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Phage display technology can be used for the discovery of antibodies for research, diagnostic, and therapeutic purposes. In this review, we present and discuss key parameters that can be optimized when performing phage display selection campaigns, including the use of different antibody formats and advanced strategies for antigen presentation, such as immobilization, liposomes, nanodiscs, virus-like particles, and whole cells. Furthermore, we provide insights into selection strategies that can be used for the discovery of antibodies with complex binding requirements, such as targeting a specific epitope, cross-reactivity, or pH-dependent binding. Lastly, we provide a description of specialized phage display libraries for the discovery of bispecific antibodies and pH-sensitive antibodies. Together, these methods can be used to improve antibody discovery campaigns against all types of antigens.

Keywords: Phage display; Antibody discovery; Antigen presentation; Selection strategy; Library design

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John McCafferty John McCafferty was one of the founders of Cambridge Antibody Technology (CAT) and a co-inventor of antibody phage display. In 2012, he formed IONTAS, an innovative biotechnology company using phage display to develop novel antibody therapeutics. During this period John developed IONTAS's proprietary mammalian display technology. John is also an inventor of a novel molecular fusion format (KnotBody™), wherein naturally occurring, venom-derived cysteine-rich peptides are inserted into peripheral CDR loops of an antibody. He recently formed Maxion Therapeutics to take advantage of this drug development opportunity with a particular focus on modulation of ion channels and GPCRs. Interspersed within this company formation John has held academic positions at the Wellcome Trust Sanger Institute and the University of Cambridge. This includes establishment in 2022 of a group in the Department of Medicine to develop recombinant antivenoms.

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Introduction

Phage display technology was first invented in 1985[1] for the display of peptides and, later, in 1990, the first antibody fragment was displayed on phages.[2] Since then, the technology has been used successfully for the discovery of many hundreds of antibodies for research, diagnostic, and therapeutic applications, including more than 14 antibodies that are clinically approved.[3] All aspects of the phage display methodology have been refined and advanced to enable the discovery of antibodies against challenging targets and antibodies with certain binding properties. One of the advantages of phage display, compared with other display technologies, such as ribosome,[4,5] yeast,[6] or mammalian display,[7,8] is that large libraries (diversity of $> 10^{11}$ unique clones) can be created and stored ready for selections, which allows for high-affinity antibodies to be discovered against a wide range of antigens. In this review, we address four major parameters that can be optimized to improve the outcome of antibody discovery campaigns: the choice of antibody display format, antigen presentation, selection strategy, and library construction.

Antibody formats used in phage display libraries

Phage display libraries can be designed using different bacteriophages, such as filamentous M13, fd, and f1 bacteriophages,[9] to display a variety of different antibody formats. The two most commonly used formats are single-chain variable fragments (scFvs) and antigen-binding fragments (Fabs).[10,11] ScFvs are small (25–27 kDa) monovalent antibody fragments comprising V_H and V_L domains connected by a short peptide linker.[12] Fabs are 50 kDa in size[13] and comprise V_H, V_L, C_L, and C_H1 domains.[14] There is potential for loss of affinity on conversion of scFv to the Fab/IgG format,[11,15] which might be less of an issue for antibodies discovered in the Fab format.[11,15,16] However, Fabs generally do have lower expression yields than scFvs[17] and typically exhibit lower display levels on phages,[18,19] making scFvs a more robust format for libraries, particularly naïve libraries.

Other antibody formats have also been used for the construction of antibody phage display libraries, including human single-domain antibodies (human V_H) and camelid and shark single-domain antibodies (V_HH and V_{NAR}, respectively). V_HHs are small (12–15 kDa[13]) and comprise the antigen-binding fragment from heavy-chain-only antibodies. With conventional antibodies, the interface that mediates pairing of V_H and V_L incorporates hydrophobic residues that are buried in the interface. In V_HHs, these are substituted with more hydrophilic residues, which results in increased water solubility and a decreased tendency to form aggregates.[20] The complementarity-determining region 3 (CDR3) loop in the V_HH is often elongated compared with conventional antibodies, which allows the V_HH to bind antigens that would be inaccessible for conventional antibodies, such as catalytic clefts of enzymes or receptor domains.[21,22] The V_{NAR} antibody fragments are similar to the V_HH antibody fragments in size, with the notable exception that they only have two CDR loops because of a deletion of a large portion of the Fr2-CDR2 region.[23]

Which of the antibody formats to choose for a phage display campaign is dependent on the final application of the discovered antibody. If the application is therapeutic and a long half-life is beneficial or engagement of effector cells is needed, an scFv or Fab library might be optimal, because they allow for easy reformatting to the commonly therapeutically used IgG format. For research reagents and diagnostic applications, or when the cost of large-scale manufacture is a major concern, a format such as the V_HH might be most optimal, although this format can also be fused to an Fc-region to create a V_HH-Fc molecule with similar properties as an IgG in terms of half-life and effector cell engagement. Taken together, it is vital to delineate the requirements for the final antibody product to select the most suitable type of library.

Antigen presentation strategies

For a successful phage display-based antibody discovery program, it is crucial that the conformation of the included antigens resembles the conformation that the antigens will have in the final application. Otherwise, the discovered antibodies could end up only recognizing the antigen in an altered conformation. Therefore, an initial and critical step for a phage display campaign is to determine the optimal strategy for antigen presentation.[24]

Antigen presentation through direct or indirect immobilization

The most widely used antigen presentation strategy is to directly or indirectly immobilize the antigen on a surface (Fig. 1a). In direct immobilization, the antigen is coated on the surface using passive adsorption. This strategy is by far the simplest for antigen presentation; however, it is not well suited for many types of antigen that alter their native conformation upon adsorption.[25] It can be particularly more problematic for small antigens that might not exhibit enough intermolecular attraction forces to exert passive adsorption.[26] For some of these antigens, indirect immobilization can be used instead of direct immobilization.

