Chapter

Alternative Methods to Animal Use for Monoclonal Antibody Generation and Production

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Abstract

Monoclonal antibody (mAb) has broad applicability in research, diagnosis, and treatment. After the introduction of hybridoma technology in 1975, the mAb market has increased dramatically, moving a large industry of more than US$ 140 billions in 2020. In 1954, the concept of the 3R's was proposed and much changed the animal use scenario, including the recent ban on inducing ascites in mice for the production of mAb. In light of this, the generation and production of antibodies had to be reassessed. In this chapter, we present an overview of the main alternative technologies to the use of animals in the generation and production of mAb. Antibody display libraries and *in silico* modeling are very promising technologies that may provide mAb genetic constructs that, in the sequence, may be expressed on mammalian, bacterial, yeast or plant systems. Although the total replacement of the use of animals in the entire process is not currently feasible, it is possible to find ways to reduce and refine the use of animals in obtaining and producing mAb.

Keywords: monoclonal antibody, alternative methods, antibody generation, antibody display libraries, *in silico* antibody modeling, antibody expression systems

1. Introduction

Animals have been used for research applications since the early centuries after Christ [1]. This practice has always been controversial. However, only in 1870, after discovering that animals feel pain, the theme began to be reconsidered [2]. Yet, the first significant milestone involving ethical issues in using animals for research occurred in 1954, when Charles Hume and William Russel proposed the concept of the 3Rs. They advocated "Replacing, Reducing and Refining" the use of animals to minimize pain or stress whenever possible [3]. This conception was further extended to the 6Rs to include "Read across", referring to the critical analysis of new results, "Relevance", which concerns ethical and educational visions, including good laboratory practices, and finally "Roadmaps," which evolves planning, communication, conference and technical implementation policies [4]. Recently, the American

Anti-Vivisection Society (AAVS) banned the production of ascites in animals, launching the "antibodies without animals" campaign [5, 6].

In view of this, the conventionally used strategies to generate and produce monoclonal antibodies (mAbs), initially proposed by Köhler and Milstein in 1975 [7] as the hybridoma technology, had to be reconsidered. This methodology is based on the immunization of animals with the antigen of interest, followed by the fusion of B lymphocytes with myeloma cells, resulting in the formation of hybridomas. After cloning and selection, the antibody-secreting stable monoclonal cell lines were used to produce mAb-enriched ascitic liquid [8], a now-banned practice. For many years, mAbs, which have wide applicability in research, diagnosis, and treatment, were generated and produced with this methodology. In this chapter, the main technologies that emerged as alternatives to the use of animals for the generation and production of mAbs are discussed.

1.1 Overview of antibody structure and most common formats

mAbs are immunoglobulin molecules with a molecular weight of ~150 kDa, made up of four polypeptide chains: one pair of identical light chains and another pair of identical heavy chains joined by disulfide and non-covalent bonds. Each chain contains a variable domain (VL and VH) at the N-terminal portion and one or three constant domains at the polypeptide's C-terminal portion. The antigen-binding fragment (Fab) has ~50 kDa and is composed of variable and constant regions of heavy and light chains. The variable fragment (Fv) includes only the two variable domains of both chains (**Figure 1a**) [9]. Each variable region is composed

