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Engineering antibody fragments: replicating the immune system and beyond

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La ingeniería de fragmentos de anticuerpos: imitando y expandiendo el sistema inmune

***Abstract*—**Since genetic engineering of humanized murine monoclonal antibodies was first demonstrated over two decades ago, antibody engineering technologies have evolved based upon an increasing understanding of the mechanisms involved in antibody generation *in vivo*, and a constant search for alternative routes to evolve and exploit the characteristics of antibodies. As a result, antibody engineers have devised innovative strategies for the rapid evolution and selection of antibodies and novel antibody designs (i.e., antibody fragments). Phage display, cell display and ribosome display technologies, which comprise the core of the currently available technologies for the discovery and preparation of such antibodies, are reviewed herein. This article intends to communicate the state-of-the-art technology available for the engineering of antibodies to a general readership interested in this important field. Therefore, important immunology concepts are introduced before detailed descriptions of the three antibody engineering technologies are presented in later sections. A comparison of these methodologies suggests that despite the predominance of phage display for the engineering of antibody fragments in the past 20 years, cell display and ribosome display will likely gain importance in the selection and discovery of the antibody fragments in the future. Finally, these technologies are likely to play an important role in the production of the next generation of antibody-based therapeutics.

***Keywords*—** Antibody engineering, Phase display, Cell display, Ribosome display, Antibody humanization.

***Resumen*—**Las tecnologías para la ingeniería de anticuerpos han evolucionado durante las últimas dos décadas, desde la demostración de la posibilidad de humanizar anticuerpos monoclonales de ratón mediante ingeniería genética, apoyadas en el creciente entendimiento de los mecanismos involucrados en la generación de anticuerpos *in vivo*, y en una búsqueda constante de rutas alternativas para evolucionar y explotar sus características. Es así como los ingenieros de anticuerpos han desarrollado estrategias innovadoras para la evolución y selección de anticuerpos y de novedosos diseños de anticuerpos conocidos como fragmentos de anticuerpos. Esta revisión se enfoca en tres tecnologías que comprenden el núcleo de las tecnologías actualmente disponibles para el descubrimiento y preparación de tales anticuerpos: la presentación en fagos, la presentación en células, y la presentación en ribosomas. Este artículo busca presentar el estado del arte de estas tecnologías a un grupo general de lectores interesados en este campo, por lo que inicialmente se introducen importantes conceptos de inmunología requeridos para comprender en detalle las tecnologías discutidas. Una comparación de estas metodologías para la ingeniería de anticuerpos sugiere que a pesar del dominio de las tecnologías basadas en la presentación en fagos durante los últimos 20 años, en los próximos años la presentación en células y la presentación en ribosomas probablemente ganarán importancia para la selección y descubrimiento de fragmentos de anticuerpos. Finalmente, es probable que estas tecnologías jueguen un papel importante en la producción de la siguiente generación de terapéuticos basados en anticuerpos.

***Palabras clave*—** Ingeniería de anticuerpos, Presentación en fagos, Presentación en células, Presentación en ribosomas, humanización de anticuerpos.

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I. Introduction

Most recent reviews in the field of antibody engineering have examined in great detail the dynamics of the clinical transfer of antibody engineering technology developed for therapeutic purposes. Substantial emphasis has been placed on the characteristics of the antibodies being used, their targets and mechanisms, and the opportunities and challenges for the continuous progress of the field, particularly the remaining limitations of the state-of-the-art technology for antibody production [1-4]. Because of this emphasis, previous reviews have been directed toward a relatively specialized audience of antibody engineers in need of constant feedback on the increasing number of antibody-based therapeutic strategies under clinical trials, since the outcome of these trials significantly affects new research initiatives and thus the evolution of the field. However, the possibility to engineer human antibodies and novel related proteins against virtually any target has broad biomedical impact, providing for a means to neutralize (i.e., render inactive through antibody binding) key soluble proteins or receptors involved in the onset or progression of disease (e.g., chronic inflammation, cancer), or develop a means to target and release additional therapeutic cargos to specific cell populations (e.g., cancer cells) in the body. Hence, this short review article is aimed at a more general readership, who may have an interest in this technology but may not be acquainted with the immunology concepts required for understanding the relevant literature in this field. This review surveys the current technologies for engineering antibodies with a focus on the methodologies for developing antibody fragments and novel engineered proteins inspired by the structural components of complete antibodies. These novel technologies provide an important alternative to traditional antibody-based technologies and are often better suited for certain biomedical applications than conventional monoclonal antibodies.

II. Key immunology concepts

This section introduces important immunology concepts essential to understanding antibody engineering strategies, their rationale, relevance, challenges and limitations. In some cases, the *in vivo* processes are contrasted with their engineered counterparts, although additional analogies will become evident throughout later sections of the article. These concepts may lie in any of three categories: (i) antibody structure and (ii) function, and (iii) diversity of the immune repertoires. Figure 1 summarizes basic information regarding antibody structure and function, and Fig. 2 and Table 1 detail the concepts related to antibody diversity.

