



Review

Selection of antibodies from synthetic antibody libraries

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ABSTRACT

More than 2 dozen years had passed since the field of antibody engineering was established, with the first reports of bacterial [1–3] and mammalian cells [4] expression of recombinant antibody fragments, and in that time a lot of effort was dedicated to the development of efficient technological means, intended to assist in the creation of therapeutic monoclonal antibodies (mAbs). Research focus was given to two intertwined technological aspects: the selection platform and the recombinant antibody repertoires. In accordance with these areas of interest, it is the goal of this chapter to describe the various selection tools and antibody libraries existing, with emphasis on the later, and their applications. This chapter gives a far from exhaustive, subjective “historic account” of the field, describing the selection platforms, the different formats of antibody repertoires and the applications of both for selecting recombinant antibodies. Several excellent books provide detailed protocols for constructing antibody libraries and selecting antibodies from those libraries [5–13]. Such books may guide a newcomer to the field in the fine details of antibody engineering. We would like to offer advice to the novice: although seemingly simple, effective library construction and antibody isolation provide best benefits in the hands of professionals. It is an art as much as it is science.

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Selection platforms for recombinant antibody repertoires

The numerous recombinant antibody display technologies available nowadays can be roughly divided into *in vitro* display technologies, including phage display which is the most common antibody display technique [14,15], ribosome display that is completely cell free [16,17], and into *in vivo* display platforms, such as bacterial, yeast and mammalian cell-surface display [18,19].

All these systems will be shortly described in this section, but first, for thematic reasons, it should be clearly stated that the purpose of antibody engineering is to mimic, direct, improve or even surpass the natural *in vivo* process of generating antibodies by the human immune system. Hence, as Bradbury and Marks [20] first pointed out, the selection platforms for recombinant antibody repertoires must enable four features and processes parallel to those occurring in nature: (1) *genotypic diversity* – the diversity *in vivo* which is primarily formed by gene rearrangement events that occurs during B-cell development through combinatorial shuffling of V-J or V-DJ gene segments of the variable domains of the light chain (V_L) and heavy chain (V_H), respectively and by non-templated nucleotide addition [14,21]. *In vitro*, this diversity is dependent on the nature of the recombinant antibody repertoire as well

as on its construction method, as will be further discussed in the next section. (2) *Genotype–phenotype coupling* – *in vivo* and *in vitro* there is a physical linkage between the binding capability of a produced antibody (phenotype) and the DNA sequence encoding it. (3) *Selective pressure* and (4) *amplification* – antigen presence and its gradual reduction in blood concentration are the driving forces that promote *in vivo* generation of high affinity antigen-specific antibodies, whereas exposure of formed recombinant antibody libraries to an antigen of choice, under restricting conditions, followed by repetitive enrichment cycles and/or screening enable the isolation of suitable antibody candidates from these repertoires. Inevitably, careful design of the selection method must address the properties of the antibody repertoire, the technical means as well as their compatibility with the selection tool of interest.

To emphasize the interrelation between the display platform and the properties of the recombinant antibody repertoires, here are two examples of the possible consequences on the outcome of the selective process: (1) *in vitro* display platforms can be applied to very large antibody repertoires (up to 10^{14} clones), whereas *in vivo* display platforms can only support modest sizes of antibody repertoires (usually $\leq 10^9$), due to limitations in the transformation efficiency and of the screening tools [14,22,23]. The antibody repertoire size significantly influences the affinity of the antibody that can be isolated. Antibodies with affinity ≤ 100 pM can be isolated from libraries with more than 10^{10} clones, as opposed to antibodies with affinity ≤ 10 nM that can be isolated from libraries with 10^8 clones [24,25]. (2) As described by Wark

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and Hudson [26], selection of the same recombinant antibody libraries against the same antigen, using phage display and ribosome display, resulted in distinct antibodies that varied in their affinities and recognized epitopes. In fact, different antibodies can be isolated from a given library and a given display technology by altering the affinity selection protocol [27,28]. Meaning, the selection tool and the way it is applied play a role in the determination of selection outcome, even when the antibody repertoires are identical.

An equally important consequence derived from the interrelation between the display platform and the properties of the recombinant antibody repertoires concerns the feasible usage of each platform. Specifically, display platforms that can support large size antibody libraries are usually used for *de novo* antibody isolation, whereas display platforms that bear modest size libraries are used in most cases for the improvement of pre-existing antibodies. This topic will receive a detailed explanation in the third section of this chapter.

In vitro display platforms

Phage display

Filamentous bacteriophage (phage) was used in the early days of molecular biology for making single-stranded DNA for site-directed mutagenesis and for DNA sequencing [29,30]. Phage display, as a molecular diversity selection tool, was first demonstrated for selection of binders from peptide repertoires by George Smith from the University of Missouri [31]. Five years later, the first reports of the utilization of this technology for recombinant antibody repertoires were published [32–34]. To date, in spite of the time elapsed, the basic concept remained unchanged: phage clones, carrying a recombinant antibody fragment fused to a phage coat protein while the antibody-coding gene is fused at the DNA level to the gene encoding that phage coat protein are subjected to selective pressure. The selective pressure is mostly for binding to an antigen, referred to affinity selection or (bio)panning. Phages, that survive the repetitive cycles of selective pressure due to favorable binding capabilities, can be isolated from a given antibody repertoire and be further characterized and manipulated [25,35–37].

In general, M13 and fd-based display continue to be the most widespread antibody phage display selection tools [14,22] and with a few exceptions (pioneering work done at the Scripps Research Institute [34] where Fab was displayed on p8), all known antibody phage display repertoires are fused to the minor phage coat protein p3 [20], which is involved in the bacterial infection process through the F pilus and is present in 3–5 copies per phage [38] and not to the major phage coat protein p8 which was found to be less efficient, though more prevalent with 2700 copies per phage [39]. In addition, there are two available systems for antibody phage display: one is based on a phage vector that consists of a gene cassette encoding an antibody fragment-p3 fusion instead of the natural gene encoding p3. This system is known as the “3 system” and it enables polyvalent display of the recombinant antibody fragment, with 3–5 copies per phage. The other system, called “3 + 3 system”, is based on two elements: the first is a phagemid, which consists the gene cassette encoding an antibody fragment-p3 fusion and a phage ss-DNA packaging signal, but lacks all other necessary components to make vital phages. The second element of the “3 + 3 system” is a helper phage carrying a defective packaging signal, while all other components necessary for replication, packaging and egress from the infected bacterium are intact. This display system results in the monovalent display of antibody-phage coat protein fusions, yet less than 10% of all phages display this chimera on their protein capsid [40]. Another key issue relates to the antibody formats used in phage display platforms: the leading formats, that were actually initially used in the pioneering studies performed by the groups of Sir Gregory Winter from

MRC, Cambridge, UK [33] and Richard Lerner from the Scripps Research Institute, La-Jolla, California [41], are single-chain variable fragment (scFv)¹ and Fab [14,23]. Furthermore, in consideration with the all existing possibilities as for the phage type, display system, antibody format, etc. (reviewed in [15,20,35,42]), it is fair enough to say that phage display was and still is by far the most diverse selection tool for antibody repertoires. Phage display is illustrated schematically in Fig. 1.