Through indirect immobilization, the antigen is captured on the surface using a capture molecule. The most popular technique exploits the strong binding between streptavidin/neutravidin and biotin, whereby the surface is coated with streptavidin/neutravidin, and the antigen is conjugated to biotin via a linker or tag.[27,28] This enables an indirect, yet stable, attachment of the antigen to the surface.[27,28] The antigen is more likely to retain its native conformation through indirect immobilization because the antigen is raised from the selection surface. However, it is crucial not to overbiotinylate the antigen, because this can obscure important epitopes or result in antigen aggregation.[29]

Two different strategies for biotinylation exist: site-specific or random biotinylation. Site-specific biotinylation can be achieved using biotinylation acceptor peptides (BAPs) comprising an enzymatic biotinylation site.[30,31] One of the most widely used BAPs is the AviTag, which requires recombinant expression of the target antigen fused to the 15-amino acid peptide tag.[32] The AviTag sequence is biotinylated at its lysine residue by the *Escherichia coli* biotin ligase, BirA.[33] The AviTagged antigen can be co-expressed with BirA in bacterial cells, yeast, and mammalian cells to achieve *in vivo* biotinylation.[34,35] Alternatively, purified AviTagged antigen can be incubated with purified BirA and biotin to achieve *in vitro* biotinylation.[36] Biotinylation using BAPs

results in site-specific addition of a single biotin per antigen, thus controlling the antigen-to-biotin ratio and avoiding overbiotinylation. Nevertheless, it might not be possible to use the AviTag system in all cases, especially when it is difficult/unpractical to express the target antigen recombinantly, or when the AviTag would interfere with a potentially important (terminal) epitope of the antigen.

As an alternative to BAP biotinylation, random chemical biotinylation can be used. In this method, purified antigen and biotinylation reagents, using a variety of possible reaction chemistries, are mixed to achieve a covalent linkage between the antigen and biotin. A variety of linkers are available, making it possible to biotinylate antigens at the primary amines (N terminus or side chain of lysine residues[37]) or sulfhydryl and carboxyl groups.[38,39] Although faster and cheaper than enzymatic biotinylation (by the *E. coli* biotin ligase), chemical biotinylation needs titration to achieve the desired 1:1 antigen-to-biotin ratio.

Indirect immobilization of an antigen can also be used based on different peptide tags and tag-specific capture molecules. This requires the recombinant expression of the antigen in fusion with a peptide tag and the selection surface to be coated with the fusion tag-specific capture molecule. Binding between the tag and the capture molecule results in immobilization of the antigen. Although less popular than the biotin–streptavidin system, His-tags and anti-His antibodies or other His-capturing molecules have been exploited for antigen presentation in phage display selections.[40] Recently, a peptide–protein ligand pair, known as SpyTag/SpyCatcher, derived from fibronectin-binding protein in *Streptococcus pyogenes,* was used for antigen presentation in phage display selections.[41,42] The binding between SpyTag and SpyCatcher occurs via an isopeptide bond and has been reported to be irreversible, specific, and robust to various conditions, such as pH, temperature, and buffer.[41].

Antigen presentation through whole-cell panning

Even though indirect immobilization is suitable for displaying many antigens, it is often not optimal when it comes to presenting antigens such as membrane proteins. Membrane proteins typically contain hydrophobic transmembrane regions and might be a part of a multisubunit protein complex; as a result,