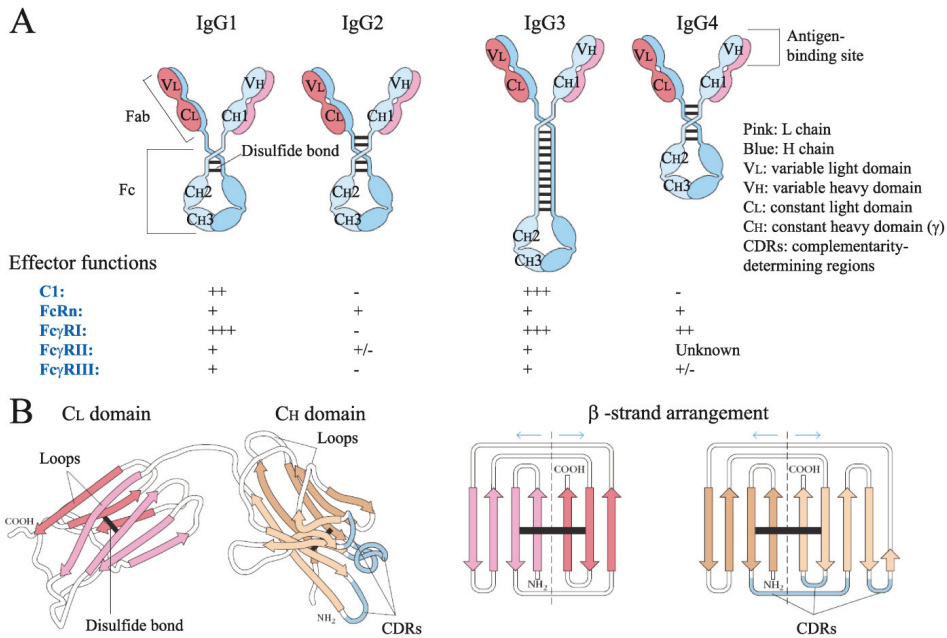

Fig. 1. **Structure and folding of immunoglobulins.** (A) Schematic of the general structure of the four immunoglobulin G (IgG) isotypes, the predominant immunoglobulin class used in antibody engineering, which differ in the number and arrangement of disulfide bonds and the heavy-chain component (γ1, γ2, γ3 and γ4, respectively; not shown in figure). The effector functions of each subisotype are indicated, since the ability to activate different receptors present in immune cells (i.e., effector functions mediated by Fc gamma receptors) plays a critical role in isotype selection for antibody engineering, and removal of constant domains can also prevent complement activation (e.g., C1) and other immune responses [5]. (B) Folding of an immunoglobulin light chain depicting the β-pleated sheet structure in each domain, the conserved disulfide bond and the localization of the hypervariable regions (CDRs) in three loops joining β-strands of the variable domain. Images modified from Goldsby *et al.* 2003 [6].

Antibodies, or immunoglobulins, are heterodimers composed of two identical light (L) chains and two identical heavy (H) chains. One light chain is covalently linked to one heavy chain by a disulfide bond, and the resulting H-L structures are joined as a dimer of dimers (i.e., H_2L_2) by additional disulfide bonds between heavy chains (Fig. 1A). The heterodimeric structure is further stabilized by non-covalent interactions, such as hydrophobic interactions, hydrogen bonds, and salt-linkages. Early investigations into the structure of antibodies using enzymatic digestion helped to elucidate the Y-shaped structure of antibodies. Digestion with papain resulted into two antigen-binding fragments (Fab) and one crystallizable fragment (Fc), while digestion with pepsin resulted in a single antigen binding fragment comprised of two antigen-binding domains ($F(ab')_2$) [6].

Genetic analysis of antibodies isolated from human subjects provided further understanding of the immunoglobulin structure and variability. The first 110 amino acids of the N-terminal segments of H and L chains are highly variable sequences called the V_L and V_H domains, which account for most of the differences in specificity displayed by native antibodies [6]. The unique sequences of the V_L and V_H for a given antibody determine its idiotype (i.e., antigenic determinants). The cleft between a V_L and V_H chain is the antigen binding pocket, and the specificity of antibody-antigen binding is predominantly controlled by 6 segmented, hypervariable loops called the complementarity-determining regions (CDRs) that extend from a highly ordered β-pleated structure characteristic of the immunoglobulin folding (Fig. 1B). While the CDRs are primarily responsible for antigen specificity, the whole variable domain serves as a scaffold for the correct presentation of the binding site, and mutations along its sequence also influence, to a minor extent, antibody affinity [4,6].

The remaining amino acids of the H and L chains are highly conserved regions known as constant domains (C_H or C_L). Heavy chains have 3 to 4 C_H domains, whereas L chains have a single C_L domain (Fig. 1) encoded by one of two light-chain genes, kappa (κ) or lambda (λ). The class of an antibody is determined by its heavy chain, of which there are five different chains or isotypes: α, δ, ε, γ and μ. Immunoglobulin G (IgG) is made up of two γ heavy chains and is the most abundant (~80% of total serum immunoglobulin) and most studied immunoglobulin class for antibody engineering (Fig. 1A). The structure and functions of the other immunoglobulin classes (i.e., IgA, IgD, IgE and IgM), which play important roles in adaptive immunity, will not be discussed due to their minor role in current antibody engineering applications. Subtle amino acid differences encoded in the C_H germ-line genes lead to a further division of isotypes into subisotypes or subclasses. In humans, for instance, there are four subisotypes of γ heavy chains (γ1, γ2, γ3, and γ4) with 90-95% homology between their genes. Additionally, different members of the same species may have multiple alleles for the same isotype genes, which determine the antibody allotype.

The isotype and subisotype of an antibody strongly impact the structure and effector functions of the Fc region. Because of this, the selection of the isotype is relevant for engineering antibodies, since different applications may require the mediation of different effector functions or, even more, their absence [4,5]. The existence of different Fc regions modulates the binding to specific Fc receptors found in immune effector cells —Fc gamma receptors (FcγR) in the case of IgG—, which trigger different effector functions upon binding of the antibody-antigen complexes, such as complement activation (component C1), antibody-dependent cell-mediated cytotoxicity (ADCC), opsonization (phagocytosis by macrophages and neutrophils) and transcytosis (crossing of epithelial layers). In the case of IgG, the Fc region also has the ability to bind to the neonatal Fc receptor (FcR_N), which plays a critical role in the regulation of IgG pharmacokinetics, since the binding to the FcR_N constitutes a salvage mechanism that recycles IgG and therefore allows for prolonged serum half-lives. Despite the importance of the Fc fragment in the modulation of effector functions, and although it is amenable to tailoring antibody pharmacokinetics (i.e., select antibodies with increased affinity to FcR_N) and has the ability to trigger specific effector functions (i.e., ADCC to tumor cells expressing the target antigen), the antibody engineering technologies discussed in this article focus on the antibody-antigen interaction, and are optimized and selected in formats devoid of Fc regions [2,7]. However, it should be noted that the modularity of the antibody structures also allows for the grafting of Fc regions into optimized antibody fragments (e.g., variable regions), although this usually requires the expression of the antibody fragment in eukaryotic expression systems [8].

The ability of the immune system to generate antibodies against virtually any antigen depends on its ability to generate a sufficient number of antibodies that can be selected based on their affinity for binding the antigen. The mechanisms involved in the generation of such diversity span different levels of cell physiology and are tightly associated with the maturation and differentiation of B cells, which are responsible for their production and secretion *in vivo* [6]. The main mechanisms involved in the generation of antibody diversity, as depicted in Fig. 2, are further explained in Table 1, which account for the tremendous diversity ($>10^{10}$) of the immune repertoire. In addition, the role of these mechanisms or their analogues in generating antibody diversity in existing antibody engineering technologies is indicated.