Ribosome and mRNA display

Among display technologies, cell-free selection platforms (reviewed in [43]), ribosome display and mRNA display (reviewed in [44]) are considered to be very powerful molecular diversity selection tools for antibody repertoires [17], since they allow the screening of very large antibody libraries ranging from 10^{12} to 10^{14} individual clones and thus theoretically and practically enabling the isolation of antibodies with pM affinities [16,45–48]. These two methodologies share almost all features, including: the use of *in vitro* transcription and *in vitro* translation, in either coupled or uncoupled systems, in the presence of either rabbit reticulocyte lysate, wheat germ extract or an *Escherichia coli* S30 extract. Each of them can result in either monoribosome or in polysome display complexes and of course selection against antigen of interest and the use of reverse transcription polymerase chain reaction (RT-PCR) in order to amplify and preserve the affinity-selected clones for the following procedures [16,17,49,50]. However, the difference between the two resides in the displaying complex: in the case of ribosome display, the displaying entity is a stable ternary complex consisting of mRNA, nascent protein and halted ribosomes caused by the presence of antibiotics (such as chloramphenicol or cycloheximide for prokaryotic or eukaryotic ribosomes, respectively) or by the deletion of the stop codon from the translated mRNA [46,51–53]. Whereas, in the case of mRNA display, the ribosome-free complexes are made up of covalently linked mRNA and nascent protein, formed by the addition of the antibiotic agent puromycin that acts as a aminoacyl-tRNA mimetic [54,55]. It should be noted that scFv is the favorable format when using ribosome or mRNA display as the selection platforms, to ensure physical linkage between the variable domains [14,44]. Ribosome display is illustrated schematically in Fig. 2.

In vivo display platforms

Bacterial surface display

In 1993, George Georgiou's group from the University of Texas at Austin, in a proof of concept report demonstrated the use of bacteria as an antibody fragment display system [56]. In detail, they used an Lpp-OmpA' chimera [57] to display two specific scFvs on the outer membrane of the Gram negative bacterium *E. coli*. A few years later they realized this technology into a competent screening tool for antibody repertoires [58,59]. Another approach to display recombinant antibody repertoires on bacteria was developed by the same group several years later [60,61]. This system, which they named APEx (Anchored Periplasmic Expression) was based on the expression of antibody libraries, in a scFv format, in the periplasmic space anchored to the inner membrane of the *E. coli* bacterium. In both approaches, antigen-specific clones were isolated using flow cytometry and their DNA is obtained by PCR for ascending procedures. These two earlier bacterial surface display attempts suffered from the technological shortcoming of the FACS

¹ Abbreviations used: mAbs, monoclonal antibodies; scFv, single-chain variable fragment; PDGFR, platelet-derived growth factor receptor; EBV, Epstein-Barr virus; IRES, internal ribosome entry site; CDR, complementarity determining region; VEGF, vascular endothelial growth factor; BCR, B-cell-receptor; HAMA, human anti-mouse antibodies; APEx, Anchored Periplasmic Expression.

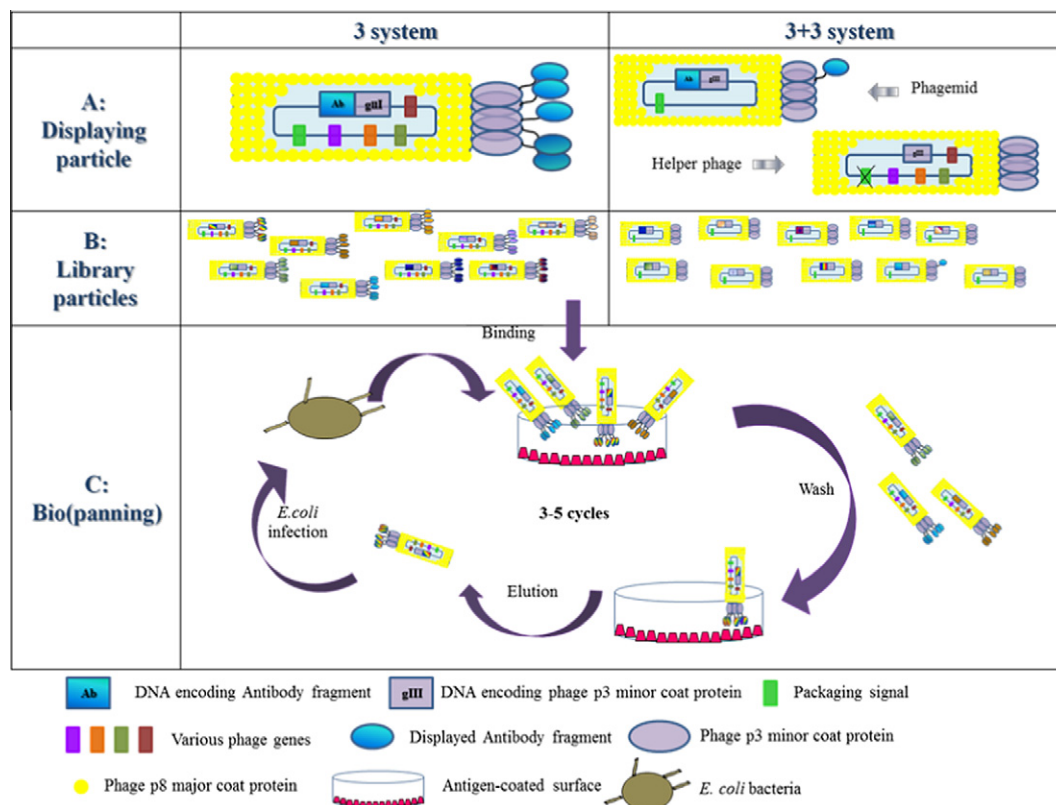


Fig. 1. Phage display platforms for antibody selection. (A) Schematic representation of displaying phage particles in the “3 system” and “3 + 3 system”. In the “3 system”, antibody fragments, usually scFv or Fab, are fused to the phage minor coat protein, p3 and displayed in a polyvalent manner. In the “3 + 3 system”, antibody fragment–p3 fusions are displayed in a monovalent manner on phagemid particles, which contain phage packaging signal but lack all other necessary components for the production of vital phages and thus are dependent on the infection of bacterial cells with helper phages. (B) Representative phage library clones. In the “3 system”, each phage egresses from the bacterial cell display the antibody fragment, whereas in the “3 + 3 system” only 10% of all produced phages display the antibody fragment. (C) Bio(panning). The selective process for specific antigen-binding clones, called bio(panning), is identical in both phage display systems and is therefore shown only for the “3 system”. Antibody displaying particles are incubated with antigen-coated surfaces and after washing away non-binding particles, the specific binders are eluted and amplified by infecting *E. coli* cells. This process is repeated for 3–5 cycles before performing any further screening steps.

technology of that time, being limited screenable library size and were therefore used for the evolution of pre-existing antibodies. However, two beneficial improvements were recently reported: the expression and capture of soluble full length IgGs in the periplasmic space of *E. coli* [62] and an additional prescreening step using phage particles that allows reduction in library size to suit the upper limit of FACS [63]. The combination of phage display and bacterial surface display expanded the traditional applications of this display platform to *de novo* selection of antibodies, from larger repertoires, as was demonstrated by Mazor and coworkers [63]. Bacterial surface display is illustrated schematically in Fig. 3.

