaphylaxis & Headache |
| Ranibizumab         | Lucentis   | Humanized      | +                | +             | +                | +                 | +          | +      | +        | +            | +                     | +           | +          | Ocular Inflammation |
| Muromonab           | CD3        | Mouse          | +                | +             | +                | +                 | +          | +      | +        | +            | +                     | +           | +          | Hepatitis |
| Certolizumab        | Cimzia     | Pegylated      | +                | +             | +                | +                 | +          | +      | +        | +            | +                     | +           | +          | Increased Liver Enzymes |
concluding that the CDR substitution offered an efficient means to construct a “Humanized Antibody” (Fig. 2C)[65].

4. Advantages of antibody fragments and their derivatives

A substantial advancement in the arena of immunotherapy and immunoassays designed for diagnostic purposes has been evoked by the development of smaller, but more efficient, antibody-derived fragments that maintained the antigen binding capacity of conventional antibodies. Facilitated by the modular structure of immunoglobulin coding genes, a large spectrum of antibody fragments emerged in the last decades including the Fragment for Antigen Binding (Fab), the Single Chain Fragment Variable (scFv), the Crystallizable Fragment (Fc), and the Single-Domain Immunoglobulins[66]. Anticipated with great hope, the cloning techniques in conjunction with genetic engineering of therapeutic proteins allowed biotechnologists to puzzle the gene segments resulting in a wide range of agents with dual or multiple specificities (bispecific and multi-specific), composed of paired or multimeric domains and providing a diverse spectrum of biological activities[67].

Antibody fragments, unlike conventional full length monoclonal antibodies, are not glycosylated which facilitated their synthesis in bacteria[68]. The ease of their production in prokaryotic host cells used as microbial expression machineries, added to the fast growing rate of the microbes in inexpensive growth media results in a larger yield at a less expensive cost and makes them more advantageous than approved conventional antibodies[23]. Clinically, the use of small antibody fragments resulted in a relatively higher tissue penetration but then a faster renal clearance which increases the requirement for higher effective doses and boosting the frequency of their administration unless alleviated by pegylation, polysialylation, or by fusion and conjugation with serum albumin[66,69]. The small size of such recombinant therapeutics has been shown to be very advantageous. Some of them acquired a more efficient binding capacity to the receptors of their targets especially those that are inaccessible to the full length antibodies encumbered by their large size and three dimensional conformations. Some derivatives are designed to have multitude of identical binding sites while some others are potentially able to bind two or even more antigenic determinants[20,70].

5. Molecular mechanisms of antibodies and antibody fragments production

Conventionally, antibody fragments are produced using recombinant DNA technologies to transform special bacterial strains with shuttling plasmid(s) bearing the coding genes. The antibody fragments encoding genes are cloned on autonomous, self-replicating plasmids inserted at a relatively high copy number. The genes of interest are traditionally placed under the control of strong, regulated promoter in order to control the timing of their induction and the level of their expression. Lac promoter, Trp-promoter, the hybrid Tac promoter (Lac & Trp) and bacteriophage T7 promoter are commonly used as they are highly manageable and fully controllable[68]. Much work was directed during the last years towards optimizing the protocols of protein production at the industrial level. Optimal strategies were not limited to upgrading of the transcription process but encompassed also the tuning of the translation and the post-translational modifications required for a protein to acquire its biological activity. A number of modifications were proposed to promote the stability of nascent proteins either in the cytoplasm or the periplasm. A number of strains were genetically modified in order to secrete the native form of the antibody fragments as membrane-bound or extracellular which facilitated their separation and then their purification[71–73]. For that purpose, a number of “optimal host cell” models were developed using prokaryotic cells (especially Escherichia coli), yeast cell strains (Saccharomyces cerevisiae, Pichia pastoris), or mammalian cell lines (Chinese Hamster Ovary cells (CHO), Human Embryonic Kidney cells (HEK), and Murine Myeloma Cells)[68,74,75]. For these later, many molecular aspects were subject to continuous improvement in the aim of increasing the success rate of their use at the industrial level. Among each other, one can include the position of the ribosomal binding site and its affinity to the translation factors, the number of antibody fragments cloned genes, the localization of the nascent peptide fragment either in the cytoplasm, or in the periplasm, at the surface of the cell membrane or even secreted in the medium which affects their intrinsic stability and purity. Some host cells showed an overwhelmed metabolism altering the efficiency of the antibody production. Metabolic burden has been related to a multitude of factors including the high plasmid copy number, the large-sized vectors, the overproduction of the proteins, the synthesis of degrading enzymes and

**Fig. 2.** (A) Murine Antibody compared to genetically engineered antibodies shown in B and C. Chimeric Antibody (B): The coding genes of the murine heavy and light chains variable regions were replaced by the human sequences yielding a chimeric protein with rodent binding sites. Humanized Antibody (C): The murine derived Complementary Determining Regions (CDR1, CDR2, and CDR3) are used to replace the human CDRs generating a protein with human properties and murine antigen binding site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
the depletion of oxygen[76,77]. Technical and molecular adjustments are continuously being adopted to increase translation efficiency, facilitate the folding of the different subunits, enhance the antibody fragment stability, decrease the degradation of cytoplasmic aggregates, reduce the formation of inclusion bodies, encourage protein secretion and facilitate purification[71,77,78]. Recentl, insight gained from cell lysates (E. coli, CHO cells, insect cells) was used to develop the well-established Cell-Free systems. These later were shown to be more flexible and reliable providing fast and scalable machinery for antibody fragments production. The total absence of the phospholipid bilayer of the plasma membrane allowed the direct addition of molecular resources facilitating the synthesis of antibody peptides such as ATP molecules, free amino acids, the bacterial disulfide bridge isomerases C (dsBC) and the chaperone proteins. Although still not fully functional, these cell free systems are expected to be, once fully optimized, preferably adopted for antibody fragments production[79,80]. An alternative technology to generate antibody fragments, known as «Phage Display» of combinatorial antibody fragments genes has been explored in the early 1990s. For that purpose, a wide range of variable cDNA coding sequences collected from antibody producing cells or engineered scFvs combinatorial sequences of VH and VL are expressed at the surface of M13 filamentous bacteriophage. The expressed scFvs are panned after reacting with antigen-coated plates to select the chains of interest that of M13 filamentous bacteriophage. The expressed scFvs are panned after reacting with antigen-coated plates to select the chains of interest that are expressed by hybridoma techniques[81-83].