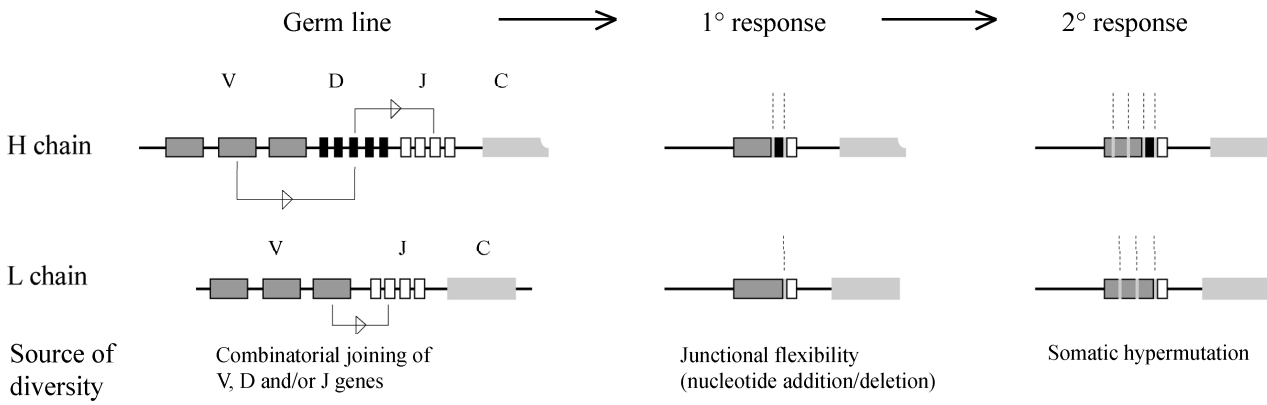

Fig. 2. **Rearrangement of immunoglobulin genes responsible for generating antibody diversity.** The cartoon depicts the three main sources of variation resulting in the antibody repertoire diversity: combinatorial joining of the germ line V, D, J (H chain) or V and J (L chain) segments; imprecise joining of the coding sequences (junctional flexibility) and random addition and deletion of nucleotides at the joint between segments; and finally somatic hypermutation along the VJ and VDJ regions for affinity maturation during a T-cell-dependent secondary immune response [9]. Dotted (vertical) lines indicate non germ line encoded residues.

Table 1. Principal sources of antibody diversity in humans. The overall diversity is believed to exceed 10^{10}. Analogue mechanisms, such as error prone amplification of the variable regions are harnessed for the generation of diversity in synthetic and semisynthetic antibody libraries. Similarly, all the mechanisms below account for the diversity available in *in vivo* models for antibody generation, such as transgenic mice expressing repertoires of human antibody genes [6,9-11].

Source of variation	Mechanism	Calculated diversity	Role in antibody engineering
Combinatorial V-J and V-D-J joining	Heavy chain: combinations of 51 V_H gene segments, 27 D_H and 6 J_H segments. Light chain: combinations of 40 V_L and 5 J_L kappa chains; and of 30 V_L and 4 J_L lambda chains.	8262 for Heavy chain and 320 for Light chain.	*In vitro* combinatorial assembly of the naïve immune repertoire (V-J and V-D-J segments). Construction of synthetic libraries with a subset of V_H and V_L gene families.
Junctional flexibility	Imprecise joining of the coding sequences during recombination of the gene segments.	Undetermined	N.A
P-nucleotide additions	Variation in the sequence of the coding joint due to imprecise cutting of the hairpin structure formed during the initial recombination process, leaving a single strand at the end of the coding sequence. A repair enzyme adds complementary nucleotides to this strand forming a palindromic (P) sequence.	Undetermined	N.A
N-nucleotide additions	Random nucleotides added during the D_HJ_H and V_H to D_HJ_H joining process by a terminal deoxynucleotidyl transferase (i.e., addition of residues not encoded in the germ line genes).	Undetermined	N.A.
Somatic hypermutation	Mutations along the whole VJ and VDJ segments, although the mutations are usually concentrated in the CDR regions probably due to their major contribution to the affinity maturation of the antibodies. The process occurs at a frequency ~ 10^3 per base pair per generation.	Undetermined	Error prone amplification of the variable regions. Site-directed mutagenesis at the CDRs. Combinatorial ligation of CDR-encoding regions. [Important for affinity maturation].
Possible combinatorial association of heavy and light chains	Combinatorial association of 8262 heavy chains and 320 light chains.	$2.64x10^6$	Direct amplification of the antibody repertoire (assembled genes) from immunized animals by RT-PCR. Ligation of synthetic variable light and heavy genes.

III. Engineering antibody fragments

The modular structure of antibodies has enabled the customization and engineering of high affinity binders in a variety of ways. Before discussing the technologies developed for the design and discovery of antibody fragments, the available antibody fragment formats are presented, since those technologies, as will be noted in section IV, are only suited for particular antibody formats.

Figure 3 depicts the available battery of antibody fragments derived from the parental IgG structure. The seminal work on the engineering of these new sets of antibody formats was conducted on Fab fragments —comprised of one antigen binding site of an IgG (V_H-C_H+V_L-C_L)—, and on single-chain variable fragments (scFv), a further simplification of the Fab structure achieved by removing the constant domains and linking the V_H and V_L fragments with a peptide linker [1]. The scFv format rapidly popularized, and is probably the most widely used antibody fragment today, mainly due to the advantages of directly linking the heavy and light domain genes. Linking these domains at the genetic level not only simplified the recombinant DNA methods involved in their processing, but significantly increased the stability of the structure and eliminated the folding problems encountered with prokaryotic expression systems (e.g., E. coli) during selection and production of antibodies with disulfide bonds [2,8]. Interestingly, the incorporation of the peptide linker and the variation of its length has been found to control the dimerization properties of the scFv fragments, with shorter sequences resulting in increasing valency (diabody, triabody and tetrabody formats have been produced). The absence of linker, which prevents the self-folding of the V_H and V_L domains of one scFv promotes the formation of bispecific scFv by noncovalent interactions between the variable domains of a second scFv [3].

The maximum simplification of the antibody structure, known as domain antibody (dAb), consists of a single V_H or V_L domain (i.e., only 3 CDRs). The initial attempts to derive high affinity binders using dAb were not encouraging, resulting in the selection of fragments displaying significant decreases in binding affinity, but most importantly, poor stability and a tendency to aggregate [1]. Nevertheless, the finding of dAb naturally occurring in camels, which displayed high affinity and stability, inspired the design of new dAb circumventing these problems, in a process termed "camelization" [12]. Despite the success of the camelization approach, the therapeutic applications of such antibodies were limited due to the potential immunogenicity associated with using non-human scaffolds in the variable region design [2]. Only recently, Winter and coworkers, in their efforts to characterize a set of dAbs produced against hen egg lysozyme (HEL), discovered an antibody domain displaying similar properties to those found in camel and llama V_HH Abs but without recurring to camel-based scaffolds (i.e., camelising mutations). The same group also devised a methodology for the generation of equally stable and aggregation-resistant domain antibodies [13]. One important realization of this work was the increased understanding of the role of the CDRs in determining the thermodynamic stability, as well as expression and purification yields, of antibodies [11,12].