Yeast surface display

A comprehensive description of yeast display can be found in the first chapter of the book, therefore we will describe it only briefly here. The initial utilization of the budding yeast *Saccharomyces cerevisiae* for the display of recombinant antibody repertoires was demonstrated by Dane Wittrup's group from the University of Illinois, Urbana [64,65]. In some aspects, yeast display resembled bacterial surface display [58,59]. Recombinant antibody libraries, usually in the scFv format, were displayed on the yeast cell wall through their linkage to the adhesion receptor α -agglutinin [18,22] and antigen-specific clones were identified using flow cytometry. Over the years, three main observations lead to a change in the way yeast display is perceived nowadays: (1) scFv mutants, originated from medium size antibody libraries (10^5 – 10^7), having antigen affinity reaching the fM range, were obtained via yeast display [66], (2) *de novo* isolation of antibodies from larger libraries, containing $\sim 10^9$ individual clones, via yeast display

was possible through the use of two screening stages comprising initial enrichment of antigen-specific population on magnetic beads followed by screening for individual binders by flow cytometry [67,68], (3) implementation of yeast biology, specifically of yeast mating types that made it possible to overcome limitations of library size that can be obtained by transformation by mating haploid yeasts that display i.e. heavy and light chains of Fabs [68,69] and efficient homologous recombination which enables *in vivo* diversification [70], has turned the generation of large antibody repertoires to be displayed on yeast much more appealing. Furthermore, when taking into consideration the antibody formats that are successfully displayed on yeast, including: scFv, Fab and full length IgG [23,71], there is no wonder why yeast display is currently the most popular cell-based display technology. Yeast surface display is illustrated schematically in Fig. 4.

Mammalian cells surface display

The use of mammalian cells as a means to display antibody repertoires was established 5 years ago by Ira Pastan's group from NIH who displayed a scFv library fused to the N-terminal transmembrane domain of human platelet-derived growth factor receptor (PDGFR) on the surface of HEK-293T cells and was able to isolate high affinity anti-CD22 antibodies [72]. Afterwards, a second system for the display of scFv repertoires on the surface of BHK mammalian cells, via a Sindbis virus expression vector, became available. This system gave rise to the isolation of antibodies targeted against influenza A virus M2 ectodomain [73,74]. An additional and more complex work by Akamatsu and coworkers [75], revealed that full length chimeric IgG-based repertoires can also

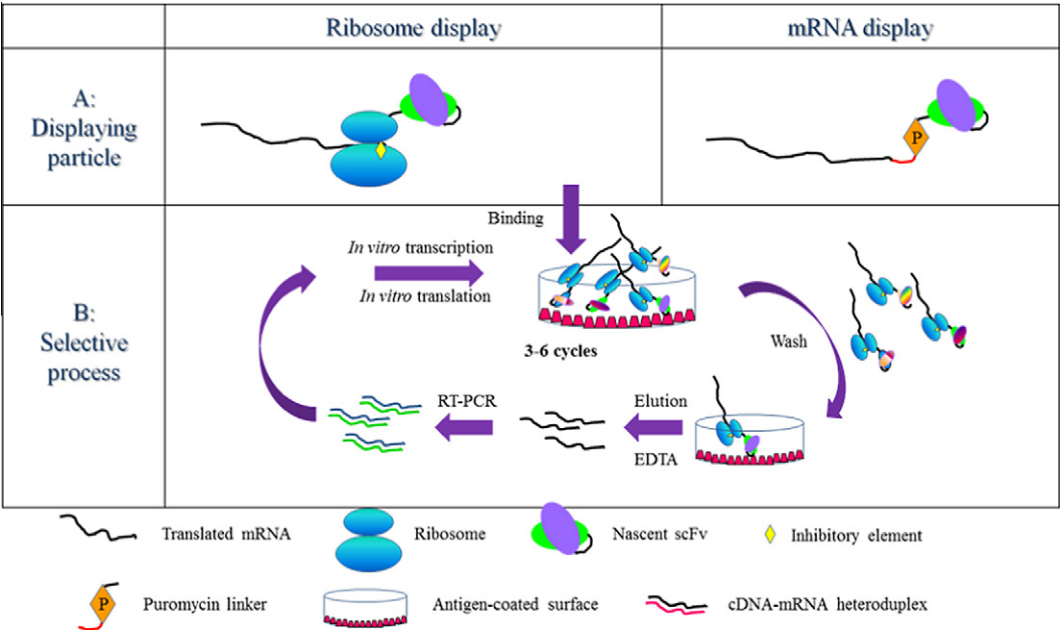


Fig. 2. Ribosome and mRNA display platforms for antibody selection. (A) Schematic representation of displaying particles used in ribosome and mRNA display platforms. In ribosome display, the displaying particle is composed of a stable ternary complex consisting of mRNA, nascent scFv and halted ribosomes (caused either by the presence of translation-inhibitory antibiotics or by the lack of stop codons). In mRNA display, the displaying particle is composed of mRNA–scFv complexes connected together by a puromycin linker. (B) Selective process. The selective process for specific antigen-binding clones is similar in ribosome and mRNA display and is therefore shown only for ribosome display. Antibody displaying complexes are incubated with antigen-coated surfaces and after washing away non-binding complexes, eluting the selected mRNAs requires the addition of EDTA for the destabilization of ribosomes. RT-PCR reaction amplifies the selected scFv's encoded DNA for additional selection cycles. It should be noted that in mRNA display the RT reaction takes place earlier, before the elution step.

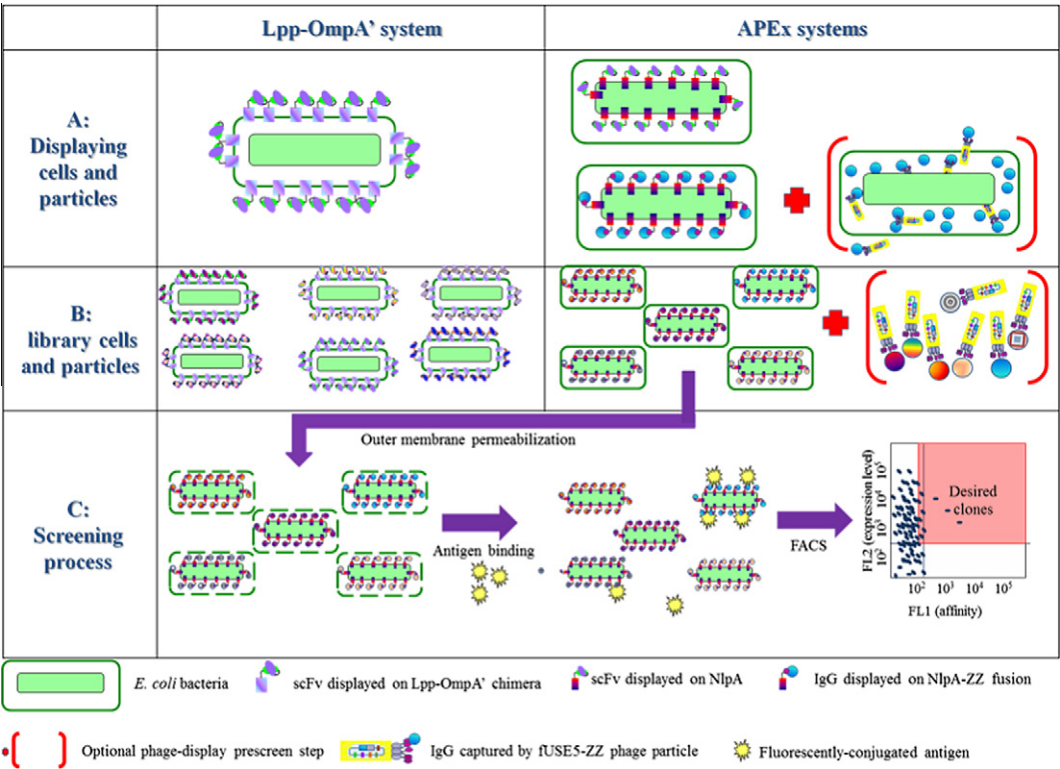


Fig. 3. Bacterial surface display platforms for antibody selection. (A) Schematic representation of displaying cells used in bacterial surface display systems. In the Lpp-OmpA' system, scFv fused to Lpp-OmpA' chimera is displayed on the outer membrane of Gram-negative bacteria. In the APEX systems, the antibody or antibody fragment are anchored to NlpA [49] or NlpA-ZZ fusion [51] and are thus facing the periplasmic space of Gram-negative bacteria. Furthermore, in the most advanced APEX system, an additional prescreen step uses phage particles to capture and display soluble antibodies [52]. (B) Representative library cells and particles. (C) Screening process. The screening process for specific antigen-binding clones, is similar in both bacterial surface display systems and is therefore shown only for APEX systems. Antibody displaying spheroplasts formed by outer membrane permeabilization are incubated with fluorescently-conjugated antigen and then sorted by flow cytometry. When using the APEX system with the additional phage display stage [52], phages undergo bio(panning) as described in Fig. 1.