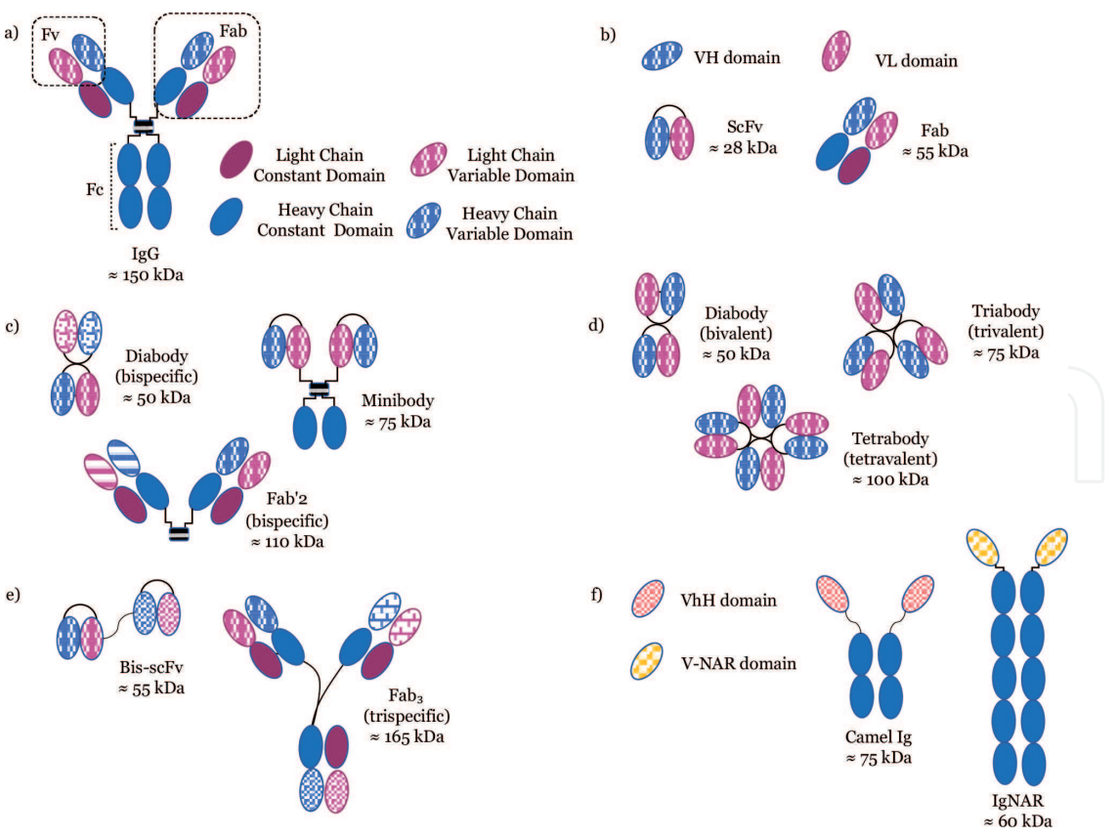

Figure 1.
Schematic representation of different antibody formats: (a) the classic IgG and its respective regions and chains; (b) monovalent antigen-binding formats: Fab and scFv; (c) bivalent antigen-binding formats: Fab'2, diabody and minibody. Different textures indicate different antigen specificities; (d) structures composed of scFv can form diabody, triabody and tetrabody; (e) other possible formats that can be constructed; (f) camelid Ig and shark IgNAR molecules.

of six complementarity determining regions (CDRs), also known as hypervariable regions: three in the light chain (L1, L2, and L3) and three in the heavy chain (H1, H2, and H3), CDR H3 being the most variable in length, sequence, and structure. These regions promote the high specificity of functional binding of mAbs with the target antigen [10]. In spite of hypervariable regions, it should be noted that these regions assume conformations in the form of loops because of the presence of some conserved residues [11].

There are several formats of antibodies used for therapeutic, diagnostic, and basic research purposes. Smaller formats were initially generated by the removal of the constant domain (Fc) by proteolysis, with the enzymes papain and pepsin, and later by genetic manipulations, giving rise to monovalent antigen-binding formats: Fab and scFv (variable single-chain fragment) (**Figure 1b**) or bivalent formats such as Fab'2, diabody, minibody, among others (**Figure 1c**). These antibodies present characteristics in comparison to complete mAbs that may be useful depending on the application: besides retaining the antigen-binding affinity of the parental antibody, they have a reduced serum half-life and are less immunogenic [12, 13]. In addition to conventional shapes, camelids and sharks produce unusual antibodies composed only of heavy chains, with just a single domain in its variable antigen-binding site. In Camelidae, it is called variable domain of heavy chain antibodies (VhHs), while in some cartilaginous fish, like sharks, it is called new variable antigen receptor (V-NARs). The smaller sizes of VhHs and V-NARs allow them to be good candidates as biotechnological tools (**Figure 1f**) [14–16].

Fab favors the crystallization of several proteins facilitating the determination of their three-dimensional structure [15]. Therapeutic Fabs have been available since 1994, starting with the chimeric abciximab Fab (ReoPro), used as an antiplatelet agent. Ranibizumab (Lucentis) was approved by Food and Drug Administration (FDA) in 2006 for treatment of age-related macular degeneration, certolizumab pegol (Cimzia) was approved in 2009 for rheumatoid arthritis, and several other Fabs are now in clinical and pre-clinical trials. This format is also useful for diagnostic imaging, like arcitumomab (CEA-scan) approved in 1996 for colorectal cancer screening [12, 17, 18].