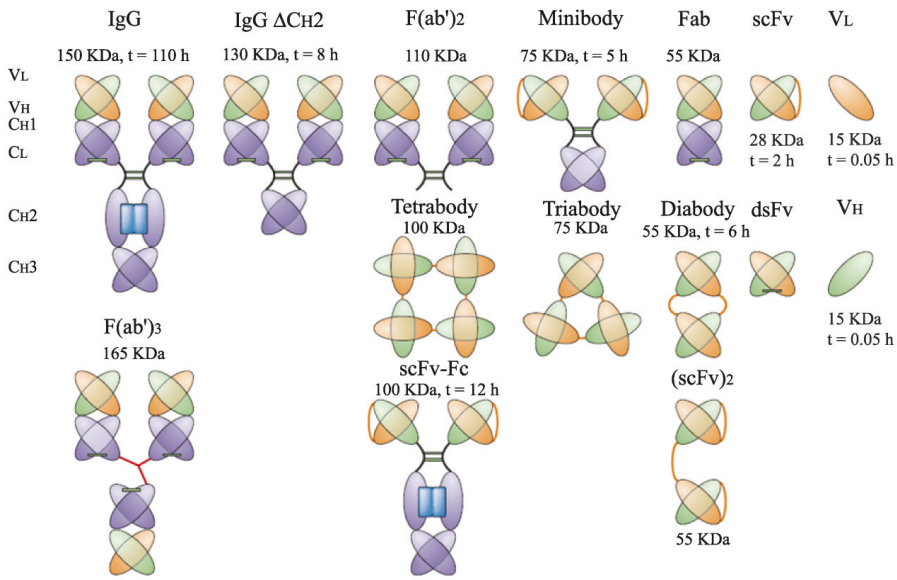

Fig. 3. **Schematic of the most common engineered antibody fragments.** The molecular weight (MW) of the fragments varies from 15 KDa for the light and heavy variable domains, V_L and V_H respectively, with serum half-lives (t) of 0.05 h, through 100 KDa in single-chain variable fragments (scFv) with a crystallizable fragment (Fc), scFv-Fc, and a half-life of 12 h, to 165 KDa in the trispecific Fab (antigen binding fragment), F(ab')$_3$. dsFv: disulphide-stabilized scFv. Image modified from Carter, 2006 [3]; pharmacokinetics and MW data was taken from Holliger and Hudson, 2005 [2].

IV. Antibody engineering technologies

The discovery of the hybridoma technology in 1975 enabled the production of monoclonal antibodies (mAbs) and paved the way for the evolution of the Antibody Engineering field [14]. The therapeutic potential of such technology became evident in 1984, when Winter and collaborators demonstrated an ability to form chimeric antibodies with murine antigen-binding domains and complete human effector functions (i.e., Fc region). In 1986, the same group developed the groundbreaking antibody humanization technology, which involves transferring the CDR regions of a murine monoclonal antibody into a human immunoglobulin scaffold, significantly reducing the immunogenicity issues associated with murine antibodies [15]. Current antibody engineering technologies have surpassed many of the challenges imposed by the selection of antibodies using murine cell lines (i.e., hybridoma technology), eliminating the need for humanization by enabling the production of fully human antibodies *in vitro* or in other engineered animal models. Hence, the technologies presented in this section will focus on these alternative methods for the selection and production of human antibody fragments.

4.1 Antibody libraries

As explained in section II, the diversity of the immune repertoire is critical for the successful isolation and production of high affinity antibodies [16]. Indeed, library characteristics, such as size (overall diversity) and quality (i.e., number of functional combinations), dictate the ability to express relevant antibody fragments against a particular antigen [1,17,18]. Therefore, the screening technologies presented in the next section are strongly dependent on the characteristics of the antibody library being used.

Due to the complexity of the immune repertoire, the initial approaches for the construction of antibody libraries followed a simple strategy: the amplification of assembled antibody genes after mice immunization by means of RT-PCR using a set of primers designed for the amplification of all antibody genes and based on the variable region frameworks (already known by the time and deposited in data bases: Kabat and V-base database) [19]. However, this approach still used mice for the generation of the assembled antibody genes after immunization, therefore presenting only partial advantages. An additional level of complexity was included by applying a similar strategy for the amplification of naïve libraries (i.e., gene segments before recombination) from non-immunized animals followed by *in vitro* combinatorial assembly of the antibody repertoire [10].

Despite these significant advances, the antibody fragment screening and production technologies relied on non-mammalian systems, which suffer from inadequate expression levels and other problems derived from differences in codon usage. As a result, the development of semisynthetic and later of fully synthetic human antibody libraries represented an important achievement for the antibody engineering field. These libraries can now be optimized for expression according to the selection technology and desired expression system, have modular designs that allow relatively easy interconversion between different antibody formats, and significantly simplify laborious DNA manipulation steps. In addition, synthetic libraries are not limited by the bias introduced in germ-line repertoires throughout evolution, such as the tolerance mechanism against selection of self-antigens, and therefore enable, at least in theory, the discovery and selection of antibodies with no representation in natural immune repertoires [4,20].

Figure 4 presents the designs of the most advanced human synthetic libraries currently available, which are known as Human Combinatorial Antibody Libraries (HuCAL) [17,21]. Initially introduced in 2000, this synthetic library implemented innovative concepts for the generation of diversity, including diversity not only in the CDRs but also in the framework regions, which are known to play a role in CDR conformation. In addition, the diversity introduced in the CDR libraries is biased towards sequences predominant in the human immune repertoire (by using trinucleotide cassette mutagenesis), which facilitates the selection of antibody fragments with minimal or no immunogenicity (human anti-human antibody, HAHA) [9,16]. It is worth noting that the diversity in the V_H and V_L gene families, as well as the families selected, were carefully analyzed by bioinformatics means to achieve sufficient diversity while preventing excessive complexity of the library. Indeed, this library only uses 7 master genes for heavy chains and 7 genes for light chains corresponding to consensus sequences for seven V_H and seven V_L germ-line families which were found to account for more than 95% of the human antibody diversity observed *in vivo*. The library was initially developed in scFv format, but is now also available for Fab fragments [10]. Some characteristics of the newest versions of the HuCAL library (Fig. 4B and 4C), HuCAL Fab 1 and HuCAL GOLD, that require special attention are: (i) the absence of cysteine residues in the constant domains (eliminated to avoid problems during expression), (ii) that only Fd (V_H + C_H) is covalently attached to pIII (for phage display, reviewed in next section), so that the system depends on the non-covalent interactions with the light chain, and (iii) the absence of cysteine residues in the CDR regions in the HuCAL

GOLD library (to avoid problems with the CysDisplay™ technology). Although (ii) may be complicated by light chain exchange in a given phage preparation, thereby losing the linkage of genotype to phenotype, the authors claim that after extensive use of the library this non-covalent interaction proved very stable [20,21].