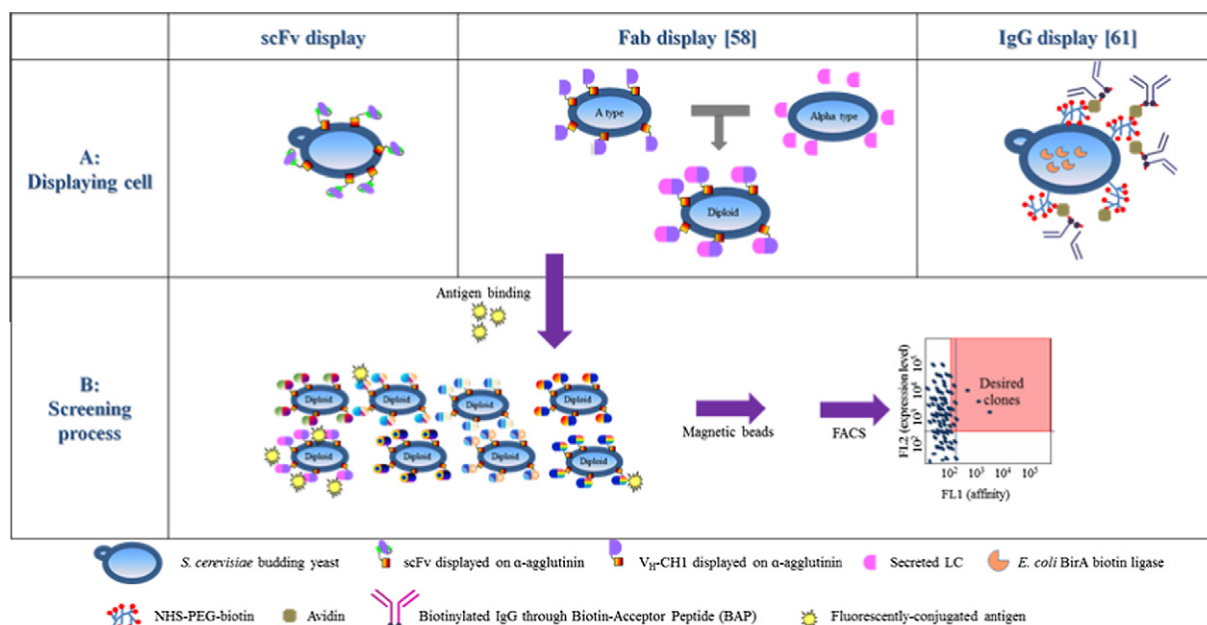


Fig. 4. Yeast surface display platforms for antibody selection. (A) Schematic representation of displaying cells used in yeast surface display systems. scFvs and Fabs were displayed on the adhesion receptor α -agglutinin. In the presented Fab yeast display, mating types of the *S. cerevisiae* were used to form diploid cells that secret LC and present HC. In the case of IgG yeast display, IgGs were biotinylated *in vivo* and secreted to culture media, where they were captured by the same cells displaying NHS-PEG-biotin-avidin complexes. (B) Screening process. The screening process for specific antigen-binding clones is identical in the yeast surface display systems and is therefore shown only for the case of Fab display. Antibody displaying yeasts are incubated with fluorescently-conjugated antigen and then sorted twice, once with magnetic beads and afterwards by flow cytometry.

be displayed on the surface of HEK293c18 mammalian cells. This system used an Epstein-Barr virus (EBV)-derived bi-cistronic vector carrying an internal ribosome entry site (IRES) signal to allow the production of whole IgGs, where the C-terminal constant region of the HC was fused to glycosidylphosphatidylinositol linkage signal (GPI anchor) derived from the human decay-accelerating factor. The use of this system resulted in the *de novo* isolation of chimeric IgGs, composed of chicken variable domains and human constant domains, neutralizing both human and mouse IL-12. As there are only a few reports of applications of mammalian cell surface antibody libraries display, the full potential of this selection platform is still ahead. The main limitation of mammalian cell display systems is small repertoire sizes. Mammalian cells surface display is illustrated schematically in Fig. 5.

Construction of recombinant antibody repertoires

Antibody repertoires are the most basic and fundamental requirement for the development of therapeutic mAbs via antibody engineering. Over the years four main library types were established: immune, naïve, semi-synthetic and synthetic libraries. These libraries vary in the origin of the antibodies variable fragments as well as in their combinatoriality, sizes, compatible display platforms and practical applications. The generation of immune and naïve repertoires is illustrated schematically in Fig. 6.

Immune antibody libraries

The term “immune library” refers to antibody repertoires originating from an immunized donor, usually murine or human, but also from other species [14,75]. Non-human immune libraries allow the isolation of antibodies against a desired immunogen of human or foreign origin, comprising the unique antibody characteristics of the immunized animal [75,76]. Humans, of course are not intentionally immunized for the purpose of raising antibodies and consequently immunized antibody libraries originating from humans are generally used for the profiling of antibody

production in infectious diseases, autoimmune conditions and cancer [77]. For example, human immune libraries enabled the identification of *in vivo* formed disease-related antibodies, such as HIV-1 neutralizing antibodies [78], celiac auto-antibodies [79] and anti-melanoma antibodies [80]. In either case, the library construction process is basically identical: separate RT-PCR reactions are used for the recovery of intact germ-line encoded LC and/or HC variable genes, consisting of VJ segments for V_L and VDJ segments for V_H, from B cells isolated either from lymphoid organs, including bone marrow, spleen and lymph nodes or from peripheral blood. Afterwards, the cDNA products are inserted into appropriate display cassettes, usually in the scFv or Fab format, but also as single domain cassettes (e.g. [81]). The combinatorial pairing of V_Hs and V_Ls in each expression cassette creates immune libraries which usually consist of $\leq 10^8$ individual clones. In spite of the limited sizes of these antibody repertoires, due to the natural *in vivo* affinity maturation processes that were activated following the immunization and boosting with a desired antigen, anti-immunogen antibodies with affinities within the nM range can be obtained [20]. Yet, the high level of enrichment with immunogen-specific clones in immune libraries, does not eliminate the possibility of isolating antibodies directed against unexpected antigens, such as self-antigens [27,82]. Basically, immune libraries, while enriched for immunogen-specific antibodies, still contain the other antibodies from the antibody repertoire of the donor. Moreover, the combinatorial assembly of antibody heavy and light chains creates within the libraries V_H-V_L combinations that were not part of the donor original repertoire. Philosophically, then, immune repertoires contain within a non-immune repertoire that may be exploited to isolate antibodies specific for antigens other than the immunogen. In reality, the limited size of immune libraries makes the likelihood of isolating high-affinity antibodies specific for such antigens very low.