6. Mechanism of action elaborated by different approved monoclonal antibodies

Monoclonal antibody mediated cancer immunotherapy is generally based on apoptotic activation induced by a number of pro-apoptotic factors mediating cancer cell death or through a number of diverse mechanisms of action elaborated by different approved monoclonal antibodies.

Table 2
Examples of FDA Approved Monoclonal Antibodies along with their Clinical Indications and Mechanism of Action.

| Monoclonal Antibody (Trade Name) | mAbs & Target Ag | Tumor disease | ADCP | ADCC | CDC | RND | CDD | SB |
|----------------------------------|------------------|---------------|------|------|-----|-----|-----|----|
| Rituximab (Rituxan)              | Chimeric anti-CD20 | Chronic & Non-Hodgkin’s lymphoma | +    | +    | +   |     |     |    |
| Ofatumumab (Arzerra)             | Human anti-CD20   | Chronic lymphocytic leukemia     | +    | +    |     |     |     |    |
| Ibritumomab tiuxetan (Zevalin)   | Murine anti-CD20  | Non-Hodgkin’s lymphoma           | +    |     |     |     |     |    |
| Tositumomab-I131 (Bexxar)        | Murine anti-CD20  | Non-Hodgkin’s lymphoma           | +    |     |     |     |     |    |
| Obinutuzumab (Gazyvaro)          | Humanized anti-CD20 | Chronic lymphocytic leukemia     | +    |     |     |     |     |    |
| Alemtuzumab (Campath)            | Humanized anti-CD52 | B-cell chronic lymphocytic leukemia | + | + | + | | | |
| Tafnetanib (Monjavi)             | Humanized anti-CD19 | Diffuse large B-cell lymphoma    | +    | +    |     |     |     |    |
| Loncastuximab tesirine (Zymblona) | Humanized anti-CD19 | Diffuse large B-cell lymphoma    | +    |     |     |     |     |    |
| Polatuzumab vedotin (Polivy)     | Humanized anti-CD79b | Diffuse large B-cell lymphoma    | +    |     |     |     |     |    |
| Daratumumab (Darsaza)            | Human anti-CD38   | Multiple Myeloma                 | +    | +    |     |     |     |    |
| Isatuximab (Sarclisa)            | Chimeric anti-CD38 | Multiple Myeloma                 | +    | +    |     |     |     |    |
| Mozamulimab (Poteligero)         | Humanized anti-CCR4 | Cutaneous T cell lymphoma        | +    | +    |     |     |     |    |
| Gemtuzumab ozogamicin (Mylotarg) | Humanized anti-CD33 | Acute myeloid leukemia (AML)  | +   |     |     |     |     |    |
| Inotuzumab ozogamicin (Beposyn)  | Humanized anti-CD22 | Acute lymphoblastic leukemia   | +    |     |     |     |     |    |
| Moxetumumab pasudotox (Lumoxiti) | Murine anti-CD22  | Hair cell leukemia               | +    |     |     |     |     |    |
| Belantamab mafodotin (BLENREP)   | Humanized anti-BCMA | Multiple Myeloma                 | +    |     |     |     |     |    |
| Brentuximab vedotin (Adcetris)   | Chimeric anti-CD30 | Hodgkin lymphoma (HL)           | +    |     |     |     |     |    |
| Eotuzumab (Eloztuzumab)          | Humanized anti-SLAMF7 | Multiple Myeloma               | +    | +    |     |     |     |    |
| Trastuzumab (Herceptin)          | Humanized anti-HER2 | Breast cancer; metastatic adenocarcinoma | + | + | + | | | |
| Ado- Trastuzumab emtansine (Kadcyla) | Humanized anti-HER2 | Breast cancer                   | +    |     |     |     |     |    |
| [fam]-trastuzumab deruxtecan (Enhertu) | Humanized anti-HER2 | Breast cancer                   | +    |     |     |     |     |    |
| Pertuzumab (Perjeta)             | Humanized anti-HER2 | Breast cancer                   | +    | +    |     |     |     |    |
| Margetuximab (Margenza)          | Chimeric anti-HER2 | Breast cancer                   | +    | +    |     |     |     |    |
| Cetuximab (Erbitux)              | Chimeric anti-EGFR | Head and neck cancer; colorectal cancer | + | + | + | | | |
| Panitumumab (Vectibix)           | Human2 anti-EGFR  | Metastatic colorectal carcinoma | +    |     |     |     |     |    |
| Necitumumab (Portrazza)          | Human anti-EGFR   | Carcinoma, non-small-cell lung  | +    |     |     |     |     |    |
| Dinutuximab (Unituxin)           | Chimeric anti-GD2  | Neuroblastoma                   | +    |     |     |     |     |    |
| Naxitumab (Danyeleca)            | Humanized anti-GD2 | Neuroblastoma                   | +    |     |     |     |     |    |
| Infotuzumab (Padove)             | Human anti-Nectin-4 | Urothelial cancer               | +    |     |     |     |     |    |
| Sacituzumab govitecan ( Trodelvy) | Humanized anti-TROP-2 | Breast cancer                   | +    |     |     |     |     |    |

ADCP: Antibody-Dependent-Cellular-Phagocytosis; ADCC: Antibody-Dependent-Cellular-Cytotoxicity; CDC: Complement Dependent Cytotoxicity; RND: Radio-Nucleotide Delivery; CDD: Cytotoxic Drug Delivery; SB: Signal Blockade.
the release of granzymes and the subsequent lysis of the cancer cells[99, 100].