Two powerful technologies for screening antibody libraries and selecting antibodies of high affinity for a particular antigen involve the display of one (monovalent) or several (multivalent) antibody particles on the surface of either phage virion, phage display (section 4.2), or on the cell surface (i.e., cell wall, cell membrane) of a prokaryotic or eukaryotic host, cell display (section 4.3).

4.2 Phage display

Phage display is a powerful biomolecular engineering technique for selecting high affinity binders to biologically relevant targets by several rounds of affinity selection. Foreign DNA encoding recombinant peptides or proteins is fused to coat protein DNA of bacteriophage such that recombinant molecules will be expressed and displayed on the outer surface of the phage. This strategy effectively links the protein phenotype and genotype (i.e., the corresponding DNA carried by the phage) thereby enabling the simple identification of the selected proteins at the DNA level. In the seminal publication of phage display [22], fragment genes of the endonuclease EcoRI were fused to the gene III protein (g3p) of *fd* filamentous phage to produce "fusion phage" capable of yielding peptides with 1000-fold higher affinity for anti-EcoRI antibody. Winter's group then demonstrated the possibility to display functional antigen-binding sites on the surface of these phage particles for their evolution [23,24], and subsequently contributed to the seminal work on the construction of large phage antibody libraries [25]. By linking phenotype to genotype, vast libraries of phage (10^9-10^{12} clones) displaying different fusion proteins can be assembled, selected for with simple affinity techniques (Fig. 5A), and quickly identified by conventional DNA sequencing [26]. Marks *et al*. (1991) prepared a library of scFv genes from peripheral blood lymphocytes isolated from unimmunized human donors by RT-PCR, which contained randomly generated heavy and light chain variable fragments. After affinity selection, phage displaying scFv demonstrated affinity for their target

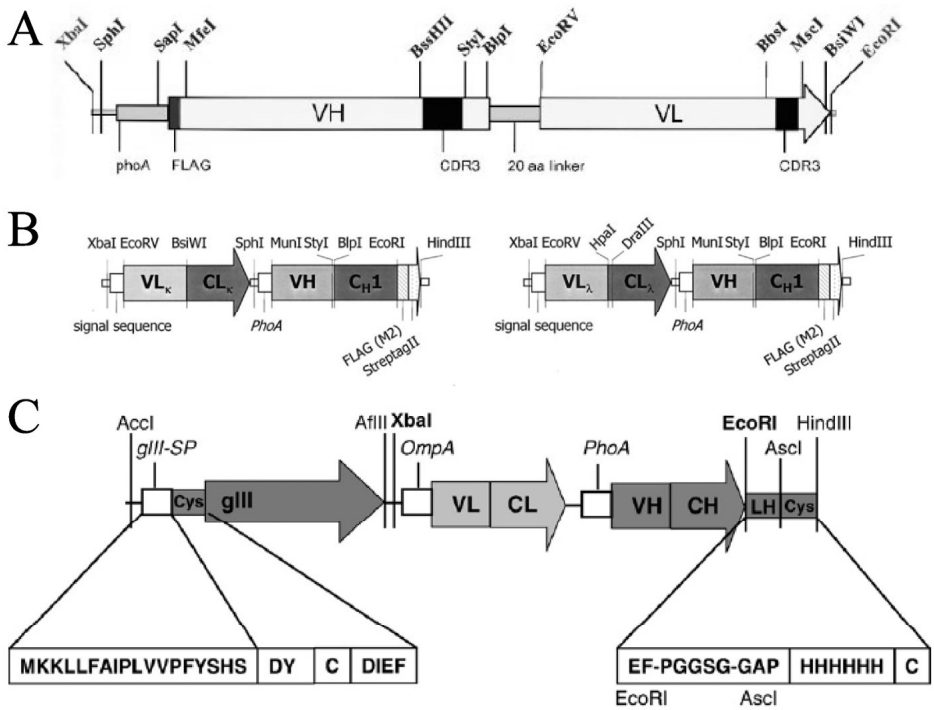

Fig. 4. **Modularity and diversity in synthetic human antibody libraries.** (A) General format of the 49 scFv master genes comprising the Human Combinatorial Antibody Libraries (HuCAL) scFv (V_H-V_L orientation), where the scFv cassette is preceded by a phoA signal sequence (reporter alkaline phosphatase for screening and expression purposes) and a FLAG tag (purification purposes), and the V_H-V_L domains are fused by a peptide linker; diversity is further incorporated by pre-built CDR3 cassettes libraries yielding a library size of $2x10^9$ (~61% functional sequences). (B) HuCAL- Fab 1 library generated from the original HuCAL scFv library, with all master genes in Fab format and library size of $2.1x10^{10}$ (~67% functional sequences). (C) Most recent version of the HuCAL library, HuCAL GOLD, incorporating diversity in all six CDRs and adapted for antibody selection by CysDisplay™. Images reproduced from (A) Knappik *et al*. 2000, (B) Rauchenberger *et al*. 2003 and (C) *Rothe* et al. 2008 [10,20,21].

on par with the affinity of native antibodies for the same target, indicating the utility of phage display for bypassing immunization and producing high affinity binders. Today, phage display constitutes the most developed *in vitro* system for the selection of antibody fragments [4,27].

Filamentous phage (Ff), bacteriophage T4, and phage λ are the three general types of phage used for phage display. Initial bacteriophage studies used filamentous M13 phage to amplify and isolate single-stranded DNA [28], which led to M13-based plasmid constructs (M13mp18/19) that have become the basis for most phage display systems [29]. While M13-based systems are the most developed, bacteriophage T4 and phage λ systems have shown equal promise. Bacteriophage T4 are able to house significantly larger quantities of DNA [30], and phage λ display has shown increased ability to incorporate larger proteins and cDNA libraries compared with Ff phage display [31].