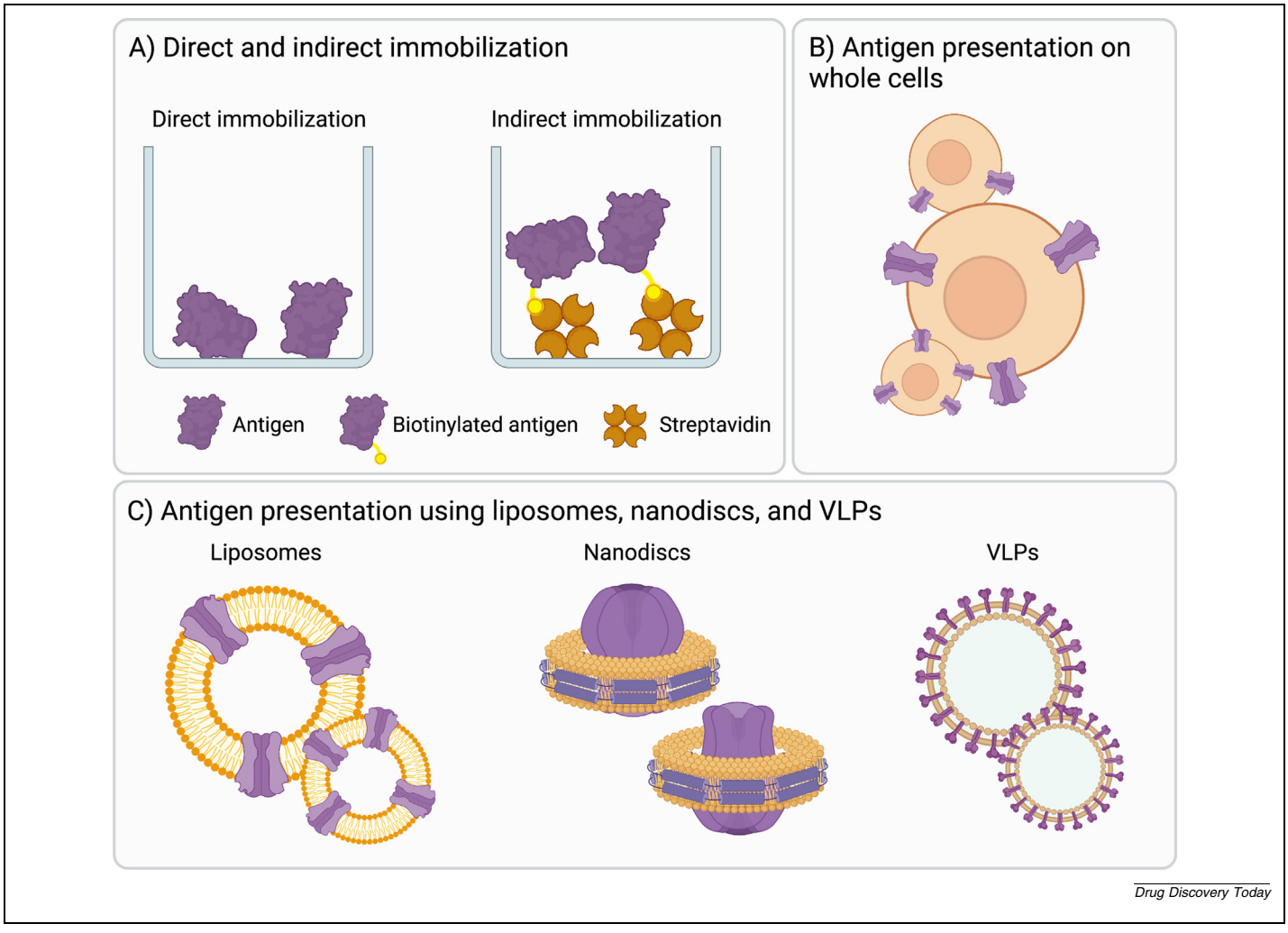

FIG. 1

Antigen presentation strategies. **(a)** Direct and indirect immobilization. **(b)** Antigen presentation on whole cells. **(c)** Antigen presentation on liposomes, nanodiscs, and virus-like particles (VLPs).

they lose their native conformation when isolated from their natural environment.[43] To conserve their conformation, membrane proteins can be expressed on a cell membrane (Fig. 1b). Mammalian cell lines, such as human embryonic kidney (HEK) cells or Chinese hamster ovary (CHO) cells, can be either transiently or stably transfected with a target protein to overexpress it and obtain a high density on the cell surface, while retaining the native conformation of the antigen.[43–46] Although selections are sometimes performed using cultured primary cells, it has been shown that cultured cells can alter their protein expression levels compared with primary cells[47]; to overcome this potential issue,[48] primary cells without culturing can be used for selection.[49] Other cell expression systems, including *E. coli*, yeast, and insect cells, can also be used to express and present membrane proteins.[50–52].

One problem with phage display selection on whole cells is that the target antigen, whether endogenous or recombinantly expressed, will represent only a small proportion of the total protein milieu presented to the library. To overcome this, deselection techniques can be used as described below. In addition, when transfected cells are used, the host cell can be altered between the selection rounds to focus selection on the recombinant antigens present on both cells.[43] Another challenge is that phage particles can nonspecifically adsorb to the cell surface via their coat protein (independently of their displayed antibody fragment). To counteract this, washing using low pH can be applied.[43,53] Furthermore, some phages can bind nonspecifically to dead cells and cell debris in the cell suspension used for panning. To reduce enrichment of such nonspecific binders, it is important to ensure that most of the cells used for selection are viable.[43,54]

Antigen presentation through liposomes, nanodiscs, and VLPs

Membrane proteins can also be presented on amphiphilic structures, such as liposomes, nanodiscs, and virus-like particles (VPLs) (Fig. 1c). Liposomes are spherical vesicles comprising a volume of aqueous solution enclosed by one or more lipid bilayer membranes, usually composed of phospholipid molecules. The phospholipid bilayer membrane mimics the environment of a plasma membrane and creates a suitable platform to present membrane proteins.[55,56] Presenting antigens on liposomes requires the formation of the liposomes, extraction of the antigen from its native membrane environment (whether isolated from natural source or recombinantly expressed), and finally transferring the extracted antigen to the preformed liposomes. When recombinantly expressed, the antigen can be fused with a tag, which can be used later for purification.[57]

Membrane proteins can also be presented on nanodiscs, which are nanometer-sized discoidal structures comprising phospholipid bilayers encircled by two amphipathic helical protein belts, termed 'membrane scaffold proteins' (MSPs).[58] Purified membrane protein can be mixed with phospholipids and MSPs to obtain membrane protein-carrying nanodiscs[59,60] (Fig. 1c). The protein belts constrain the size of the bilayers, resulting in a more monodispersed and consistent size distribution of nanodiscs compared with liposomes. Furthermore, nanodiscs provide a more stable environment for the membrane proteins and can be stored for a longer period compared with liposomes.[61,62] Moreover, because of their discoidal structure, proteins incorporated in the nanodiscs are accessible from both sides of the membrane. This is beneficial when access to both the extracellular and intracellular domains of membrane proteins is required. Both liposomes and nanodiscs can be used to present ion channels and multitransmembrane proteins, such as ion channels and G-protein-coupled receptors, which have until recently proved difficult to express/purify. However, both liposomes and nanodiscs rely on detergents to extract membrane proteins, which can alter the structure of the protein. As an alternative, a detergent-free approach, using styrene maleic acid (SMA) copolymer, can solubilize membranes into lipid nanodiscs, which are nanometer-sized discoidal structures comprising a phospholipid bilayer encircled by SMA copolymer resulting in a structure called a 'styrene maleic acid–lipid particle' (SMALP).[57,63,64] The detergent-free extracted protein can also then be incorporated into liposomes for antigen presentation.[57].