Another variant is scFv antibodies, with a molecular mass of ~30 kDa, composed of VH and VL domains joined with a peptide ligand. These structures can be presented as dimers, trimers, tetramers (**Figure 1d**) or other formats (**Figure 1e**) through genetic or chemical manipulations. Diabodies can present two identical antigen-binding sites, when it is called a bivalent diabody, or have two different antigen-binding sites, a bispecific diabody [19, 20]. Numerous scFvs have been constructed against haptens, proteins, carbohydrates, receptors, tumor antigens, and viruses for applications in therapy and diagnosis [21]. In 2019, the FDA approved the humanized scFv brolucizumab (Beovu) to treat neovascular age-related macular degeneration. Other scFvs are already in a pre-clinical study for targeted cancer therapy [22].

2. Alternative methods for monoclonal antibody generation

2.1 Antibody display libraries

Antibody display libraries are powerful tools to isolate high-affinity antibodies for therapeutic and/or diagnostics applications. They can be divided into two groups: cell surface and cell-free display libraries. In the first case, the antibody is expressed on the surface of bacteria, yeasts, or mammalian cells, using own internal machinery [22] and, in the second, extracts of prokaryotic or eukaryotic

cells, mainly from rabbit or wheat germ, are used to transcribe and translate genetic information contained in the library [23, 24]. Although they use different paths, these methodologies have a common property: they are potential alternative methods to the use of animals for antibody generation.

These methodologies involve two main steps: the construction of the library and the selection of the antibody of interest. According to the origin of the genetic material used in their construction, the libraries are classified as naïve, immune, synthetic, or semi-synthetic. The immune library is obtained from animals or humans that have been immunized and developed antibodies against a particular antigen [25, 26]. The other types are known as universal libraries: naïve is cloned from non-immunized donors; the semisynthetic is created using both naturally and synthetically (*in silico*) randomized CDRs, which increases the diversity of the library without requiring a large number of donors; and synthetic, based only in *in silico* design and gene synthesis to optimize individual amino acids, hence expanding its diversity, expression, and stability [27].

2.1.1 Cell surface display

Among the display libraries, the phage display stands out for being the first described and currently more used in the generation of antibodies [28]. The technique was developed by George Smith in 1985 and it uses the bacteriophage's ability to infect bacteria. In this way, a foreign DNA sequence is inserted into the genes III or VIII, which encode the pIII and pVIII coat proteins, respectively. The recombinant protein is displayed on the outer surface of the phage as a fusion protein in an immunologically accessible form [29]. The displayed antibodies can be of either Fab or scFv formats. The phage display library is generated by assembling DNA sequences that encode antibody fragments in the phage or phagemid vectors. The phage vector has a complete phage genome, but it is not effective for large proteins. A phagemid vector is a plasmid that contains phage coat gene (gIII or gVIII), and phage and plasmid's origin of replication. The vectors are used to transform *E. coli*. The phage vector has all the ability to produce phage particles and display the fusion antibody, while the phagemid needs to infect the bacterium with a helper phage to enable the recombinant DNA package, as a single-strand DNA into virion particles, and to display the antibody fragments [25, 28, 30]. The screening of displayed antibodies is performed by biopanning, a process in which the phages are incubated with the immobilized antigen, and the non-binding phages are removed by extensive washing. The bound phages are then eluted and enriched by reinfection of *E. coli* and thus successive rounds of selection can be carried out as many times as needed [31].

Bacterial display, an alternative to phage display, allows libraries of greater diversity. In this system, the expression of recombinant proteins is easier and the transformation by DNA is more efficient than phage display. The methodology is fast, easy to handle, and eliminates the stage of infection by the phage. The library can be displayed on the membrane in the periplasmic space or fused to the filament flagellar or fimbrial adhesin proteins [32, 33]. To generate the bacterial library, the sequences of DNA, encoding scFv or Fab fragments, are inserted into the appropriate display vector used to transform *E. coli*, the most common bacterial strain used in this technique [34]. The target antigen, adsorbed on magnetic beads or fluorophore-labeled, is used for the screening of antibody libraries by cell sorting [35].

In the cases of yeast and mammalian displays, eukaryotic systems, folding and post-translational modifications, which are relevant to the function and stability of the antibody, are more effective when compared to what occurs in the prokaryotic system [22]. Briefly, the yeast library is created by linking the antibody

gene sequence into suitable yeast display vectors. The transformed yeast by the plasmid generated is induced to express the recombinant library. The system can display scFv, Fab, or full-length antibody formats [36], that are expressed in fusion with anchor proteins of the glycosylphosphatidylinositol (GPI) family, such as α-agglutinin and a-agglutinin. The screening is performed by cell sorting [37].