Phage display has also been used for epitope mapping [32] and discovery [33], identifying new receptor-ligand pairs [34] and drug discovery [35]. In each case, multiple rounds of selection with increasing stringency should yield a small set of champion peptides. The hope, and ultimate goal, of phage display is that a consensus sequence from selected peptides will emerge. Often, the consensus sequences are non-obvious amino acid sequences that could not have been predicted by rational design methods [22]. The success of phage display depends on understanding phage biology, methods of display, random peptide library limitations, and affinity selection schemes.

Phage display methods are named depending upon which phage protein has been fused, how many copies of the fusion are present in one phage particle, and what type of phage vectors are employed, phage and/or phagemid vectors [26]. One important characteristic of the phage display technology is the control it offers over valency of display, since this parameter determines the avidity—functional affinity determined by the number of binding sites—of the selection strategy and significantly affects the affinity of the selected clones. For instance, high valency often results in antibodies with moderate affinity, since the higher, uncontrolled avidity increases the stability of the antigen-antibody complex independently of the antibody affinity, whereas monovalent display ensures the selection of the antibodies with the highest affinity for the antigen [36]. Type 3 phage display uses phage vectors with one copy of recombinant gene III protein (g3p) and will generate phage that display 3 to 5 copies of a recombinant p3. Type 33 phage display systems contain two copies of g3p gene, one recombinant and one wild type and will yield multivalent phage particles with recombinant and wild-type p3 proteins displayed on the phage surface. Type 3+3 phage display systems use two different vectors, helper phage vectors and phagemid vectors. Phagemid vectors are small plasmids with high transformation efficiency that contain all the necessary components for infection, house recombinant DNA encoding the antibody-g3p fusion protein, but lack assembly and export genes. Phagemid systems, therefore, require helper phage, which retain the genes for packaging proteins and wild-type protein genes. Phagemid vectors are packaged in preference to helper phage vectors, but both wild-type and recombinant proteins will be displayed. In fact, more than 90% of the recovered phagemid particles do not display antibodies. However, this mechanism ensures that phage displaying recombinant g3p generally display a single copy on the virion surface [16,36].

Phage and phagemid vectors have been engineered extensively and now include antibiotic resistance genes for selection, a multiple cloning site (MCS) for easy generation of libraries in frame, and optimal promoter and packaging signals [22,37]. Phagemid systems are generally more stable than phage vectors, which can spontaneously delete foreign DNA fragments. Phagemid systems are also more tolerant to larger peptide inserts and generation of larger libraries is easier [38]. Phagemid display, which presents a single copy of a recombinant g3p protein, is required for optimal affinity maturation studies like antibody engineering, as display of single proteins results in the selection of fewer unique binders without interference from the effects of avidity [24]. However, phagemid display requires the addition of helper phage at specific points of the bacterial growth cycle, and therefore presents operational difficulties not characteristic of pure phage display.

Recently, a variant of the traditional phage display strategy, CysDisplay™ (http://www.morphosys.com/), was introduced that facilitates the recovery of antibody fragments with ultra-high affinity [16]. In CysDisplay™, the antibody fragment is linked to the coat protein through a disulfide bond instead of the direct peptide linkage when expressed as a fusion protein. Because of this, the recovery of the phage particles is easily achieved by adding reducing agents and is rendered independent of the antibody affinity to the antigen.

4.3. Cell display

Cell display, on the other hand, uses an analogous system wherein the antibody gene is fused to a protein naturally displayed on the outer membrane of a cell, as shown in Fig. 5B. Although cell display has been used with prokaryotic cells, the most current technology mainly uses eukaryotic systems such as yeasts and mammalian cells [18,39]. This strategy, in opposition to phage display, only

allows for multivalent display, whereby a large number of antibody copies are displayed on the outer membrane of the cell. While this strategy may be complicated by increased avidity compared to monovalent display systems, it benefits from existing technologies for studying eukaryotic cells, such as flow-cytometry and fluorescence activated cell sorting (FACS) ,which enable simultaneous selection and characterization of antibody fragments kinetics [40]. The basic strategy incorporates additional tags in the fusion protein in order to quantify the expression levels of the antibody fragment (i.e., the valency of the display), thus allowing the normalization of the antigen-antibody fluorescence signal to discriminate avidity effects, and also for selecting antibody fragments already optimized for high expression and the early removal of truncated products during the selection process [40].

The implementation of the cell display technology in a yeast model may also offer additional advantages compared with screening in prokaryotes by phage display. For instance, expression biases and growth selection introduced by bacteria can skew library diversity. In contrast, yeast models have been shown to propagate full library diversity along the selection process [18]. Figure 5B includes additional details about the construction of the display system, and compares the design of the fusion proteins involved in the two variants of the cell display technology herein discussed with the phage display method. In addition, a comprehensive comparison of the phage display, cell display and ribosome display (reviewed in next section) technologies is presented in Table 2.

4.4 Ribosome display

The development of the ribosome display technology by Hanes and Plückthun in 1997, based on earlier work by Mattheakis *et al.,* 1994, represented a breakthrough in the protein engineering field by demonstrating the ability to screen libraries of unprecedented size in a cell-free system [41-43]. The advantages and disadvantages of this novel technology, in contrast with phage and cell display, are presented in Table 2; cell-free systems, for instance, overcome the basic limitation of library size imposed by transformation efficiency for the previously discussed methods [44]. Ribosome display, which has evolved over the past 10 years, is now actively used for the development of new antibody fragments in various applications [17,45,46].