Cytotoxic proteins can cause growth retardation and toxicity to the host cells when overexpressed, making them difficult to express.[65] Cytotoxic and membrane proteins can be synthesized in a cell-free manner in a reaction comprising modified cell lysates, which provide a suitable environment for the target protein expression,[66] potentially combined with membrane-mimicking structures, such as liposomes and nanodiscs, which capture and present the newly synthesized proteins.[67] Membrane protein presentation on nanodiscs has been successfully implemented for phage display.[62,68].

VLPs are another alternative for presentation of membrane proteins suitable for phage display.[69] VLPs are non-infectious, virus-like multiprotein structures that lack the viral genome, but contain the viral capsid proteins.[70,71] Target membrane proteins can be transiently overexpressed on the surface of the capsid-expressing host cell. The self-assembling viral capsid protein directs the budding of the plasma membrane, resulting in the formation of VLPs studded with target antigens (Fig. 1c). It is also possible to first synthesize the VLPs and then covalently attach the target proteins to their surface.[72] Compared with liposomes, VLPs are more stable and can present antigens at higher density. However, VLPs have a high cost, because commercially available VLPs are expensive, and their production in the lab can be laborious.[72].

Advanced phage display selection strategies

An antibody discovery campaign using phage display selection can be conducted using various strategies and protocols. These strategies should be carefully selected to maximize the chance of discovering an antibody with the desired characteristics. Here, we present different strategies that can be used to discover antibodies with binding characteristics, such as cross-reactivity, high selectivity, or pH dependence.

Deselection strategies: Antigen tags and carrier material

During a selection process, binders will potentially be selected against all antigens, including tags or fusion partners, as well support matrices, such as streptavidin beads. To overcome this, a deselection step using a nontarget is typically included to limit

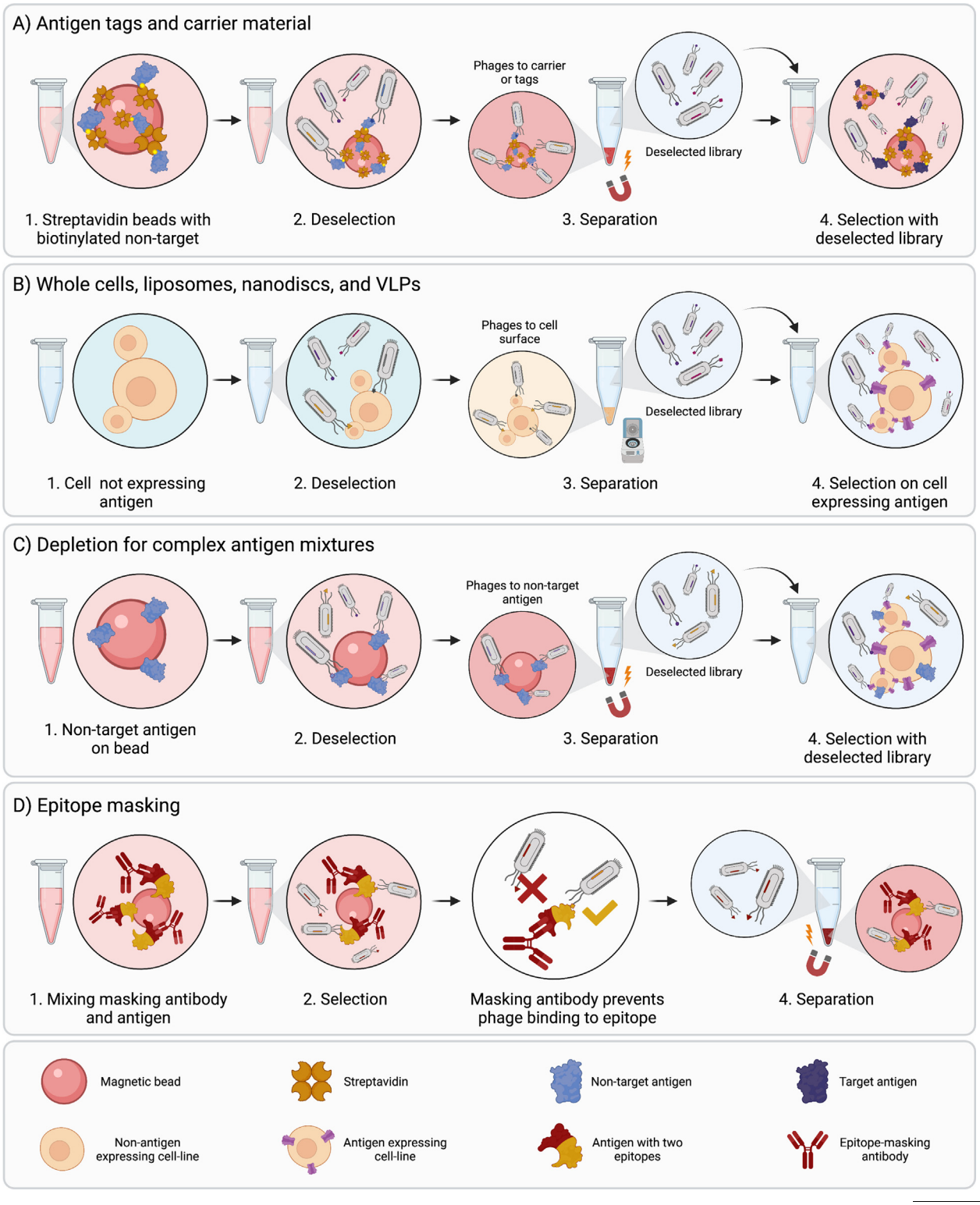

FIG. 2

Deselection strategies. **(a)** Deselection of antibodies against antigen tags or carrier material. **(b)** Deselection when presenting antigens on cells, liposomes, nanodiscs, and virus-like particles (VLPs). **(c)** Depletion of phages when selecting on complex antigen mixtures. **(d)** Using antibodies for masking specific epitopes.

enrichment of antibodies against antigens other than the target. For example, selection on a biotinylated protein using streptavidin beads can be preceded by exposing the library to a biotinylated nontarget protein coupled to streptavidin beads to reduce the proportion of such unwanted binders progressing to the selection step on the intended target[73–75] (Fig. 2a).