Naïve antibody libraries

Naïve antibody repertoires are constructed similarly to immune libraries, with the only exception being the source of the B cells,

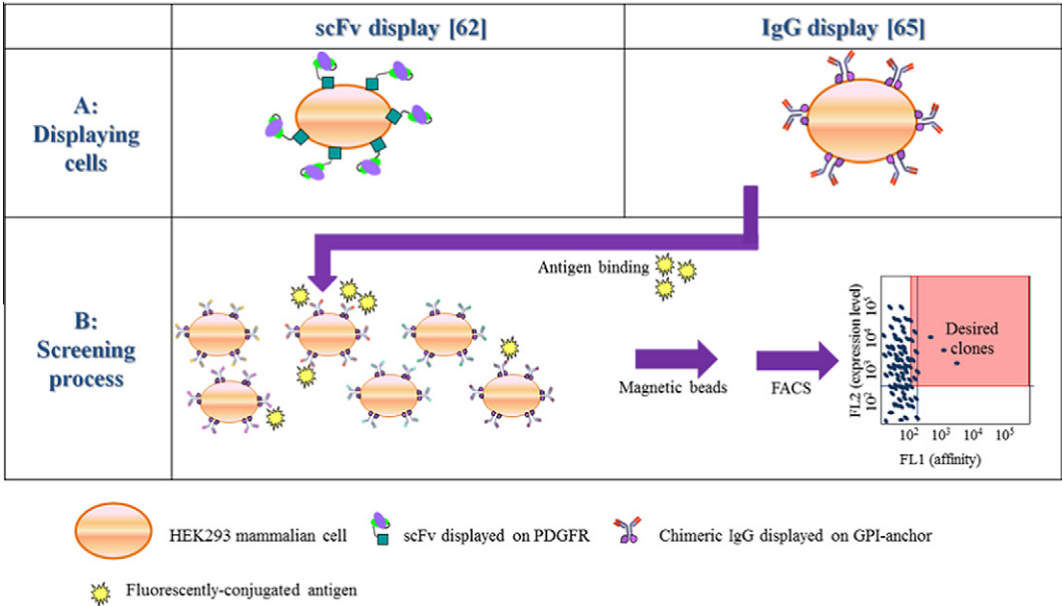


Fig. 5. Mammalian cells display platforms for antibody selection. (A) Schematic representation of displaying cells used in mammalian cells display systems. scFvs were displayed on human platelet-derived growth factor receptor (PDGFR) on the surface of HEK-293T cells. IgGs were displayed on the surface of HEK293c18 cells via HC–GPI anchor derived from the human decay-accelerating factor and LCs were secreted and captured by the HC–GPI. (B) Screening process. IgG displaying mammalian cells are incubated with fluorescently-conjugated antigen and then sorted twice, once with magnetic beads and afterwards by flow cytometry.

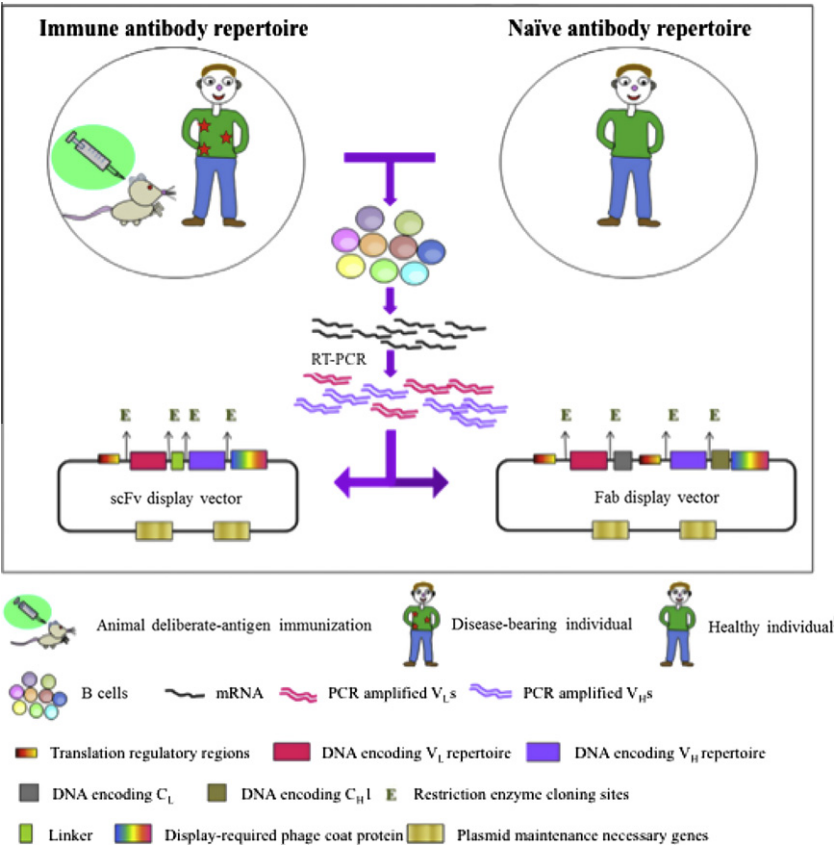


Fig. 6. Immune and naïve antibody repertoires. mRNAs encoding antibody variable domains are purified from B-cells of immune donors (intentionally immunized animals or disease-bearing humans) or from healthy individuals. After RT-PCR amplification reaction, the PCR product is cloned into a desired display cassette, mostly in a scFv or Fab format.

which is from non-immunized donors (or ones that were not immunized intentionally for the purpose of constructing a library). It should be noted that even though in theory, to be truly naïve, the population of B-cells contributing to the library construction must be restricted to cells carrying binding potentials only in IgM/IgD formats, libraries formed by RT-PCR including all possible isotypes

were equally good [36,83]. However, since the variable regions of the HC and LC were not subjected to antigen-driven *in vivo* affinity maturation processes, the library size that should compensate for that becomes most important for the determination of the library quality [14]. Typical naïve libraries consist about 10^9 – 10^{10} clones [84,85], but recently the upper limit has been extended to 1.29×10^{11} individual clones in the phage display scFv library named CAT2.0 (MedImmune, Cambridge, UK) [86]. Analysis of the germ-line diversity represented in the CAT2.0 library revealed that the natural variable regions coverage in this library is well above 90% (48/49 V_H , 28/30 V_K , 31/35 V_κ) of the human repertoire. Moreover, this highly productive library enabled the isolation of 3340 different antibodies against 28 antigens, each of them with inherent biological activity.

Semi-synthetic and fully synthetic antibody libraries

The next evolutionary step in the creation of antibody repertoires was the introduction of synthetic components into the natural diversity of antibodies. There are illustrated schematically in Fig. 7. By replacing one or more of the complementarity determining region (CDR) loops with artificial elements, the resulting antibody repertoire offers unnatural variability that evades biases and redundancies of natural *in vivo* formed antibodies [87]. Two types of synthetic antibody libraries were developed over the years: semi-synthetic libraries that combine natural CDRs with artificial ones and fully synthetic libraries in which all CDRs are man-made [88]. In contrast to immune libraries and similar to naïve libraries, synthetic libraries should be very large to yield high-affinity binders. Some estimates claimed that to yield single nanomolar antibodies, a non-immune library should include no less than 10^9 antibodies [14,25]. The construction of such large libraries involves brute-force solutions, involving carrying out many transformations and using a large amount of ligated DNA and automation where it is available. Several elegant studies involved using site-specific recombination as a means of increasing the size of synthetic repertoires [89–91]. In such studies, the heavy and light chain components are introduced via two different constructs that, owing to the inclusion of site-specific recombinogenic sequences, recombine within the transformed bacteria to form very large libraries.