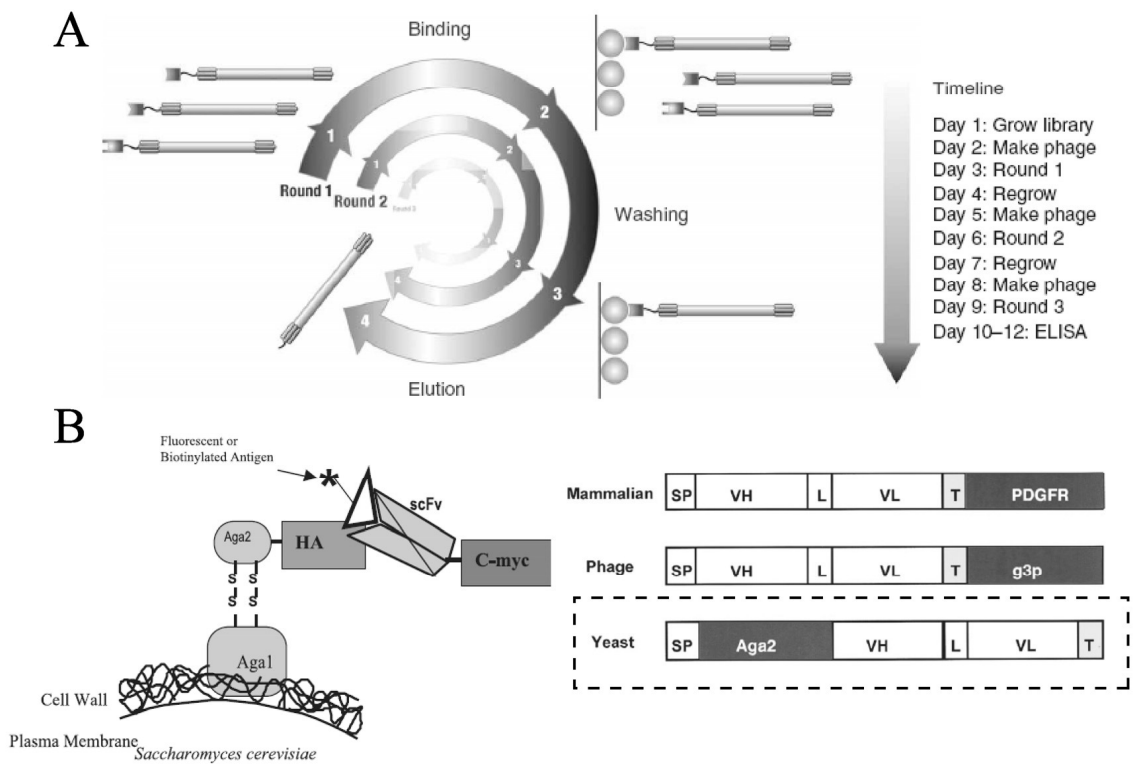

Fig. 5. **Schematics depicting the phage and cell display systems.** (A) In phage display, clones carrying antibody fragments with specificity to the desired antigen are bound to the column, whereas the nonspecific clones are removed by washing; after elution (e.g., by using protease cleavage) the selected clones are amplified by infection, followed by about 2 additional panning rounds. (B) Cell display uses fusion proteins to localize the antibody fragments to the cell wall (yeast) or cell membrane (e.g. Aga2 for yeast, PDGFR in mammalian cells), and selection is achieved by FACS of a fluorescently labeled antigen. Images modified from (A) Lee *et al*. 2007 and (B) Feldhaus and Siegel, 2004, and Ho *et al*. 2006 [27, 39, 40].

Although cell-free transcription and translation systems were already established, applying cell-free systems to screen antibody repertoires was only feasible when Hanes and Plückthun (1997) were able to form stable mRNA-ribosome-antibody complexes by removing the terminal stop codon in the mRNA, which prevented the release of the nascent peptide from the ribosome during protein synthesis [41]. By including a linker or spacer sequence between the peptide and the ribosome, they could correctly fold the protein and use stabilized mRNA molecules (by modification of the 5' and 3' ends loops) for a completely *in vitro* affinity selection process [46]. Figure 6 shows a detailed diagram of the ribosome display strategy recently published by Plückthun and collaborators, depicting the major steps of the process and important methodological aspects [46].

Ribosome display has been developed using either prokaryotic gene expression machinery (e.g., bacterial extracts) [17] or eukaryotic systems [44,47]. In both cases, the technologies are almost indistinguishable, although the use of eukaryotic ribosome display allows direct cDNA synthesis from the Antibody –Ribosome-mRNA (ARM) complexes by *in-situ* RT-PCR, as demonstrated by He and Taussig in 1997, and avoids some technical issues of prokaryotic ribosome display related to ARM complex disruption during elution of mRNA [44,48]. Hence, this approach reduces loss of material during ribosome disruption and mRNA recovery, which is critical to maximize diversity.

In 2007, Contreras-Martínez and Delisa proposed a variation of the ribosome display technology for the evolution of intracellular antibodies [49]. Intracellular ribosome display, which makes use of the recently discovered E. coli SecM translation arrest mechanism to allow the formation of mRNA-ribosome-antibody (fused to SecM signal), enables stable stalling of the ribosome (intracellularly) at the SecM sequence (serving also as spacer for appropriate folding) and subsequent recovery of the complexes by centrifugation. Additional selection steps proceed in a similar fashion to standard ribosome display [49].