7. Antibody drug conjugates

In order to compensate the inefficiency of monoclonal antibody-mediated cell toxicity using naked mAbs, a novel design, termed Antibody-Drug Conjugate (ADC) has been developed[101,102]. Each ADC is composed of a target-specific monoclonal antibody, chemically linked to a highly potent cytotoxic payload which synergistically enhances the therapeutic ratio of the construct[103]. Conventionally, an effective ADC should maintain its serum stability, readily reach its target cell, and ultimately release the associated cytotoxic payload in the proximity of the target cell[102,104]. Ideally, the target tumor marker should be expressed at the surface of the cell rather than being intracellular, soluble, or secreted in the blood circulation which risks inciting the cytolysis to take place outside the context of the tumor site. In order to engender the highest cytotoxic effect, the ADCs should be internalized by receptor mediated endocytosis followed by the release of the cytotoxic payload causing the cell death by apoptosis to be induced as a result of the cytoskeletal microtubules and DNA alteration[105-107]. During the last two decades, the FDA approved 14 different ADCs designed for the treatment of a wide spectrum of hematological and non-hematological malignancies[108,109]. Recently, a novel format of the ADCs termed Antibody-Antibiotic Conjugates has been designed to counteract against bacterial infections as a novel modality to limit the emergence of antimicrobial resistance[110]. Beside their immunogenicity, first generation ADCs such as BR96-Doxorubicin failed to engender a significant rate of tumor cell death[111,112]. A second generation of ADCs has been developed to overcome the drawbacks of the first line of products. These novel designs (Bretuximab-Vedotin and Adotrastuzumab-Emtansine) carry smaller but more toxic, water soluble payloads with optimized linkers and enhanced tumor cell targeting capacity[113-115]. Although, significant improvements were reached, many purposes were unmet; essentially, ADC biased toxicity, aggregation and rapid clearance which paved the road for the third generation. Based on the technology of Site-Specific conjugation, innovative consistent formats were introduced in the market (Polatuzumab vedotin, Enfortumab vedotin, Fam-trastuzumab deruxtecan). These molecules provoke high anticancer activity with less off-target toxicity, establish improved pharmacokinetic features, and cause lower immunogenicity based on the use of fully human rather murine or chimeric entities[116].

8. Transgenic xenomice for human antibody production

In order to overcome the molecular difficulties associated with the Phage Display technology and to avoid the time-consuming conventions yielding low-affinity antibodies, a genetically engineered strain of mice in which the murine antibody coding genes were inactivated while a set of human heavy and light chains encoding sequences were incorporated into the transgenic mouse genome. This novel success owes in much to the success of the transgenesis technology using the Yeast Artificial Chromosome (YAC) as a shuttle due to their extremely high transfer capacity into the murine germ line cells (Fig. 3). The YAC bearing human antibody coding genes are introduced into the mouse germ cells following the fusion of yeast spherocytes (devoid of cell wall) with embryonic stem cells (ESC). The aforementioned manipulation generates a large number of transgenic ESC where human coding genes are found to be integrated in a stable alliance with the chromosomal material. Some of the transfected cells used to produce mice carried a combination of the human and the murine immunoglobulin coding genes capable of producing both types of antibodies. Later, two of the resulting transgenic progeny, one carrying the two types of genes, the other with only the knocked-out murine DNA were crossbred producing a transgenic strain able to synthesize fully human immunoglobulins with great potential for approval as therapeutic monoclonal antibodies. Ongoing research is focused on growing up the repertoire of human antibodies and antibody fragments to be generated by this promising technology. Similarly, efforts are being devoted to introduce lambda coding genes into the mouse genome or inferring this technique in cattle using microcell-mediated gene transfer technology.

![Fig. 3. Genetic engineering of Xenomice. Murine antibody coding genes are disabled by site-directed mutagenesis. The mutated stem cells are used to create transgenic disarmed strains (left-up) that are unable to synthesize antibodies. Human heavy and light chains coding sequences were then cloned into Yeast Artificial Chromosome (YAC) and introduced into ESC used to yield another strain able to produce both, murine and human antibodies (right-up). These strains are crossbred and Xenomice able to synthesize human antibodies are screened and selected. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)](image-url)
9. Antibody fragments and related derivatives

9.1. Fragments of antigen binding (Fab & F(ab’)2)

The Fragment of Antigen Binding (Fab) was the first molecular format adopted as a therapeutic antibody fragment followed by a dozen of novel molecules that were respectively introduced in clinical trials starting from mid-1990s where they represented about 49% of therapeutic fragments under investigation[67]. Originally, Fab fragments could be easily generated by enzymatic cleavage of IgG using papain, a protease that cleaves the heavy chains at the sequence located just above the so-called “hinge region” resulting in the production of two Fab fragments in addition to a stretch of CH2-CH3-HR (Fig. 4)[117]. Each Fab fragment weighing about 50 KDa is the result of the association between a full length light chain (CL & VL) joined by a disulfide bridge to the cleaved heavy chain comprising a variable domain (VH) with the first constant domain (CH1) where the protease has cleaved the chain [118,119]. Fab fragments are perceived as “Natural Fragments” since they are generated by enzymatic cleavage of the immunoglobulins which alleviates the need for genetic engineering required for the production of their counterparts with enhanced stability gained from reciprocal stabilization naturally existing between the variable and constant domains of the heavy and light chains[23,120]. The lack of the crystallizable fragment (Fc) hinders the ability of these fragments to cause the bystander activation of undesirable “opsonized” immune reactions mediated by the effector function of the antibodies and their subsequent cytotoxicity (namely the Antibody Dependent Cell Mediated Cytotoxicity (ADCC), Antibody Dependent Cellular Mediated Phagocytosis (ADCP), and Complement Mediated Cytotoxicity (CDC))[39].