The first semi-synthetic antibody library, designed by Hoogenboom and Winter [92] was intended for scFv display on phages and it consisted of only a single V_L (from the $V_{\lambda 3}$ germ-line) that was shuffled with rearranged V_H domains. All natural 49 human V_H gene segments (contributing CDR1 and CDR2) were assembled with a synthetic PCR-based CDR3 comprising 5–8 residues. This small size library consisted of 10^7 clones and enabled the isolation of anti-hapten antibodies, with affinities in the μ M range. Later, this group generated a second “single pot” library that was constructed similarly to the first one, with two main changes: it consisted of 50 natural human V_H gene segments that were assembled with a synthetic CDR3 comprising 4–12 residues. Despite the medium size of this library that consisted only of 10^8 clones, it enabled the isolation of antibodies targeted against various self- and foreign-antigens, haptens and proteins altogether [93]. de Kruif and coworkers [94] further developed and expanded these first generation semi-synthetic repertoires, as they randomly paired 7 V_L s (3 V_κ and 4 V_λ) with 49 germ line V_H gene segments that were rearranged with a synthetic CDR3 comprising 6–15 residues. In an attempt to increase the number of functional clones in this library, the synthetic CDR-H3 was designed to possess low variability at its ends contributed by abundant antibody amino acids as well as high degree of diversity, created by full randomization, in the center of the CDR. This scFv phage display library consisted of 3.6×10^8 clones and it enabled the isolation of antibodies with

affinities ranging from 2 μ M to 100 nM. A large Fab phage display library of 6.5×10^{10} clones expanded the level of synthetic elements even further [89]. This library was constructed on the basis of the “Nissim library” [93] and it included the replacement of the single V_L with 21 germ-line V_κ gene segments, encoding CDR-L1 and CDR-L2 rearranged with CDR-L3 loops which consisted of 0–5 random residues within the 8–13 residues constituting CDR-L3 as well as 26 germ-line V_κ gene segments rearranged with CDR-L3 loops which consisted of 1–3 random residues within the 8–10 residues constituting CDR-L3.

The first generation of semi-synthetic libraries confirmed the interrelation between library size and antibody's affinity. In addition, it was shown that in spite of the great natural variability these libraries possessed, some V_H families were overrepresented in the population of selected antigen-specific clones [89]. This observation implied that the heterologous expression of antibody fragments in the library context (usually as a phage display library) depended heavily on its folding properties. These properties are determined by the antibody's framework region, since it provides the structural support for the CDRs, and are therefore dependent on the V_H family classification. Hence, the functional size of the first generation semi-synthetic antibody repertoires was probably smaller than the calculated size, as there was no significant meaning to the inclusion of poorly folded V_H families in the repertoires [95].

The second generation of semi-synthetic and fully synthetic libraries was based on a limited set of frameworks, originating from well-expressed V_H families, and the introduction of synthetic elements in the natural space between them. The first semi-synthetic scFv phage display library of the second generation was indeed based on the most frequent HC and LC germ-line gene segments, DP47 and DPK22, respectively. The artificial elements were introduced in the antigen binding site in a PCR-based method at positions 95–98 in CDR-H3 and at positions 91, 93, 94 and 96 of CDR-L3. The resulting library was $>3 \times 10^8$ clones and the affinity of isolated anti-ED-B domain of fibronectin antibodies ranged from 10^{-7} to 10^{-8} M [96]. A different strategy for the generation of semi-synthetic antibody repertoires used the n-CoDeR® (BioInvent, Sweden) principle. This approach shuffles *in vivo* formed CDRs into one master scaffold, meaning the artificial component in the antibody libraries constructed according to this principle is the combinatorial assignation of natural germ-line CDRs (clustered to CDR1, 2 and 3) in a specific framework context to provide optimal structural support. The first study that established and utilized the n-CoDeR® principle constructed a single domain V_H library, also based on the DP47 framework, with 9×10^6 members [97]. Two years later, the first scFv phage display library designed according to the n-CoDeR® principle was formed, with DP47 and DPL3 as the master frameworks for V_H and V_κ , respectively. This library consisted of 2×10^9 members and enabled the isolation of antibodies with affinities in the sub-nM range, targeted against haptens, peptides, carbohydrates, and proteins [98]. A similar scFv phage display repertoire was constructed by the author's group, with an additional V_L master framework DPK22 from the V_κ germ line. This library included 2×10^9 individual clones as well and it enabled the isolation of antibodies with affinities ranging from 10^{-8} to 10^{-10} M directed against peptides and proteins [24] and later haptens and semiconductors [99]. Libraries construction based on a single master framework was also performed by Genentech [100]. They constructed several Fab phage display repertoires on the framework of humanized 4D5 antibody (Herceptin®), with the obvious advantage being the implementation of an already optimized scaffold for the presentation of diverse CDRs. These synthetic libraries were carefully designed to mimic the natural diversity of HC and LC CDRs in an attempt to form large functional libraries, on the basis of an initial scFv library [101]. It was found