Deselection strategies: Whole cells, liposomes, nanodiscs, and VLPs

The same principle can be applied to more complex targets, such as whole cells, liposomes, nanodiscs, or VLPs. When panning on whole cells, a cell transfected to express e.g. a surface receptor of interest is used to display the antigen, and antibodies specific to the receptor can be enriched using a mock-transfected cell or possibly an untransfected cell for deselection.[43,44,54] Moreover, for discovery of antibodies against viral targets, selection can be performed using an infected host cell as antigen and lysate of uninfected host cells for deselection.[76] When cells with an endogenous expression of the target are used, ideally, the same cell knocked down or knocked out for the antigen of interest can be used for deselection (Fig. 2b). For liposomes, nanodiscs, and VLPs, a similar strategy can be used, in which deselection is performed using the particle used for presentation without the antigen embedded, before selection on the antigen-displaying particle.[77]

A more complex scenario is when whole cells are used without knowing the target *a priori* in a phenotypic discovery campaign using mammalian[49] or bacterial cells.[78] Under such circumstances, deselection can be performed on a nontarget cell similar to the target cell to avoid enrichment against common cell surface antigens. However, the perfect match, as in the example with transfected cells, is impossible.[49,79] An example includes deselection on T cells when the goal is to identify antibodies targeting B cells.

Deselection strategies: Depletion for complex antigen mixtures

Deselection through depletion can be used for complex targets, such as whole cells or impure protein samples, also when the target is unknown. An example is selection on whole cells without knowing the target beforehand. In such cases, even though the target antigen is unknown, the nontarget antigens might be known, which allows for protein depletion to be performed. During protein depletion, the phage display library is incubated with recombinant proteins corresponding to nontarget antigens coated or captured on immunotubes or beads. Thereafter, the unbound phages are transferred to the target antigen and used for selection[80–82] (Fig. 2c).

Deselection strategies: epitope-specific deselection

For therapeutic antibodies, it is often crucial which epitope of a target antigen an antibody binds, because this can determine whether the antibody is of therapeutic value. To direct antibody binding to a specific part of the antigen, different techniques can be applied. To find binders against the ligand-binding site of a receptor, the elution step can be performed by adding high concentrations of the ligand, which will elute only antibodies competing with the ligand for binding.[83] However, a major drawback with this strategy is that mainly low-affinity antibodies are eluted, which makes it possible to use the method specifically for the reduction of the amount of low-affinity binders.[84] Antibody blocking,[81] also called epitope masking,[85–88] is another strategy for directing antibodies against a specific part of an antigen. During the selection, previously discovered antibodies binding undesirable epitopes of the antigen are included for blocking. These antibodies bind and block certain epitopes of the antigen, making some epitopes nonaccessible for new antibodies displayed on phages during the selection step. Thereby, antibodies binding new epitopes can be enriched (Fig. 2d). In addition to antibodies, receptor–ligand complexes can also be used in a similar way to deselect binders that do not recognize the same site as the receptor or ligand does.[89]

Selection with competition

In many cases, it is desirable to reduce binding to antigens that are related to the target antigen. Thus, the goal is to focus selections on epitopes that are unique to the target antigen and reduce the proportion of binders to epitopes shared with related antigens. This is achieved by prior deselection on the related antigen.[74] However, deselection is not 100% efficient and is related to the target concentration and the affinity of the binding to the shared epitope. The binding between an antibody and an antigen is an equilibrium reaction following the law of mass action. Therefore, not all antibodies are bound to their antigens at a given time point. Thus, in all deselection strategies, several antibodies that have specificity for an antigen used for deselection will not be bound to the antigen at the time point at which deselection is concluded. Consequently, these antibodies with specificity to the antigen used for deselection will be carried through to the selection phase and might bind the target antigen here. To circumvent this, as an alternative to (or in combination with) deselection, selections can be performed in the presence of competing antigens (selection with competition).

In a selection with competition, target and nontarget antigens are mixed with the antibody library, allowing for competition for antibody binding between the target and nontarget. Use of a large excess of the nontarget antigen drives binding to epitopes shared between target and nontarget antigen, increasing the fraction of recovered antibodies that bind target-specific epitopes. Therefore, after the selection step, antibodies binding to the target antigen are enriched. Strategies to collect the target with binding antibodies include labeling the target with, for example, biotin, while leaving the nontarget unlabeled (Fig. 3a). This strategy can be used both for whole cells and purified proteins.[75] Another alternative is to present the target and nontarget antigens in different ways, such as having the target antigen immobilized or coated on a plastic surface and adding nontarget antigen in solution[90] (Fig. 3b). For whole-cell selections, the nontarget cells can also be presented as membrane particles, resulting in different densities of target and nontarget antigens, which allows for separation through centrifugation.[91].