In contrast to the Fab, the F(ab’)2 is a bivalent, monospecific fragment (Fig. 4) essentially composed of two individual Fab fragments assembled together by the means of the flexible hinge region. The production of F(ab’)2 is easy, natural and straightforward. A direct treatment of IgG with Pepsin cleaves the full length antibody, just beneath the hinge region, generating combined Fabs with a detectable Fc fragment[121,122]. Their molecular mass is about 110 KDa, double that of a separate Fab which reduces their tissue penetration compared to Fab; but still higher than a full length antibody[123]. Since they possess two binding sites for the same epitope, therefore, their avidity is amplified giving them an advantage over other monovalent fragment in terms of their binding capacity to the antigens[120,124]. Size reduction comes with a shorter Fab serum half-life that could be addressed by their pegylation or the traditional conjugation to albumin and XTEN fusion proteins[69,125]. A number of antigen binding fragments were successfully designed and introduced into clinical trials. Ranibizumab (Fab) and Certolizumab Pegol (Fab’) were produced in their definitive forms in the E.coli expression system while Abciximab has been first generated as a full-length IgG before being enzymatically digested with Papain to cleave the Fc region and generate the Fab fragment[126]. These fragments were mostly used for the treatment of acute conditions; some patterns however were designated for the management of the chronic inflammatory diseases. For example, the Fab Certolizumab Pegol is indicated in the framework of rheumatoid arthritis and Crohn’s disease. In order to prolong their serum half-life, the cysteine residue located in the proximity of the C-terminal end is adapted as the specific site for pegylation[127].

9.2. Single chain fragment variable (scFv)

In the issue released on October 21st, 1988, Bird and his colleagues described the Single Chain Fragment variable (scFv) for the first time. The recombinant polypeptides are antigen binding agents composed of the variable regions of both, light (V\text{L}) and the heavy (V\text{H}) chains, tethered by the mean of a linker peptide sequence (conventionally rich in glycine and serine with scattered hydrophilic residues) that joins the carboxyl terminal ends of V\text{L} and V\text{H} (Fig. 5)[128]. In order to optimize their thermal stability and their affinity towards the antigen, the number of amino acid residues making the linker peptide may differ from one scFv into another. It has been postulated that a linker peptide must spatially extend over a distance of 3.5 nm between the V\text{L} and V\text{H} domains outside the context of their binding site formation[129]. Stretches of thirty to thirty eight residues were engineered in such a way the cysteine residues involved in creating disulfide bridges between constant domains of the heavy (CH1) and light chains (CL) were deleted [130,131].

The scFv maintains the same antigenic specificity with equivalent affinity to the epitopes for which the parental full length antibody has been generated. Their simple structure allows their production by expressing individual genes in the phage or in the form of combinatorial library of surface protein within a yeast cell surface display. This
molecular modality permits in vitro screening and selection of target chains among a full repertoire of proteins. The genes of interest could be expressed inside the cell where they are potentially subject for a substantial change of conformation at the issue of their folding and various subcellular localization of the target fragments[132]. Compared to the full length antibodies, the scFv confer several advantages structurally, functionally and clinically. Their largely reduced size (25–30 KDa) makes them optimal for mass production in microbial bio-reactors systems[133]. Since VH and VL are designed in series in the same recombiant system, the scFv would be produced faster, in greater yield, and at reduced costs when compared to conventional mAbs traditionally produced in higher expression systems. The clinical efficiency of the immunotherapy, especially in oncology, witnessed a substantial success with the advent of the scFv that facilitated a greater access to the ma-lignant tissues and enhanced the tumor penetration and receptor binding [134]. The physical absence of the Fc region from scFv reduces the risk of bystander activation of the immune cells but allows the agent to bind the specific receptor on the target cell without activation of the immune reaction. Although the lack of the Fc region might be often advantageous for tumor access, yet it decreases the thermostability, increases the propensity for aggregation, which promotes their immunogenicity and reduces their circulatory half-life[135,136]. In such cases, the required effective dose is conventionally increased and the pharmaceutical must be administered more frequently. This drawback is consistently addressed by scFv pegylation[125] or their fusion with albumin[135]. A humankind scFv, anti-VEGF (Vascular Endothelial Growth) factor (Brolucizumab) has been designed for the treatment of Age Related Macular Degeneration (AMD). Instead of the conventional direct injection of Anti-VEGF drugs, Brolucizumab showed an extended durability with great effectiveness based on a 12 weeks treatment scheme[137]. In order to enhance their therapeutic efficacy for oncological applications and their in vivo retention frequency, scFv antibody fragments were conjugated with toxins and/or other immune stimulators to target ma-lignant cells. Oportuzumab montax, an anti-neoplastic scFv intended to bind an epithelial tumor cell-adhesion protein (EpCAM), fused with truncated Pseudomonas aerogenosa Endotoxin A has been approved for the treatment of BCG resistant urothelial bladder carcinoma[138,139]. An anti-CD22 (Highly expressed by B cell lymphomas and Non-Hodgkin’s lymphomas) fusion protein conjugated with apoptin has been designed to bind the lymphoma cells, cross their plasma membrane, and induce their apoptosis [140].

9.3. Tandem single chain fragment variable (Ta-scFv)

Designed to string on tandem, two, three or even more scFv bodies are linked together by a flexible linker composed of a helical strand of amino acids. The tandem construct (Fig. 6) may include identical sub-units, generating mono-specific, bivalent (two subunits), or multivalent (three subunits or more) fragments, or non-identical subunits, generating bispecific (two subunits), or multispecific, multivalent (three subunits or more) fragments[141]. The order of the cloned gene seg-ments defines the format of the tandem scFv based on the orientation of VH and VL cloned sequence (VH-VL or VL-VH), the number of subunits, and the length of the linker peptide that would affect the folding, and the antigen binding capability of the tandem chain[142].