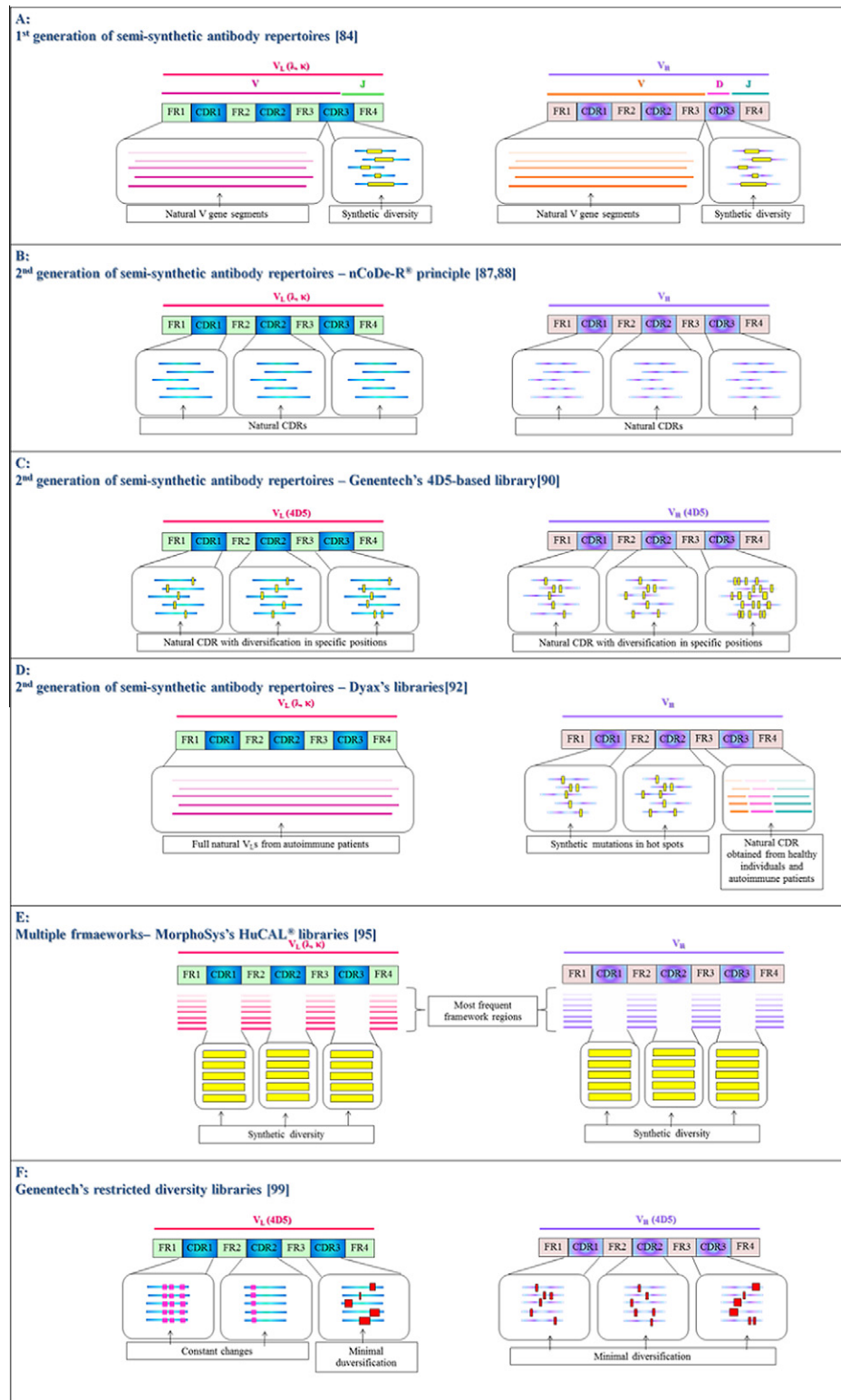


Fig. 7. Synthetic repertoires. (A) First generation of semi-synthetic antibody repertoires [84]. The presented repertoire, consisted of LC and HC natural V gene segments, encoding CDR1 and CDR2, whereas artificial elements, in both length and sequence diversity, were introduced into CDR-H3 and CDR-L3 via PCR-based assembly method. (B) Second generation of semi-synthetic antibody repertoires – nCoDeR[®] principle [87,88]. Natural LC and HC CDRs are combinatorially inserted into one LC or HC master framework. (C) Second generation of semi-synthetic antibody repertoires – Genentech's 4D5-based library [90]. Based on the n-nCoDeR[®] principle, the LC and HC frameworks of the 4D5 antibody (Herceptin[®]) were used as the master frameworks and natural CDRs with diversification in specific positions, created via tailored codons, were inserted into them. (D) Second generation of semi-synthetic antibody repertoires – Dyax's libraries [92]. In the most sophisticated library, LC variable regions were obtained from autoimmune donors, whereas the HC was made up of synthetic and natural elements at once. Hot spots mutations were introduced into CDR1 and CDR2, while the natural diversity of CDR3 that was obtained from autoimmune patients and healthy individuals, remained unchanged. (E) Multiple frameworks – MorphoSys's fully synthetic HuCAL[®] libraries [95]. Fully synthetic CDRs, formed through gene synthesis techniques, were inserted into multiple master frameworks (7 HCs and 7 LCs, both λ and κ). (F) Genentech's restricted diversity libraries [99]. On the basis of 4D5 framework, synthetic limited randomization, biased in favor of Serine and Tyrosine, was introduced into paratope and CDR conformation determining residues in HC CDRs and CDR-L3. In CDR-L1 and CDR-L2, constant changes were made.

that Fab libraries performances benefitted from carrying restricted diversity of CDR-H1 and CDR-H2 combined with high variability in both length and in residues composition of CDR-H3. In practice, tailored codons enabled the diversity restriction of CDR-H1 and CDR-H2 and when all 20 natural amino acids were allowed in CDR-H3 in combination with LC CDRs specific randomization, the affinities of anti-murine vascular endothelial growth factor (VEGF) reached low pM values. A year later, Dyax (Boston, MA, USA) reported the construction of semi-synthetic second generation Fab phage display libraries [102]. The LC repertoire included both $V_L\lambda$ and $V_L\kappa$ obtained from donors with autoimmune diseases. The HC repertoire combined both synthetic and natural elements: DP47 was used as the V_H master framework, specific hot spot positions (contributing to the antigen-combining site, surface exposed or usually diversified by *in vivo* affinity maturation processes) in CDR-H1 and CDR-H2 were subjected to codon-based mutagenesis, based on *in silico* design, CDR-H3 was obtained from IgM displaying B-cells from both healthy and autoimmune donors. The antibody repertoires ($\geq 1 \times 10^{10}$ clones) were displayed in both “3 system” and “3 + 3 system” (phage and phagemid systems, respectively) and antibodies with sub nM affinities were isolated. A few years later, this semi-synthetic Fab library, in its phagemid display format, was altered to enable the isolation of antibodies against small charged carbohydrates. The alternations were limited to CDR-H3 and included the introduction of basic amino acids at specific positions, based on sequence alignment with CDR-H3 of anti-carbohydrate antibodies. This library included 2×10^9 members and it gave rise to antibodies targeted against sialyl-Lewis X glycan and heparan sulfate [103]. In an attempt to construct synthetic libraries that enable maximal structural diversity of antibodies, MorphoSys had developed the “HuCAL[®]” (Human Combinatorial Antibody Libraries) phage display series of libraries. These libraries are fully synthetic antibody repertoires, as they were constructed via total gene synthesis techniques and they are based on the consensus sequence of each of the 7 V_H and 7 V_L (3 V_{λ} , 4 V_{κ}) germ-line families that cover more than 95% of human antibody natural diversity. In other words, while the above mentioned second generation semi-synthetic libraries are based on a single master framework, the HuCAL[®] libraries are based on 49 consensus framework combinations, optimized for *E. coli* expression, for providing the CDRs with the optimal structural support. The first HuCAL[®] libraries included synthetic diversification of only CDR-L3 and CDR-H3, using mixed trinucleotides biased towards natural human antibody CDR3 sequences. These libraries of $\sim 10^9$ members enabled the isolation of antigen specific antibodies with affinities in the nM range [95]. In the second version of HuCAL[®] libraries, called HuCAL Gold[®] [104], all 6 CDRs were diversified, Fab library size increased to $>10^{10}$ individual clones and accordingly the affinity of isolated antibodies reached pM values. Furthermore, library Fabs were displayed on the tip of the phage coat using CysDisplay technology. The most recent third version, HuCAL Platinum[®], includes the novel design of CDR-H3 which is now assembled from 3 length-dependent sub-designs that take the natural biases of human CDR3 sequences into consideration. In addition, the latest library design includes the replacement of unproductive frameworks with more promising ones, in order to maximize the library functionality, and also the new design avoids undesired motifs (such as N-linked glycosylation sites, cryptic splice sites and RNA instability motifs) that complicate and extend industrial-scale antibodies production [105].

The first and second generations of semi-synthetic and synthetic antibody libraries utilized various approaches to compare the level of diversity in the synthetic CDRs to the diversity level formed *in vivo*, or even exceed it. An opposite approach developed at Genentech aims at the reduction of the diversity level in the CDRs to the minimal level necessary to produce high quality

synthetic antibody repertoires. This approach is supported by CDR sequences and structural data that points at the existence of biases in amino acids diversity throughout the CDRs as well as biases in paratope compositions [106]. At first, Fab libraries which were constructed on the framework of humanized 4D5, included restricted randomization of solvent accessible CDR positions, limited to Tyrosine, Serine, Alanine and Aspartate. These libraries enabled the isolation of anti-human vascular endothelial growth factor (hVEGF) antibodies with affinity of single digit nM values [107]. Further reduction in the diversity level of residues composing the antigen binding site in CDR-H3, allowed randomization with a binary code consisting of only Tyrosine and Serine. Surprisingly, this minimalistic synthetic antibody repertoire resulted in the isolation of anti-hVEGF Fab with affinity in the nM range [108]. A third minimalistic Fab library, based on the former binary code synthetic repertoire, included additional diversification in the LC, diversification of CDR conformation determining residues excluded from the paratope as well as an expansion of the restricted diversity in CDR-H3 now designed to allow usage of all amino acids, except Cysteine, with biases toward Tyrosine, Serine and Glycine, enabled the isolation of high affinity antibodies, targeted at various antigens [109,110].