When using the mammalian system, the post-translational modifications are still more effective than those possible in the yeast system. The antibody library can be usually displayed on the surface of Human Embryonic Kidney 293 T (HEK 293 T) and Chinese Hamster Ovary (CHO) cells after transient or stable transformation [38]. The antibody expressed is fused to the transmembrane domain of human platelet-derived growth factor receptor (PDGFR), which anchors the antibody on the outer surface of the cell membrane [39]. The library screening is performed as already described for other cell-surface display systems [40].

2.1.2 Cell-free display

The cell-free display libraries, unlike previously described ones, do not depend on the efficiency of transduction or transfection. Among them, the ribosome and mRNA systems have been the most described, offering around 10^{12}–10^{14} variants, a wider diversity over other display techniques, like phage (around 10^{9}), eukaryotic (10^{6}–10^{7}), and prokaryotic (10^{8}–10^{10}) systems [41].

While the ribosome display connects nascent proteins to their encoding mRNA through the generation of stable protein–ribosome–mRNA complexes, the mRNA system uses an antibiotic, puromycin, that mimics the structure of an aminoacylated tRNA, to modify mRNA that also is linked to its respective nascent protein. In a brief description, a ribosome system display construct is designed to be used with cell extracts to allow the downstream mRNA synthesis. In the construct, it must be present a ribosome binding site to the start codon where protein synthesis begins, for recruitment and pairing of the ribosome. The open reading frame is followed by the library of binding proteins and a spacer. The spacer provides flexibility to the display library in order to fold outside of the ribosome tunnel. Another important point is that the ribosome is stalled at the 3′-end by deleting the stop codon to couple the nascent polypeptide with its encoding mRNA [23, 42, 43].

The mRNA display stands out in relation to the ribosome display for offering a large degree of control over experimental conditions [41]. Briefly, the DNA antibody library is *in vitro* transcribed in mRNA. In a second step, a covalent interaction between mRNA and puromycin is produced, providing after translation the formation of the mRNA-puromycin-protein complex, which is reverse transcribed into cDNA to obtain the heteroduplex cDNA-mRNA, more stable than mRNA alone. After screening, the selected cDNA is amplified by PCR. The amplified constructs are subjected to a new cycle to obtain the mRNA-puromycin-protein complexes, and then the heteroduplexes obtained are ready for a new round of screening [41, 43].

Both methodologies enable the generation of different formats of the antibodies, including the full-length ones. The selection of the antibodies of interest is performed by binding to an immobilized antigen. Ribosome and mRNA systems have gained relevance for allowing efficient and low-cost antibody production and for their advantageous ability to screen large libraries. Although promising, so far there is no commercially available antibody generated by cell-free technologies.

2.2 Antibody design via *in silico* modeling

Advances in DNA and protein sequencing techniques associated with X-ray crystallography approaches to evaluate the antibody structure at an atomic level

and the increasing availability of the generated data in public domains provided a fundamental basis to the in-silico generation of mAbs.

Computer-assisted design of new mAbs consists of high-throughput algorithm analyses of antibody structures modeled from query residue sequences. These models are typically obtained by homology with precompiled antibody scaffold templates [44], which is possible because, despite the unique spatial identity of mAbs, the geometry of their variable regions is well conserved, with most CDR loops having a limited number of conformations, known as canonical classes [11]. In general, the established modeling tools coupled with refined protein–protein docking [45] and machine learning methods have been found useful for predicting the VH and VL domain arrangements and the potential antibody electrostatic complementary interface [46].

Examples of platforms available for antibody modeling are the "Prediction of ImmunoGlobulin Structure" (PIGS) [47], the Rosetta Antibody Modeling [48], and the "Web Antibody Modeling" (WAM) [49]. These servers comprise fully automated homology-based modules that predict with high accuracy the tridimensional antibody structure, including most of the hypervariable regions of the antigen-binding site [50]. An exception is the H3 loop. Unlike the other CDRs, the H3 structure has unique conformations that do not follow a canonical form and are also not found in any described protein, with ~75% of its fragments not having structural neighbors in the known non-immunoglobulin protein world [51]. Therefore, the H3 loop cannot be predicted by selecting templates from a database and this is an important obstacle for the *in silico* antibody design. Some alternative algorithms, based on candidate conformations obtained computationally and energy functions, have been developed, but they often fail to produce sub-angstrom structure models [50, 52] and the problem persists.