Homopolymers of Alanine (Ala-3), hydrophilic amino acid based stretches, Glycine-Serine-Rich spacers, and sequences derived from conventional immunoglobulins or other globular proteins were used to scheme the linkers that join the tandem repeats of single chains providing flexibility and resistance to protease degradation[142,143]. The length of connectors did not show a significant difference in their in vitro bioactivity demonstrating that short linkers retain adequate flexi-bility to intensely bind the nearby accessible antigens. Connectors composed of 12 residues and above define the native orientation of the antigen binding sites. The genetic reduction of the scFv linkers peptides by reducing the length of their coding sequences leads to a spontaneous assembly of the subunits in the form of multimeric complexes such as diabodies (see next paragraph)[144–147]. The resulting products are used to target one, two or even more antigenic determinants with improved avidity and extended half-life when compared to single chains [148]. Similarly, this technique opened the way to produce bispecific full length antibodies able to recognize two different epitopes or anti-genic determinants (Fig. 7). These products are featured by the acqui-sition of novel functions that do not initially exist in their parental antibodies[149].

In 2018, Eggrenreich and his collaborators[150] introduced a re-combiant Ta-scFv designed to manage Celiac Disease. The consump-tion of Prolamins triggers the secretion of many autoantibodies, pro-inflammatory cytokines, and tissue trans-glutaminases leading to the development of a chronic inflammation associated with long term immune complications of the gut[151,152]. The unique effective treatment of Celiac Disease has been limited to a strict gluten-free diet that might be hindered by the traces of prolamins in the consumed food products[153]. The Ta-scFv serves as a neutralizing agent able to form a complex with Prolamins in the lumen of the gut, outside the context of the epithelium, preventing the formation of immunogenic agents and lead to their excretion[150,154].

9.4. Multimeric scFv fragments - Diabodies, triabodies, & tetrabodies

Different strategies were devised to improve the pharmacokinetic attributes and enhance the residual affinity towards antigenic de-terminants of the scFvs introduced in preclinical studies and clinical trials. The most effective approach resides in multimerizing the single scFvs bivalents dimers (Diabodies, 55–60 KDa) (Fig. 8A), trivalent trimmers (Triabodies, 80–90 KDa) (Fig. 8B), and/or tetravalent tetra-mers (Tetrabodies, 110–120 KDa) (Fig. 8C)[146,147].

Diabodies (Dbs) (Fig. 8A) are bivalent conjugates, that could be either mono-specific or bispecific whether their scFv forming units are identical or not. With a five residue long spacer (G,S), the VH and VL of each variable chain subunit are interconnected together. Instead of flattering intrachain assembly of the antigen binding sites, the shortness of the linker peptide brings about the dimerization of the two chains in a head-to-tail format generating a compact configuration [145,155,156]. The transfected cells are supposed to yield two different chains formats (VH–A–VH–B & VH–B–VH–A) or (VL–A–VL–B & VL–B–VL–A) (where letters A and B correspond to the different antigenic specificities) [155,157]. Since the different chains of the bispecific diabodies are simultaneously expressed.
within the same cell, it is very likely that identical single chains assemble together yielding unproductive homodimers that lose their specificities and might be more susceptible to instability [158]. Molecular studies demonstrated that the linker’s extension is critical for the conformation of the multimeric complex. When the latter is reduced to less than three amino acid residues, higher amount of triabodies (Fig. 8B) is generated in the periplasm since the shortness of this sequence affects the variable domain’s flexibility and prolongs the multimeric assembly time what brings about additional subunit yielding more triabodies conjugates [145]. When it comes to the antigen binding capacity, the spacial
distribution of the individual binding sites and the orientation of the variable modules affect the accessibility of the diabodies and triabodies to the surface antigens [145, 159].

10. DART, bite, & tandem antibodies

Over the last decade, a number of molecular modifications were devised to the diabodies format to improve thermal stability, clinical efficacy, and tumor availability. Based on their structural configuration, the bispecific diabodies could be classified into either “Fc-less antibody fragments” with a minimalistic antigen binding domain or Immunoglobulin-like antibodies [155]. Therefore, they are either conventional IgG-like or unorthodox non-conventional IgG-like molecules. Cross-Mab® [160, 161], Knobs-in-Holes [162], Dock-&-Lock [163], Quadroma® [164], Duobody® [165], and Dual-Variable-Domain [166] were developed by a number of research groups in the aim to harvest different IgG-like formats, namely the Dual Affinity Re-Targeting (DART) proteins, the Bi-specific T Cell Engagers (BiTE), and Tandem Diabodies (Tand-Ab) [167, 168]. These synthetic fragments are manufactured by assembling a number of building blocks (i.e. Fab, Single Chain Fragment Variable (scFv), Single Domains Antibodies (sd-Abs)) merged together by the mean of a peptide linker [169].