Antigens that are upregulated in diseased tissue, cells, and fluids compared with healthy samples commonly serve as relevant targets for therapy or diagnosis.[92] An alternative for discovery of both such targets and antibodies targeting these is to use phenotypic discovery. However, in this case, a classical deselection strategy using healthy samples is suboptimal, because binders

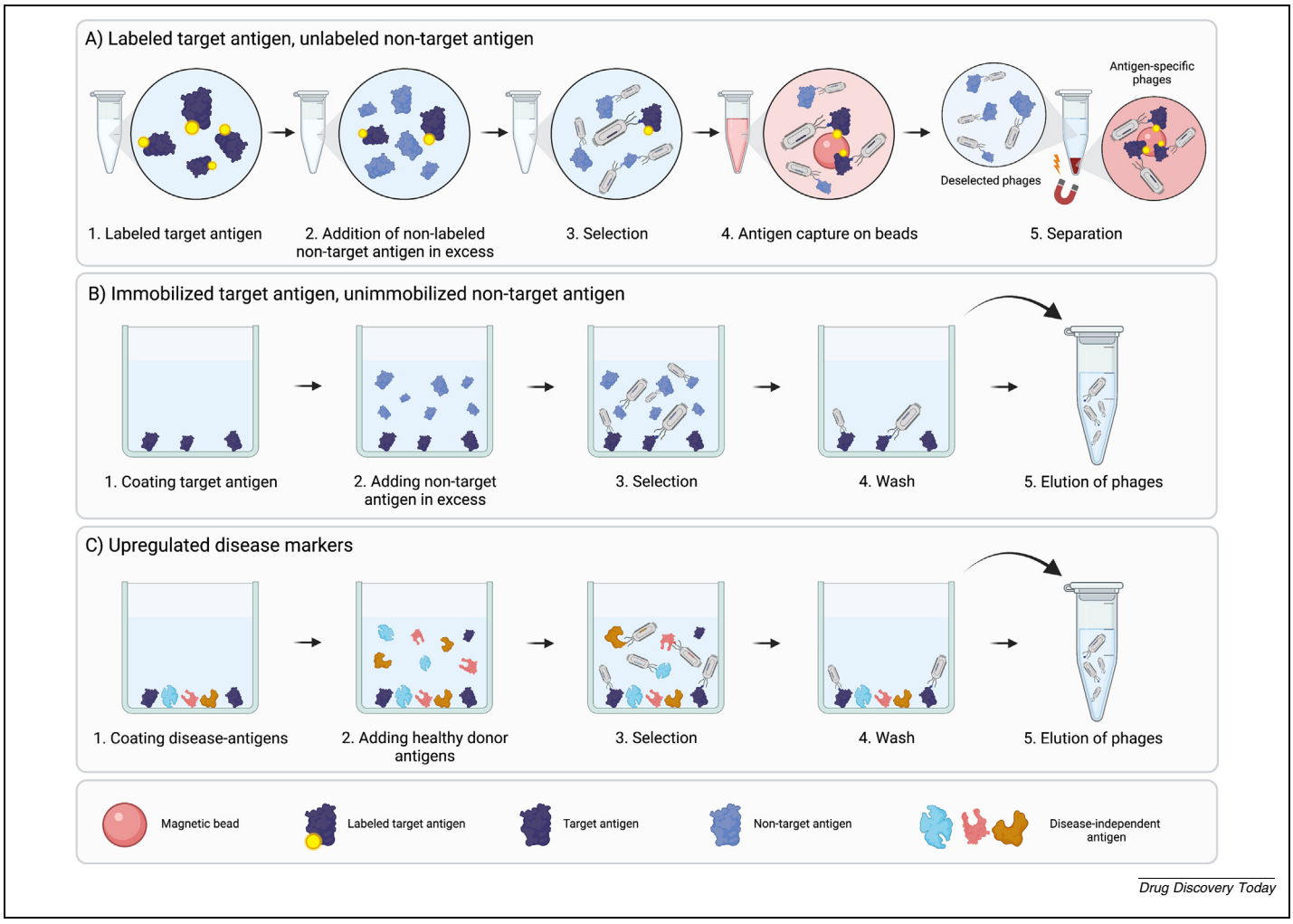

FIG. 3

Selection with competition. **(a)** Competitive selection using labeled target antigen and unlabeled nontarget antigen. **(b)** Competitive selection using immobilized target antigen and non-immobilized nontarget antigen. **(c)** Selecting for upregulated disease markers using competitive selection.

against all antigens present in the deselection step (on the healthy sample) will be reduced. Instead, including a competition during selection allows for the discovery of antibodies against these types of upregulated targets, not only the ones uniquely expressed. By varying the amount of added nontarget antigen for competition, the selection can be guided for the discovery of antibodies against a target that is upregulated to a certain degree.[93] Antibodies will compete for binding to the antigen present on both target and nontarget antigens, and the expression levels will determine whether antibodies are mainly collected and enriched or removed (Fig. 3c).

Strategies to generate cross-reactive antibodies

Antibodies are highly specific molecules, and selections are typically designed to find specific antibodies against one target antigen and avoid any binding to other molecules. However, in many circumstances, although the antibodies must be highly specific for their target, they should preferably also bind homologs or different mutated versions of the same target. For example, preclinical studies of a therapeutic antibody binding a human target will be significantly easier to conduct if the antibody also recognizes the murine and simian version of the antigen. Other examples are infectious diseases and antivenom development, where it is beneficial if broadly neutralizing antibodies recognizing several different viruses, bacteria, or toxins can be discovered.[94]

Cross-panning

A way to achieve cross-reactivity is to perform cross-panning, in which antigens are alternated between the different rounds in the selection process[95] (Fig. 4a). This technique has been used to find antibodies against conserved epitopes of HIV,[96] Influenza A strains,[97] and against cytotoxins in snake venoms from multiple species.[98] Success depends on the degree of conservation between related targets. A requirement for broad cross-reactivity against orthologs or paralogs with low conservation might result in finding low-affinity antibodies, nonspecific binders, or no antibodies.