Other concerns also affect antibody modeling. The limited number of high-quality X-ray crystal structures of mAbs in public protein databases may not be sufficient to allow a proper antibody shape prediction [50]. Regarding the docking protocols, it should be noted that, despite the great advances in the bioinformatic field, most of the antibody algorithms still need to be optimized to consider the molecular backbone flexibility and the transient conformational changes following protein–protein interactions [53, 54]. Another relevant point is the time needed for antibody modeling. High-throughput computational design of mAbs can still be as time-consuming as experimental cellular approaches, even when well-consolidated prediction systems are used. As an example, the Rosetta Antibody server was previously found to take 570,000 CPU hours to generate ~2,000 antibody models [55].

With many challenges ahead, currently there are few reports of functional antibodies completely designed by in-silico approaches. A successful attempt in this field is the mAb described by Nimrod and co-workers, which was based on robust predictions of specific residue-residue interactions rather than modeling the entire antigen–antibody complex [56]. On the other hand, computational protocols have been used with increasing frequency to improve the physicochemical properties of previously generated mAbs, as well as to engineer humanized versions of murine full-length immunoglobulins, making them like those found in humans [57]. Molecular structure-based iterative algorithms have been shown to optimize the generation of humanized antibody scaffolds without a significant drop in affinity and specificity toward the antigen, compared to the original murine one, and with reduced occurrence of structure failures, important drawbacks commonly found following conventional humanization techniques, which are mostly guided by linear antibody residue sequences [58].

The overall computational antibody discovery scenario is promising and, although the design of new biologically active mAbs is still deeply dependent on

living animals, the advances in structure prediction methods set the scene for an ongoing technological evolution that should potentially lead the future generation of these molecules using only in-silico approaches.

3. Alternative methods for mAb production

3.1 Mammalian production systems

As previously mentioned, one of the major utilities of the mammalian expression systems is to produce complex biomolecules such as antibodies that require post-translational modifications like glycosylation [38]. Though other eukaryotic systems do provide this modification, their capability of doing so is limited and might result in the addition of glycans that are not common in human proteins [59]. This event might result in misfolding and biologically inactive immunoglobulins, undesirable features in human therapeutic and diagnostic monoclonal antibodies [60]. Also, expression in prokaryotic systems might lead to contamination with endotoxins, which increases downstream processes to clear these endotoxins from the final product. Thereby, the mammalian expression systems are valuable tools to produce monoclonal antibodies as well as other proteins with proper structure and activity. Indeed, there are numerous FDA-approved mAbs produced in mammalian expression systems in contrast to prokaryotic systems and other eukaryotic cells [61].

The primary technique for mAb obtention was already originally dependent on a mammalian cell: the hybridoma cell [7]. As the high specificity of the monoclonal antibodies was making these molecules increasingly useful for various applications, a hybridoma large-scale cultivation became a great demand in the industry. Therefore, the ascites method production was no longer enough to supply bulk production, nor feasible due to ethical matters. That way, most research and diagnostic proposed mAbs are now produced *in vitro,* through the harvest and following purification of mAb-enriched media obtained in dynamic or non-dynamic cell culture systems [62].

In a therapeutic context, although the hybridoma cell lines are still responsible for the generation of more than 50% of the FDA-approved mAbs [63], these antibodies are bulk produced in other mammalian host systems [64]. This is due mainly to the highly immunogenic nature of murine mAbs for humans, demanding the antibodies to be genetically modified (humanization or generation of fragments) for human therapeutic use [65]. Besides, many of these mammalian cells had their expression machinery highly optimized for recombinant protein production [66].

In the mammalian expression system, cells are readily transfected or transduced to introduce foreign DNA that codes for the target protein and then, they are cultivated preferably in suspension in a chemically defined serum-free media [59].