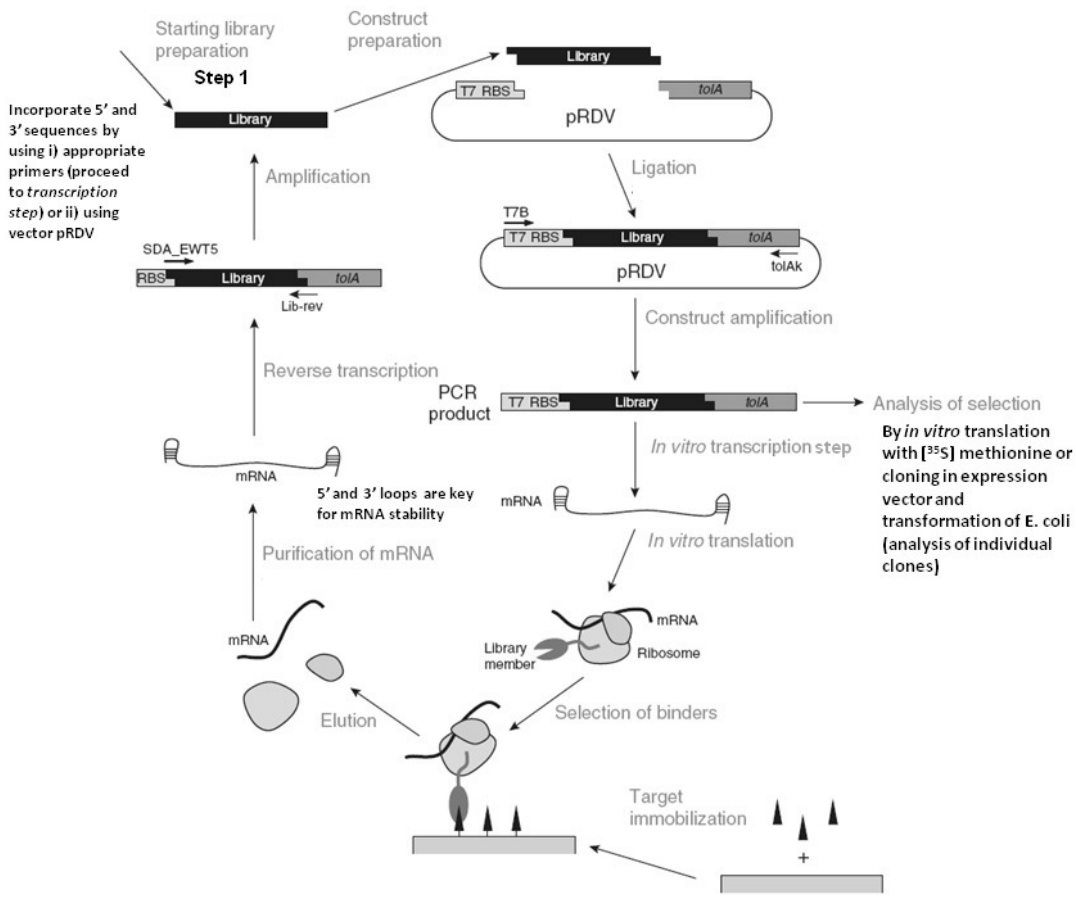

Fig. 6. **Antibody selection by ribosome display.** A library comprising the antibody repertoire (in the form of PCR product) is ligated into a Ribosome Display Vector (pRDV) to incorporate required 5' and 3' sequences (e.g. spacer, promoter, etc.), followed by *in vitro* translation. Stable mRNA-antibody complexes are formed due the absence of a stop codon, which stalls the ribosome at the end of the mRNA molecule, then, selection is performed by binding to an immobilized antigen and washing unbound complexes. The eluted mRNA is reverse transcribed and amplified (with or without incorporation of headers and tails) and used for a new round of selection or the analysis of single clones by cloning into expression vectors and expressing them in a suitable system (i.e., cell-free, yeast, bacteria, etc.). Image modified from Zahnd *et al,*. 2007 [46].

Table 2. Comparison of phage, cell and ribosome display technologies for antibody engineering.

Technology	Valency	Library size	Selection methods	Applications	Suitable formats	Advantages	Disadvantages	References*
Phage display	**Monovalent** - *phagemid+ helper phage* **Multivalent** - *no helper*	10^{10} to 10^{11}	**Versatile** - immobilized antigen, tissue sections, cells displaying antigen, subcellular fractions, etc.	**Selection of Ab** fragments from natural and synthetic libraries. **Affinity maturation. Stability maturation.**	**scFv (scFv)$_2$ Fab F(ab')$_2$ dAb: V_L and V_H Diabody**	**Automated. Multiple** formats available (display modes, selection methods, etc.). **Most established** system for production of Ab fragments.	**Monovalency** is only partial. **Library** size limited by transformation efficiency. **Slow introduction** of diversity by cloning.	[7, 16, 27]
Ribosome display	**Monovalent**	10^{12} to 10^{13}	**Limited** - Immobilized antigen.	**Selection** of Ab fragments from synthetic libraries. **Affinity maturation. Stability maturation.**	**scFv dAb**	**Fastest** method to increase library diversity during selection. **Amenable** to automation. **Enables** selection of otherwise eliminated toxic Ab fragments. **Potential** incorporation of modified amino acids.	**Selection** under conditions/buffers different from the intracellular milieu (folding issues) †. **Some** technical difficulties in the stabilization of the mRNA-ribosome-antibody complexes †. **No optimization** for expression.	[46, 47, 49]
Cell display ‡	**Multivalent** – although allows for data normalization according to valency.	10^7-10^9	**Cell sorting** - magnetic beads, FACS.	**Selection** of Ab fragments from synthetic libraries. **Affinity maturation. Stability maturation. Expression optimization.**	**scFv dAb Fab**	**Selection** of highly expressed proteins. **Multiplex screening. Kinetic** parameters (e.g. affinity constants) directly measured in cells (by flow-cytometry). **Conservation** of library diversity along selection process (due to tight control of promoters).	**Technological** limitations in sorting speed (~10^9 cells/day). **Transformation** efficiency.	[18, 39, 40]

‡ Yeast display is the most common format of cell display nowadays. Display in mammalian cells is equivalent and may help circumventing problems from selecting in microorganisms, especially if posterior expression in a mammalian cell line (e.g. Chinese hamster ovary cell, one of the main systems for production of therapeutics antibodies in the pharmaceutical industry) is required. However, the seminal work by Pastan's group needs further validation [39].

¤ The general information herein provided is well-applicable to both eukaryotic and prokaryotic ribosome display.

† This limitation has been recently addressed by the development of Intracellular Ribosome Display [49].

* Reference to the most relevant and recent review articles summarizing the characteristics and methodological aspects of each of the different antibody discovery technologies mentioned. Readers are strongly encouraged to review the reference that is underlined. For more references to the information presented in this synthesis refer to the text.

4.5 Other aspects of the antibody engineering process

The flexibility in the design of antibody fragments (Fig. 3) and the variety of technologies available for their discovery and optimization, as discussed in Table 2, allow for myriad possibilities when tailoring the properties of these unique proteins according to the design parameters dictated by the intended application. The readers are strongly encouraged to examine excellent recent review articles discussing some important aspects of the antibody engineering process that were not the subject of this review, including: antibody characterization (i.e., quantification of kinetics, stability, immunogenicity, etc.) [1,4], pharmacokinetics of monoclonal antibodies and antibody fragments (of particular relevance for imaging applications and cancer therapy) [50,51], current expression systems for the production of antibody fragments [8,52], and finally, design of antibody-based therapeutics, an active area of research that involves engineering of the antibody fragments at all levels, particularly tailoring the effector functions of the antibody fragments which may involve the engineering of the Fc region [3,53-55].

V. Conclusion

The ability to rapidly engineer antibodies against virtually any antigenic biomolecule, from mRNA to small haptens to big antigenic particles or even molecules with cryptic epitopes (as in the case of dAb), with unprecedented affinities, effector functions (i.e., bispecific antibodies, catalytic antibodies, intrabodies, etc.) and stability, has been the result of the powerful antibody discovery technologies implemented in the past 20 years. Although phage display has governed the production of antibody fragments since its introduction, the new advances in ribosome display technology and cell display will probably continue to gain importance for the selection of the antibody fragments. Expected improvements in cell sorting technology might significantly increase the throughput of the cell display system, its major limitation as of today, although the recent demonstration of antibody maturation by mammalian cell display may also stimulate the use of this technology in combination with phage display in the late stages of the antibody maturation process (i.e., stability, expression, etc.). Finally, these technologies are likely to play an important role in the production of the next generation of antibody-based therapeutics.

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