11. DART antibodies

Dual Affinity Re-Targeting proteins (Fig. 9) consist of a pair of engineered variable chains in which the VH domains are swapped with the opposite one. More explicitly, VH from anti-A antibody is exchanged with the VL from anti-B antibody. The first binding site will consists of VH-A paired with VL-B and the opposed binding site will consists of VH-B paired with VL-A [170]. The interchange of the variable domains yields a number of diverse fragments as a result of the three dimensional conformation resulting from individual peptides interaction affected by the shortened connecting linker similar to the natural interaction occurring in conventional antibodies. A promising T cell recruiting diabody-like molecule (Flotetuzumab, MGD006) has been designed to bind CD3 and CD123 clusters of the Acute Myeloid Leukemia cells or in the case of refractory myelodysplastic syndrome inducing the engagement and the activation of T cell killing of the malignant clones [171]. Besides, MGD007 is directed against GPA33 protein that characterizes gastrointestinal malignancies. The fusion with Fc region prolongs the serum half-life of MGD007 in contrast to the Fc-less format of MGD006 which is cleared more rapidly from the patients’ serum [172]. DARTs were shown to yield an enhanced B and T cell in addition to their contribution in recruiting the CD16 Natural Killer cells engendering the secretion of potent cytokines resulting in an effective cytolytic activity of targeted malignant cells [173]. In 2020, Tebotelimab (MGD013) has been investigated in a Phase-I clinical trial as a new format in the class of DART designed as a bispecific tetravalent to target PD-1 and LAG-3. The tested novel DART has been shown to provoke efficient and coordinated blocking attributes of PD-1 and LAG-3 leading to the enhancement of the T cell activity [174]. Clinical data shows that MGD013 demonstrates adequate safety attributes with promising anti-tumor efficacy [175].

Characterized by a relatively small size, DARTs are amenable to rapid renal excretion, high resistance to aggregation, and extreme potential following their administration in vitro and in vivo [170, 176]. To overcome their short serum half-life, Fc bearing DARTs were developed providing the clinicians a wider spectrum of amended protocols with different drug dosage and some products were stabilized by introducing a disulfide bridge [168].

12. BiTE antibodies

One of the key immune reactions directed towards eliminating the cancer cells involves the enhancement of T cells interactions with the tumor cell receptors [177]. BiTEs were designed to bridge the cancer cells surface antigens to the T cell Cluster of Differentiation (CD3) situated in the close proximity of the TCR which potentiates the unreactive T lymphocytes and reinforces their cytotoxic activity against the targeted tumor cells (Fig. 10). This way, the opsonizing BiTEs bring about a more specific and efficient T cell mediated immune response [167]. To manufacture BiTEs, a first scFv, directed against the CD3, is coupled to another single chain directed towards the tumor cells surface antigens (BiTEs could be expanded by adding a third scFv to become trivalent and multispecific). This conformation stands behind the proliferation of T lymphocytes inducing a T-cell mediated cytotoxicity [178].

Fig. 9. Genetic engineering of Dual Affinity Re-Targeting proteins (DART).
Engineered Recombinant DNA carried by a plasmid is used to transfect CHO or HEK cells that synthesize the fragments to be selected for the desired pharmaceutical properties. Like many other recombinant antibody fragments, the defective Fc and the reduced molecular size of BiTEs shorten their serum half-life. However, this does not hinder their efficiency in inducing an extremely potent cytotoxic effect against tumor cells even if used at picomolar concentrations [167]. Blinatumomab (AMG103, MT103), an anti-CD19 is one of the most promising Bispecific T Cell Engagers (BiTE) in clinical trials, has garnered FDA approval for the treatment and eradication of B-Cell Precursor Acute Lymphoblastic Leukemia (ALL) [94, 179]. Cases of Chronic Lymphoblastic Leukemia (CLL) showed a good prognosis when treated with Blinatumomab. Tumor regression has been also documented in the cases of Non-Hodgkin’s Lymphomas [92, 180]. Further clinical studies are conducted to assess the efficiency of using a multitude of BiTEs designed to target EpCAM (Colorectal Cancer), CEA (Gastric Carcinoma), HER-2 (Breast Cancer), PsMA (Prostate Cancer) and other tumor markers [180]. In 2019, a bicistronic model has been constructed to overcome the limitation of Chimeric Antigen Receptor (CAT) T Cell treatment of solid glioblastoma. The construct involves a CAR expression system for EGFRvIII antigen with a bispecific T Cells Engager (BiTE) directed against EGFR. The combined system (CAR-T # BiTE) has been shown to be more effective than either separate treatment modalities [181].

13. Tandem antibodies

Bearing four antigen binding sites directed against two different antigenic determinants, the Tandem Antibodies (TanAbs) are tetravalent bispecific complexes of about 100–110 KDa. The genetically engineered constructs encode a single chain of tandem subunits consisting of four different variable modules assembled together by a short peptide linker (Fig. 11). Compared to their analogues (BiTEs and DARTs), the TanAb bridges the molecular pattern of target cells to the receptors expressed at the surface of the Natural Killers (NKs) and/or CD8+ cytotoxic T cells. Owing to their bivalent domains, TanAbs preserve a comparable avidity as their conventional antibodies with the optimal affinity to the target antigens. Their relatively larger size prolongs their serum half-life that can reach up to 24 h, comparatively higher than the half-life of all other antibody fragments [159, 178].

Tandem Antibody platform has been used to design a CD19-CD3-
AFM11 bisespecific humanized tetravalent construct, extremely powerful immune cell recruiter in the case of B cell malignancies[182]. AFM11 exhibits substantial avidity to the highly expressed CD3 which turns it into a serial-killer of CD19 expressing B cell lineage. Through its anti-CD3 domains, AFM11 recruits effector T cells stimulating the destruction and lysis of Hodgkin's lymphoma cancer cells[185]. In 2021, Watanabe et al., from University of Tokyo, developed a series of constructs including a tandem scFv-Fc, a diabody-Fc, and a fusion protein scFv-Fc-scFv, each of which possess four scFv domains and able to recognize and bind the extracellular domains of ROBO1[186].

14. Minibodies

Pairs of single chain variable fragments joined together by the mean of their CH3 heavy chain’s constant domains (2 x scFv-CH3) were coined the term Minibodies (Fig. 12)[187]. These substitutes of diabodies, are bivalent dimers with an intermediate molecular weight of about 80 KDa. Minibodies could be designed to be either monospecific or bispecific according to whether their binding sites are identical or not[188]. The multivalence of Minibodies allows them to readily bind their targets, with a rather higher affinity than the monovalent counterparts. Due to their moderately small size, the Minibodies could be easily expressed at a high yield in prokaryotic host cells and therefore, when clinically used, they show a good tumor-to-normal tissue ratio as a result of their eased penetration into cancerous tissues[189].