The preferred mammalian cell lines for protein expression in research and industrial fields are CHO and HEK-293 cells [67]. CHO cells are dominant in heterologous protein production in industry, mainly because of advantages like the property to provide complex post-translational modifications similar to those of humans, their ease to scale-up, and for being easily adapted to grow in serum-free suspension cultures [65]. CHO cells are more suited for stable expression, for its transfection renders low yields of recombinant protein secretion in this lineage. Since establishing a stable cell line is time and labor-consuming, transient transfection is a suitable option to gather high amounts of proteins in a shorter period. In this case, HEK cells might represent an interesting option, since they are well-known for being rather suitable for transient transfection. This cell line also has rapid doubling time and grows in high-density concentrations, just like CHO cells,

and presents productivity of grams of protein per liter of culture [67, 68], though they have somewhat more tendency to clump [69, 70].

The PER.C6 cells are human embryonic retinal cells, and like HEK cells are pointed out to promote human glycosylation profiles. They were projected to be grown in high-density conditions [71], with stable expression and also offer production yields similar to CHO cells, indicating that human cell lines will be more economically viable and more easily scalable options for antibody productions [72]. There is even description of a production with titers of 27 g/L of antibody, astounding yields when compared to a medium CHO cell production of around 12 g/L of antibodies [73]. Although there is still no FDA-approved mAb produced in this system, there are already some ongoing clinical and preclinical studies carried out with mAbs and other biological products purified from this system, like vaccines for influenza, HIV, and Ebola [74–77].

There are other suitable host cell lineages such as murine lymphoid cell lines like NS0 and Sp2/0-Ag14, derived from BALB/c mice plasmocytomas, corresponding for almost 25% production systems of FDA-approved monoclonal antibodies. One of their major advantages is being originated from naturally high immunoglobulin producing parental cells. Though, their murine origin is not to be underestimated, for there are reports that they do generate immunogenic glycoforms of the expressed antibodies [78].

Concerning the expression vectors for mAb production, usually the plasmids carrying the heavy and light chain genes are constructed based mainly into two kinds of systems: the dihydrofolate reductase (DHFR) system or glutamyl synthetase (GS) based system, both acting as selection markers [65]. In DHFR, selection occurs through glycine, hypoxanthine, and thymidine depletion from the cell culture medium. Selected clones are subjected to the addition of methotrexate, a folate analog that poisons the cells deficient in DHFR, obliging the cells to further synthesize the enzyme with consequent co-amplification of the IgG genes. In the GS system, the selection is done in the absence of glutamine, in a way that only cells with GS can survive by synthetizing glutamine from glutamate and ammonium. Here, the selective pressure is made through increasing doses of the GS inhibitor methionine sulfoximine, pushing the cells to amplify GS and IgG genes [79]. Promoter characteristics, inclusion of antibiotic resistance genes, transcription termination sequences [poly(A)], and translation control sequences should also be taken into account when designing these vectors [80].

In comparison to other production systems, mammalian cells are more fastidious to culture than bacteria and fungi, for they are larger and do not possess tough cell walls like other microorganisms, making them more sensitive to impurities naturally occurring from the production system itself. Having them to thrive and reproduce in culture after modifications to turn them stable and in conditions to secrete the aimed molecule with high yields is a challenge in itself [61, 67]. If one is not choosing for the transient transfection, having the stable lineages may also be costly and time-consuming.

Independent of the expression system, the correct choice of the production scale should be made accordingly to the given necessity. The simplest culture system is the static culture, consisted of T bottles with screw caps kept horizontally in an incubator. Because of its low maintenance profile and low costs, it is the most widely used culture method in the academic research context. It is possible to use this system for clone screening and determining experimental conditions, but its small-scale nature might not render enough mAb quantity for some other types of assays. An option to circumvent this matter might be the use of the rolling systems that offer a medium-scale mAb yield. In this condition, roller bottles are positioned in a rotation system that causes all cells to be in constant movement, and therefore,

all of the components of the culture (cells, nutrients, dissolved gases, and metabolites) are uniformly distributed throughout the volume of the medium. This system requires gradual adaptation to cell growth in suspension, starting from very low rotation speeds [81–83].

Currently, the bulk production of mAbs in agitated bioreactors is the predominant cell culture system in the industry because it allows constant control and monitoring of the process. The area of research for innovation in these bioreactors has advanced dramatically. In general, bioreactors are used to achieve high cell densities and thus increase the production of monoclonal antibodies, biopharmaceuticals, and vaccines [84]. Different types of agitated bioreactors have been used for the cultivation of mammalian cells, both on a pilot and industrial scales.