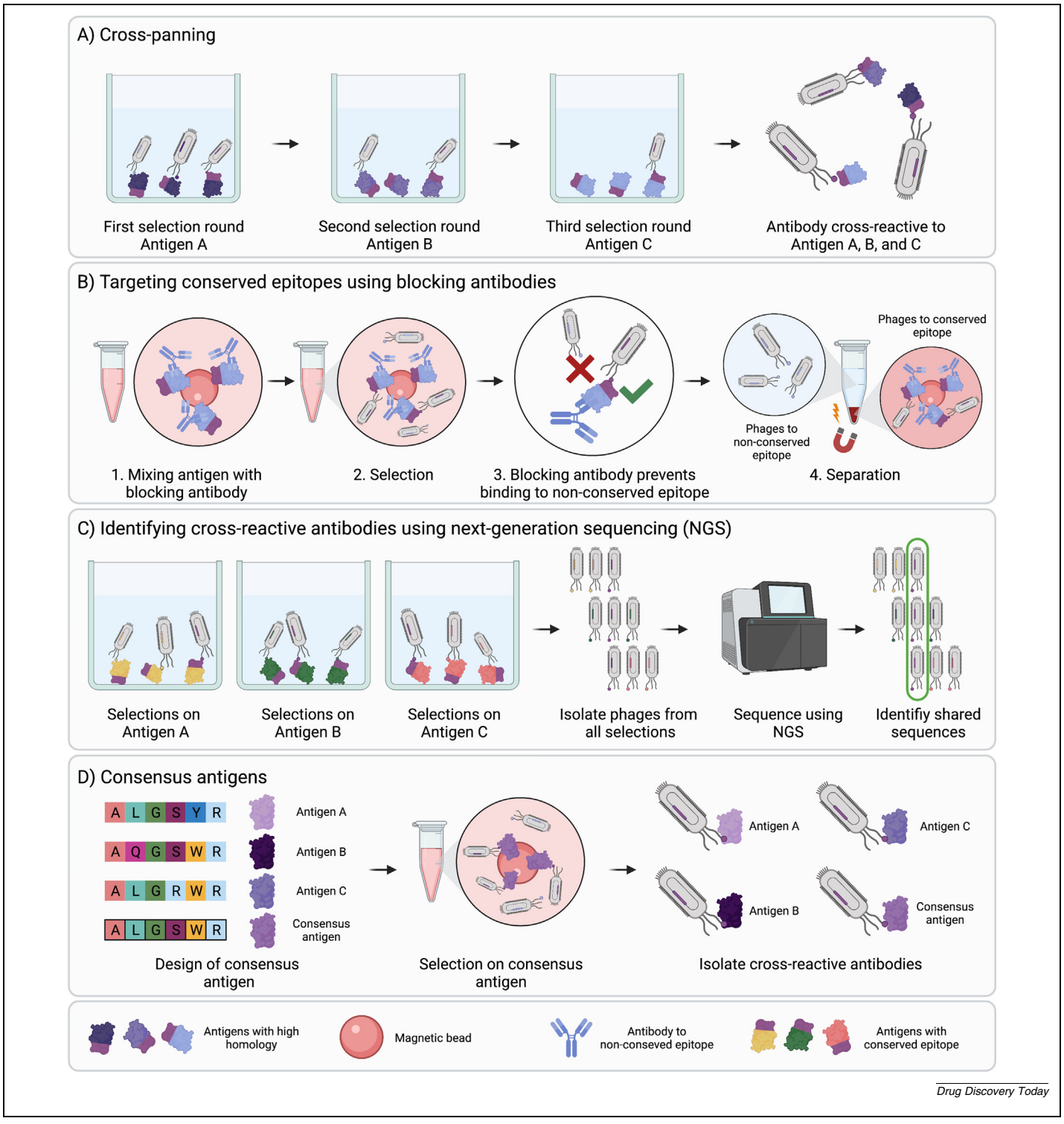

FIG. 4

Strategies to generate cross-reactive antibodies. **(a)** Cross-panning. **(b)** Targeting conserved epitopes using blocking antibodies. **(c)** Using next-generation sequencing (NGS) for identification of cross-reactive antibodies. **(d)** Consensus antigens.

Antibody blocking and next-generation sequencing

For the discovery of cross-reactive antibodies when the same antigen is used in repetitive selection rounds, selections can be guided towards conserved epitopes of the antigen using antibody blocking, as described above (Fig. 4b). Another alternative is to evaluate the output from parallel pannings on different homologous proteins, with next-generation sequencing (NGS) to identify antibodies that are enriched and found in all output pools (Fig. 4c). This has been used to identify binders against serum albumin.[99]