Although widely used in clinical trials, the approval of Minibodies is restricted by their low thermostability and their propensity for aggregation and denaturation in vivo due to the weakness of the variable chains’ interaction[190]. The extension of the linkers joining the scFvs from 15 to 18 aa improved the strength of the thermal stability in the 48 h that follow their injection[191]. Their solubility has been also affected by their variable domains orientation where VH-VL and shows an increased solubility when compared to the opposite orientation (VH-VL) [192].

Many different formats of minibodies were engineered to target a number of tumor markers in oncology. Among these, the scFv-CH3y1 dimer, was designed to target and block the Carcinoembryonic Antigen (CEA). This molecule showed a high specificity in CEA binding and short serum half-life[193]. A11 minibody has been engineered to target the prostate stem cells specific antigen. The minibody-conjugated form combined to polypeptide-based gold nanoshells has been tested in the context of thermal therapy. The minibody-conjugate demonstrates a substantial localized reduction in metastatic prostate cancer cells[193, 194]. Tositumomab, a radionuclide-coupled anti-CD20 antibody (131I labeled) and Ibritumomab (90Y conjugated) have been FDA approved for the management of Non-Hodgkin’s Lymphoma[195].

15. Heavy chain antibodies and nanobodies

In 1989, an unexpected finding, discovered at the issue of the whole and fractionated serum analysis derived from Camelidae (camels, llamas and alpacas), has perpetually changed the conceptual organization of conventional antibodies, primarily perceived as “dimers of dimers” composed of two heavy chains assembled to two identical light chains [196]. Hereinafter, a new format of non-canonical antibodies has been found to lack the light chain while only two heavy chains (lacking CH1 domains) are revealed. The expanded forms give rise to additional peaks featuring the electrophoretic profile of the serum Camelid globulin fractions. These latter were coined the term Heavy Chain Antibodies (HCAbs) containing VH,H domains with relatively lower molecular weight (90 KDa) compared to conventional IgGs (150 KDa)[197]. The DNA coding sequences of Camelid HCAbs were deciphered using molecular sequencing techniques. It has been demonstrated that HCAbs are encoded by the same conventional locus but with a different set of gene segments. The camellia genome consists of about 40 genes, assuming the same genetic organization of conventional antibody coding sequences. Although present on the sequence of coding genes, the CH1 domain coding exon bears a point mutation located at the boundary between CH1 and the proximate hinge region intron which hinders the consensus splice site between the G and T and omits the coding sequence[198]. The diversification of HCAbs antigen binding repertoire is triggered by a set of molecular mechanisms including the formation of disulfide bridges, the extended surface area of the hypervariable domains and the formal reshaping of paratopes of the binding groove. It has been postulated that a number of signal sequences stretched over the VH,H stand behind the multitude of diversity mechanisms. The lack of the light chain enables such changes to take place generating novel specificities[199]. Structurally, VH,Hs are composed of four conserved

![Fig. 12. Schematic representation of Minibodies composed of two single chain variable fragments linked together by the mean of a CH3 heavy chain’s constant domains (2 x scFv-CH3).](image-url)
Derivatives of the camelid, the variable domains of the HCabs, establishing the antigen binding sites, the naturally occurring Nano-bodies are miniaturized fragments with extended CDR3 loops and low molecular weight of only about 15 KDa[200]. Alternatively, the Nbs could be engineered using a convenient recombinant system and optimal host cell which facilitate their synthesis and multimerization to generate multivalent therapeutic agents. The three dimensional conformation of Nbs paratopes allows them to easily access the hidden epitopes or cryptic determinants on the surface of antigen. Nanobodies are characterized by a substantial stability, higher solubility and a significantly improved affinity, with a critically reduced immunogenicity
[200,201]. Their small size facilitates their tissue penetration, distribution, and targeting. Owing to these strategic attributes, Nbs are considered as one of the main promising diagnostic and therapeutic agents with a great potential use for a diversity of illnesses (oncology, neurological disorders, inflammatory, and infectious diseases)[200]. Nanobodies were proven to be amenable for protein fusion and conjugation with toxins or even radioactive elements transforming them into clinically efficient drug delivery systems for active drugs, enzymes, toxins, or inactive prodrugs which promoted their selectivity and efficacy[200,202]. An ongoing multicentric Phase I/II evaluates the therapeutic efficacy and safety of Zenocutuzumab (MCLA-128) an Anti-HER-2 x Anti-HER-3 Bispecific Heavy Chain antibody designed to treat the patients harboring solid tumors with NRG1 fusion. The molecule is able to mediate antibody-dependent cellular cytotoxicity as it binds and blocks HER-3 as the NRG-1 and NRG-1 fusion proteins counterparts[203,204]. Among those nanobodies that showed a significant clinical efficacy, the FDA-approved Caplacizumab, is an anti-von Willebrand factor designed to manage the Aquired Thomobotic Thrombocytopenic Pur-pura (aTTP) by blocking the A-1 domain of the coagulation factor and inhibits their interaction with the platelets by 67% the risk to develop an aTTP[205,206]. Ga-HER2-Nanobody, directed against HER-2 tumor marker of breast carcinoma, showed high kidney filtration, fast blood clearance after a short serum half-life (2.5 h), and a substantial capacity to identify the majority of the tumor cells locations and distribution[207,208].