3.2 Bacterial and yeast production systems

The use of microorganisms such as bacteria and yeasts is widely used in science for several purposes, generally related to antibiotics and probiotics [73]. However, both bacteria and yeasts have been getting space in the production of mAbs for immunological therapy due to the biopharmaceutical demand and technological advances about their ability to produce antibodies by reducing the use of animals in the manufacturing process [73]. The motivation behind investments that seek to optimize the means of production of mAbs in alternative models stems from the manufacturing disadvantages presented in the traditional method with mammalian cells, which have been predominantly employed in the expression of these antibodies due to their ability to introduce post-translational modifications similar to those human cells [85]. The mammalian expression system is expensive and time-consuming, and efforts have been made to express them in different systems. Microbial cells of yeasts and bacteria have many advantages, such as typical rapid growth, low cultivation costs, and genetics well known in the literature [86]. Microorganisms can produce high molecular weight compounds like proteins, perform highly selective reactions by their native enzymatic machinery, and also allow the repeated introduction of enzymes or immobilized cells [87]. In addition, finally, processes that use microorganisms do not generate organic and inorganic pollutants, such as mercury and toluene [88]. Still, it was complicated to produce complete antibodies in prokaryotes to the detriment of the insecurity of microbial products for human use [73]. Fortunately, the FDA published a special set of rules called "Generally Recognized as Safe" (GRAS), which guarantees the human safety of microbial products and the production of monoclonal antibodies [89]. Thus, several microorganisms were explored. In the case of gram-negative bacteria, *Escherichia coli* stands out, once it has two compartments for protein expression - the cytoplasm and the periplasmic space [86]. Gram-negative bacteria also have an oxidizing environment which allows the correct formation of disulfide bonds [90].

Whole antibodies can be produced in bacteria and this process is dependent on periplasm, which is an essential region for folding the proteins and chains that make up the structure of antibodies. Unfortunately, studies reveal very low levels of periplasm, which limits the yield for mAbs production [91]. Efforts to produce antibodies in the cytoplasm have not been successful until recently [92]. Gram-positive bacteria are more advantageous than gram-negative bacteria because they do not produce endotoxins - a highly immunogenic lipopolysaccharide (LPS) produced by gram-negative bacteria. Fewer complex eukaryotes such as yeasts have also been exploited for the production of mAbs. They have the advantage over prokaryotes in similarity with the mammalian protein expression system, allowing the expression and folding of complex proteins more easily, and yet, as well as gram-positive bacteria, do not produce endotoxins [93].

Among yeasts, *Pichia pastoris* and *Saccharomyces cerevisiae* dominate the field in the production of antibodies [86]. *S. cerevisiae* is promising due to the advantage of being well characterized, but the correct folding of chains and proteins and low yields are problems to be faced. On the other hand, *P. pastoris* does not secrete many endogenous proteins that need to be removed in the mAbs production process [94]. Yeasts have cellular glycosylation machinery, however, their proteins exhibit types of glycosylation completely different from human proteins, and this results in a significant reduction in therapeutic effector functions [95]. Whether from yeasts or bacteria, native full-length mAbs need to be glycosylated during their synthesis, but this is an obstacle that has yet to be overcome for better production efficiency in microbial hosts. The glycosylation status of the Fc region is critical for the recruitment of serum proteins from the complement system and the destruction of target cells by complement-dependent cytotoxicity (CDC) cascades [95]. This is the main reason why the method of producing mAbs in mammalian cells is still the most applied [91]. Until 2020, there are 151 recombinant therapeutic proteins approved by the FDA, one-third of them are mAbs but there are many other mAbs under development. Among these mAbs, only two are antigen-binding fragments (Fabs) that are produced in the periplasm of the bacteria *E. coli*: ranibizumab and certolizumab pegol. The first, ranibizumab, approved in 2006, is an IgG1 Fab fragment used to treat neovascular age-related macular generation and macular edema after retinal vein occlusion. Certolizumab pegol is also a humanized Fab fragment, approved in 2008 for the treatment of Crohn's disease and rheumatoid arthritis. Therefore, since the advent of mAb therapy, the biopharmaceutical industry has been investing considerable resources in new bioprocesses for the manufacture of glycosylated antibodies that attach human IgG-like glycans through alternative host expression [95].