Applications of recombinant antibody repertoires

This third and final section of this chapter is devoted to the various applications of antibody repertoires. In general, there are two main purposes for the construction of antibody libraries: one is *de novo* isolation of antigen-specific antibodies and the second is improvement of a pre-existing antibody. As the majority of the examples given in this chapter represent the former application of antibody repertoires, this section will focus mainly on the latter.

De novo antibody isolation

Originally, construction of antibody repertoires was intended for the supply of *de novo* isolated antigen-specific antibodies. All the above-mentioned library types are suitable for this purpose and traditionally the preferred selection platform for this application was phage display. However, successful attempts using ribosome display [48] and mammalian cell display [75] were also reported. Nonetheless, although phage display is the most widely accepted, one cannot say with certainty which repertoire type and which display platform is most likely to yield the best antibodies for any particular target antigen. There is justification therefore for many formats and platforms to co-exist.

Improvement of a pre-existing antibody

Ideally, a *de novo* isolated antibody would possess all the required properties to become a successful drug candidate. A lot of effort was invested by leading professionals to combine large optimized repertoires with robust display platforms to make it possible [105]. Still, in most cases, the introduction of improvements in an already existing antibody is currently considered as an almost obligatory step for the generation of an antibody as a therapeutic drug lead. In many cases on the basis of a characterized antibody lead, a secondary repertoire is built and selected for a desired improvement. The most common improvements include affinity maturation, antibody humanization and manufacturing-related features that are described herein:

Affinity maturation

In vivo formed antibodies acquire their high antigen affinity through somatic hypermutation events that introduce diversity

focused mostly at the CDR regions [111]. This takes place within proliferating B cells that display the antigen binding capability in the form of a B-cell-receptor (BCR) combined with the process of clonal selection that ensures the survival of B cells in an environment with a decreasing antigen concentration [112]. Although many properties weigh into the chances of an antibody to make it to the clinic, the antibody affinity is apparently the most important property that influences the probability of the clinical implementation of that antibody, as it determines its potency, pharmacokinetics, safety and toxicity [22]. Hence, *in vitro* affinity maturation approaches are aimed at the affinity elevation of an already selected antibody to the sub nM range [113] en route for its commercial application. All the many *in vitro* affinity maturation strategies that were developed over the years are based on the same notion: secondary repertoires created by the introduction of sequence diversity into a pre-existing antibody lead are evaluated under stringent conditions and clones bearing improved properties are isolated. The *in vitro* affinity maturation strategies are grouped into targeted and non-targeted approaches. The targeted approaches, like CDR walking [114,115], site-directed mutagenesis [116], hotspot mutagenesis [111,117], introduce randomness into positions that are assumed to be beneficial to its antigen recognition according to structural data and *in silico* design. On the other hand, non-targeted strategies, including error-prone PCR [60], chain shuffling [118] and mutator *E. coli* strains [119], diversify random positions throughout all the pre-existing antibody-coding DNA sequence [26], and can therefore lead to the formation of non-functional clones within the secondary library. Various combinations between diversification methodologies were also reported and to-date the highest benefit in affinity (of 3 orders of magnitude) was achieved by combining error-prone PCR and DNA-shuffling via a yeast display selection tool for an anti-fluorescein scFv lead [66].

Usually, cell-surface display platforms are more suitable for the generation of secondary affinity maturation libraries, as they are smaller in size and display secreted and folded antibodies or antibody fragments. In addition, the gating applied during flow cytometry is very useful in fine tuning specificity during the affinity selection process. Presently, yeast display is considered the most powerful platform for affinity maturation [120–123].

Proponents of ribosome display herald the possibility of improving antibodies during the process of *de novo* selection. This is provided by carrying out the PCR amplification step of the RT-PCR used to amplify the affinity-selected mRNA population under error-prone conditions. Indeed, phage or cell display systems do not regularly include re-construction of the enriched repertoire into the expression vector after each panning cycle as part of the standard operating procedure (APEX selection does require re-cloning after each selection cycle [60]). Technically it is feasible, which could make it possible to re-introduce diversity into the affinity-enriched population as is done in ribosome display. In reality, since including such a step requires re-introduction of the mutated population into bacteria of mammalian cells after each affinity selection cycle, it will become laborious and less likely to be adopted.

Antibody humanization

Secondary antibody repertoires can also be used for the humanization of a lead non-human antibody. The realization of the need for humanization of non-human antibodies is almost trivial nowadays, as it has been acknowledged that non-human antibodies evoke T-cell and B-cell responses (like the generation of human anti-mouse antibodies (HAMA) in the case of rodent antibodies) that result in the rapid clearance of the therapeutic antibody and its lower efficacy [124,125]. In contrast, therapeutic humanized antibodies or fully human antibodies are well tolerated by the

immune system. Antibody humanization can be achieved either by rational methods such as CDR grafting [126], resurfacing [127] and superhumanization [128], or by empirical approaches that utilize antibody repertoires for the selection of the best humanized clone (reviewed in [129]). In the FR library approach [130], CDRs were grafted to a human HC and LC framework donors, and a combinatorial phage display library was formed after diversity introduction into crucial positions. An optimized humanized clone with 125-fold increase in its antigen affinity, compared to the initial grafted clone was obtained from that library. Another method called “Framework Shuffling” [131,132] generates a combinatorial phage library comprising the six non-human CDRs fused to human germ line frameworks. This humanization approach enables the selection of the best chain-framework combinations.

A different approach, called “Guided Selection”, uses antibody repertoires in the humanization process and was actually employed for the generation of the first FDA-approved human antibody, Humira® (Adalimumab) [133,134]. This approach used the non-human antibody as a template for the identification of human antibodies, directed against the same antigen (but not necessarily with the same paratope). According to that approach, a non-human V domain of the original antibody (V_H , for example) is combined with a human V_L library to form chimeric antibodies that are displayed on phages or ribosomes and affinity selected on the original antigen. Next, the selected V_L domains are shuffled with a library of human V_H domains which substitute the non-human V_H domain and the phage or ribosome displayed variants are once again affinity selected on the original antigen. Fully human correlative antibodies can be isolated via this methodology, without any prior assumptions.

Manufacturing-related features

Effective mass manufacturing of therapeutic antibodies in the pharmaceutical industry relies upon the efficiency of the production process. Hence, when moving from small- to large-scale production processes of antibodies, the level of expression, solubility and stability of the antibody receive more attention. Secondary antibody repertoires have been used for the optimization of such manufacturing-related features, as they enable the examination of antibody lead variants under more harsh selection conditions, not limited to affinity. In an exemplary report, secondary scFv-ribosome display libraries formed by DNA shuffling were selected under decreasing redox potential in the translation step for the isolation of stabilized clones. After five rounds of increasing selective pressure, stabilized clones were isolated and a thorough analysis revealed sequence differences and empirically confirmed the energetic stability gain [135]. Another representative example, employed a synthetic human V_H phage display library for the isolation of non-aggregating V_H enzyme inhibitors. By repetitive cycles of selecting heat-denatured phage particles after they were cooled and regained their folded structure, non-aggregative V_H domains with reversible unfolding were isolated [136]. In theory it is possible to carry out affinity selection of an antibody repertoire under a combination of selective pressures, such as affinity and stability at the same time. In reality this could severely limit the odds of isolating and leads at all.

Conflict of interest

The authors declare no conflict of interest.

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