16. Antibody mimetics

The structural and functional limitations encountered with the full length antibodies and many of their derivatives triggered research to develop alternative platforms to create novel agents, able to “mimic” the binding capacity of the conventional antibodies. For that purpose, the monovalent “Antibody Mimetics”, a new class of minimalistic binding proteins, were manufactured outside the context of the immune system panels, generally composed of non-immunoglobulin alpha helices and beta sheets or even randomly looped stretches and are intended to include a range of antigenic binding sites[209]. A number of protein engineering strategies were devoted to yield and then select these antibody mimetics including site-directed mutagenesis, multisite directed mutagenesis, and random mutagenesis. The rationale behind designing antibody mimetics depends on the accurate selection of the proper framework suitable for the integration of recognition domains. Conventionally, this framework is a folded protein domain amenable for mutational manipulations. It should possess a substantial level of flexibility tolerating the insertion of mutations without affecting the secondary structure of the protein, without deterring the antigenic specificity and affinity to the target epitopes[210]. The isolation of the suitable mimetic could be achieved using a directed evolution cycle. Presumably, the designated mutation sites of the adopted binding domain are subject to a process of mutagenesis to construct the DNA library that will be displayed on the surface of the expression system. An error-prone PCR along with DNA shuffling process are adopted to promote the diversification of the yield and mature the desired antibody mimetics[209,211]. Commonly, the selected products should be soluble, stable, resistant to degradation by proteases, thermostable, tolerating extreme pH values, and composed of a single domain peptide without disulfide bridges or need for a process of glycosylation. Preferably, the antibody mimetics are to be synthesized in the cytoplasm of the host recombinant bacteria without being transformed into inclusion bodies or being degraded[212,213]. Despite the fact that the recognition and binding of the epitope to the conventional antibodies (and most of their fragments) involves the contribution of the loops protruding from the six different complementary determining regions of the heavy and light chains, it has been proposed that CDRs-FRs-based mimetic peptides (about 3 KDa) may be manufactured and used for a large number of medical purposes. The CDR-FR peptides are connected by alternating two CDRs by the means of a framework region selected from VH and VL coding sequences. The CDR3 is the most obvious component of the peptide mimetic due to its great accessibility, diversity, and its critical contribution to the antigen binding. The C-terminal end of CDR1/2 loops and the N-terminal end of the CDRH/L3 are interconnected with a trailed FR randomly chosen from VH and VL coding sequences[210]. A number of various versions of antibody mimetics were developed, namely the Nanofitins (Nanofitins), Anticalins (Lipocalins), Affibodies (B-domain of SPA), DARPin (Designed Ankyrin Repeat Proteins), DARPins (Designed Ankyrin Repeat Proteins),...
Avimers (A-domain), and Fynomers (SH3 Domain of Fyn Kinase). Many of these later are currently under rigorous clinical investigation suggesting a very promising future with unlimited application tenacity of such human proteins derivatives for diagnostic and therapeutic purposes [214]. Unlike conventional antibodies, the antibody mimetics exhibit a short half-life associated with a high kidney clearance rate due to their reduced molecular weight (5–10 KDa). The absence of the Fc region alters their ability to bridge and activate the immune system cells. This drawback could be overcome by combining the mimetics to free Fc domains and reestablish their functional activity. Alternatively, antibody mimetics could be further restructured by PEGylation, PASYlation (Proline-Alanine-Serine), or by fusion to proteins conjugates (Albumin Binding Domain) without affecting their affinity and their tumor infiltration power [215–217]. The management of Acute Myeloid Leukaemia (AML) has revolutionized during the last decades. A number of drugs were clinically tested and FDA approved. Among these, is Venetoclax, an orally administered antibody mimetic that selectively targets BCL-2 but not the BCL-XL. Preclinical studies revealed a promising efficacy of Venetoclax in the management of AML when combined with hypo-methylation agents (HMA) and chemotherapeutic drugs taking it to clinical trials. Data collected from Venetoclax-based therapy combined to HMA and Cytrabine in elderly (75 years and beyond) with AML (unfit for conventional intensive chemotherapy) in addition to its safety profile secured a fast FDA approval and widespread incorporation of such antibody-mimetic based therapy in the clinical context [218,219].

17. Conclusions

It is commonly said that “The smaller pieces and details make up the big broader picture.” When it comes to the mosaic puzzle of therapeutic antibodies it can be fairly said “The smaller pieces make the larger ones look better”. The great potential of antibody fragments and their mimetics has no limits with wide range of formats and the unprecedented access to uncharted purposes and overcome the new challenges coming upon in with the fast and limitless technological breakthroughs and new era of medicine. This review provides summary of the molecular mechanisms governing the genetic engineering of antibodies and their derivatives, starting with murine, chimeric and humanized antibodies to the different fragments generated in vitro using either prokaryotic host cells, eukaryotic expression systems, cell free systems, or transgenic mice. Conventional antibodies show a number of disadvantages due to their large size and immunogenicity. Antibody derivatives and modifications aim to increase tissue penetration and reduce undesired activation of autoimmunity or side-immunity for therapeutic purposes. On a side note, tissue penetration and yet quick enough body clearance represent a challenge to balance effectiveness and toxicity. Developing antibodies with multiple targets and/or acting via multiple mechanisms would definitely enhance treatment of highly mutating tumors. This can include activation of T-cells, better engagement of innate and adaptive immune system components and suppressing multiple oncogenic pathways in cancer treatment. The advancement of therapeutic options provided with antibody fragments and modifications can be further elevated with use of combination treatments along with immunomodulators such as immune checkpoint blockers. Individual assessment of each particular antibody treatment remains a “best” practice to reduce side effects. The use of suitable in vitro and in vivo and perhaps 3D organoid systems to assess toxicity can drive more rigorous exploration of new mimetics or derivatives prior to clinical trials and human administration.

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FDA approved mAbs data available on

https://www.antibodysociety.org/resources/approved-antibodies

Declaration of Competing Interest

None

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