3.3 Plant-based antibody production systems

To produce antibodies in plants, a transformation is mediated by a bacterium that infects plants, called *Agrobacterium*. The bacterium then loads the expression vector with the antibody gene, thus generating the transgenic plants that express the desired antibody. The transformed Agrobacterium is inoculated into the leaf slices of the plants. These slices regenerate in 3–4 weeks. Small shoots are then formed from the callus and transferred to a plant cultivation box *in vitro*. For the production of biomass, *in vitro* transgenic plants are transferred to a soil pot and grown in a greenhouse [96]. The most used plant systems are tobacco and alfalfa because they are the most accessible and common sources of leaf biomass. Tobacco has great advantages, such as high leaf biomass yield and rapid scaling up through easy seed production compared to other plant species. However, tobacco contains nicotine and other toxic alkaloids that need to be removed through an additional extraction step [97].

The plant system offers important advantages, such as high production capacity, low cost in the large-scale cultivation process, in addition to avoiding ethical problems associated with animals [98]. Another important advantage of using this system is found in post-translational protein modifications, which occur in plant cells in a similar way to animal cells, as well as in the correct assembly of complex molecules, such as antibodies, are aided by chaperones that mediate folding and the formation of disulfide bonds, while the addition of N-glycans is carried out by specific cellular glycosyltransferases. In fact, while core N-glycans are similar in plants and mammals, complex N-glycans show substantial differences with sialic acid [99–101].

In addition, there is a possibility to design a custom antibody glycosylation profile, and production can be enlarged simply by increasing the number of plants [102]. In comparison with the systems described earlier, the use of plants for the production of antibodies offers several irreplaceable benefits. Plants are

widespread, abundant, and develop more quickly because they normally mature after a growing season. It is possible to put the product on the market quickly, which ends up decreasing the cost of production. Plants also reduce screening costs for bacterial toxins, viruses, and prions because they are less likely to introduce animal pathogens than mammalian cells or animals [98].

The disadvantages of this system are found in the low yield of protein expression, the downstream processing problems related to the extraction of proteins from leaves, and some regulatory obstacles [103].

The first pioneering study on the production of full-size IgG in plants dates back almost 30 years ago [104]. Since then, different antibody formats have been expressed in plants, such as IgA, Fab fragments, minibodies, and scFvs [103]. The first drug from plant cells to receive FDA approval for human use was the enzyme β-glucocerebrosidase, commercially called ELELYSO, indicated for the treatment of patients with a confirmed diagnosis of Type 1 Gaucher disease [105]. Thereafter, Medicago Inc. developed a quadrivalent plant-derived seasonal influenza vaccine that recently completed Phase III clinical trials [106]. A study published in September 2020 positively demonstrated the expression of a scFv 13F6 antibody with binding activity against Ebola virus-like particles in a plant system [107, 108]. Of the antibodies produced by plants, there are already 6 against viruses, 5 against tumors, and 3 against bacteria [97].

Therefore, given the data presented and the clear advantages, we can say that the plant system is quite efficient and may, in the future, be widely used in the production of antibodies both in basic research and on an industrial scale.

4. Conclusions

Bearing in mind that obtaining high specificity and affinity mAbs is not trivial, there is a great race to develop methodologies that can meet the most varied demands. An overview of the main technologies clearly shows that the total replacement of animals' use in the generation and production of mAbs is not possible for the moment. We believe that this will only be reached when the in silico technology is fully dominated. But as the implementation of alternative methods must be seen as a process, reducing and refining the use of animals are achievements. Thus, the different types of antibody display libraries represent a major breakthrough. As described, the source of genes for building the libraries may imply greater or lesser use of animals and only synthetic display libraries completely dispense the use of animals. In the same way, for the production of mAbs, several possibilities are currently available. The important thing in the production stage is that the use of ascites, a proceeding that brings pain and stress to animals, may already be eliminated in most cases. Invariably, the purpose and amount of the mAb to be produced will determine the choice of obtaining and production methodologies. Given the great utility and diversity of mAb uses, ranging from therapeutic application to essential research tools, and the wide range of technologies available today for obtaining and producing them, it seems a fact that it is always possible to choose or design a path that meets the concept of 3Rs.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

T.P. and F.C. wrote the "Overview of antibody structure and most common formats" section; J.O. and J.M. wrote the "Antibody display libraries" section; R.A. wrote the "Antibody design via in silico modeling" section; B.H., G.S., and C.B. wrote the "Mammalian production systems" section; E.S. wrote the "Bacterial and yeast production systems" section; C.B. wrote the "Plant-based antibody production systems" section; and J.M. drafted the "Conclusions" section. All authors contributed critically to the chapter preparation.

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