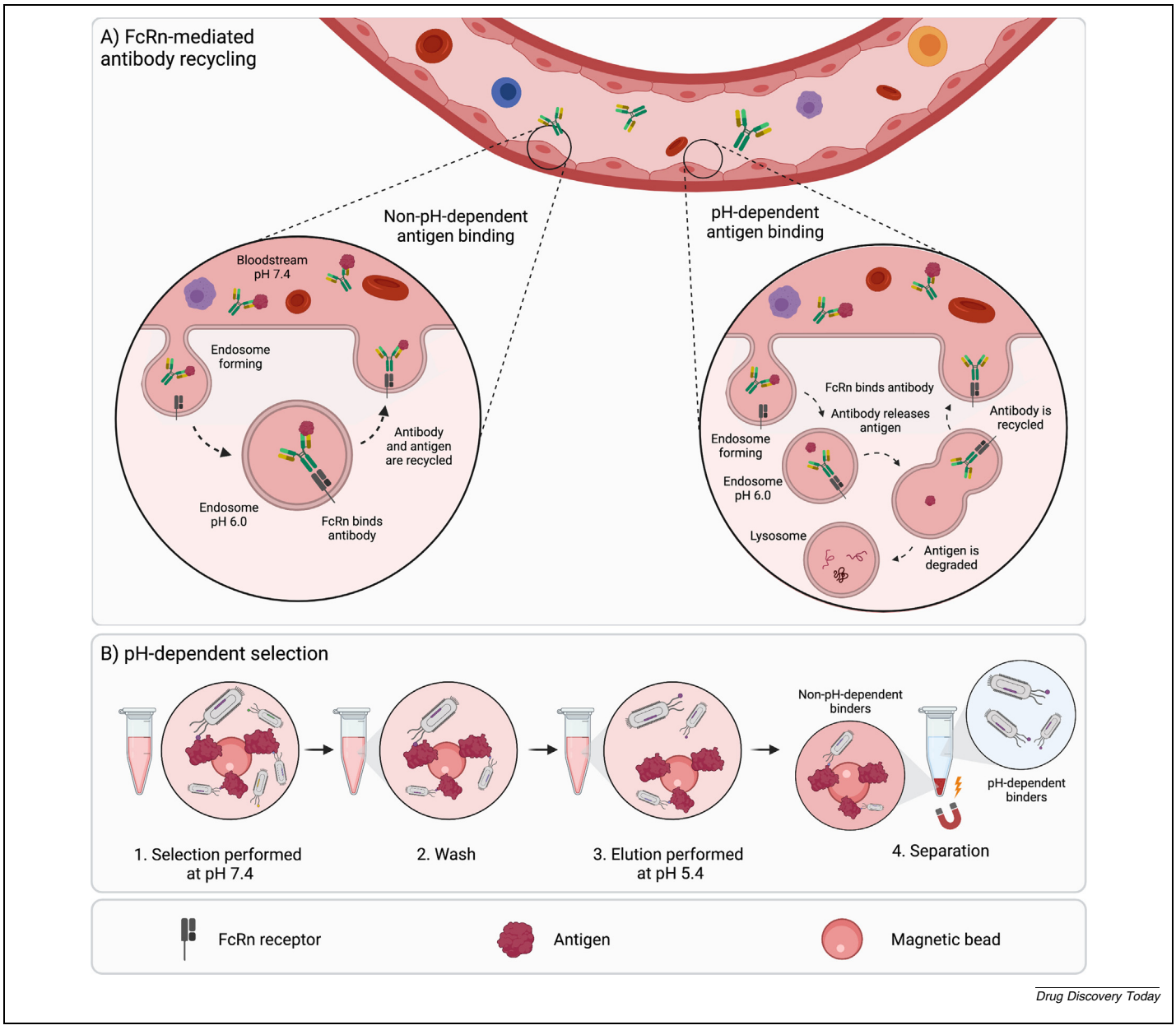

FIG. 5

Environment-sensing antibodies. **(a)** Neonatal Fc receptor (FcRn)-mediated recycling mechanism shown with and without pH-dependent binding to antigen. **(b)** Selection strategy for isolation of antibodies with pH-dependent binding.

Consensus antigens

Another alternative to identify cross-reactive antibodies is to use consensus antigens in the selections (Fig. 4d). A consensus antigen is designed by sequence alignment of multiple homologous antigens and construction of an 'average' consensus antigen, containing the most abundant amino acid in each position. In positions where multiple alternatives exist, different approaches can be taken, such as selecting amino acids based on similar chemical properties or the one with the greatest predicted immunogenicity.[100] Polyclonal broadly neutralizing antibodies against short neurotoxins from various snakes have successfully been generated using consensus toxins for immunization of horses,[101,102] and it has been hypothesized that the use of consensus antigens might also be useful in phage-display-based antibody discovery campaigns.[103]

Selection of environment-sensing antibodies

When administering therapeutic antibodies, the antibodies remain in circulation until they are endocytosed by cells. After endocytosis, the antibodies are directed to the lysosomes, where they can be recycled to the circulation via binding to the neonatal Fc receptor (FcRn). This significantly increases the half-lives of the antibodies.[104] However, when an antibody is bound to an antigen, the antigen-antibody complex is internalized and either degraded in the lysosomal compartment or recycled. To avoid recycling of the antigen as well as unnecessary antibody degrada-

tion, antibodies can be engineered to dissociate from their antigens within the acidic endosomes, allowing the antigen to be degraded while the antibody is recycled[105] (Fig. 5a). For a therapeutic antibody, this enables the antibody to be administered less frequently and/or at a lower dose to the patient. Given that the pH differs between circulation (pH 7.4) and inside endosomes (pH 5.8), antibodies binding their antigens with different affinities at different pH are desirable when the antibody is to be recycled without its cargo. It has been shown that the plasma antigen concentration was decreased by using a pH-dependent antibody, also engineered to have increased FcRn affinity, compared with a conventional antibody.[106,107] In addition, pH-dependent binding can enhance the cytotoxicity of antibody-drug conjugates[108,109] and possibly promote antibody transcytosis across the blood-brain barrier.[109] For discovery of pH-dependent antibodies, the phage selection protocol can be modified to enrich for this property. During the selection, binding is allowed to occur at neutral pH, following elution of pH-dependent binders by decreasing the pH to 5.4.[110] To optimize the chances of finding pH-dependent binders, libraries enriched for histidines can be used, which are described in more detail below.

Another strategy for increasing the half-life of antibodies is to have antibodies with binding properties dependent on the presence of ions. The calcium concentration differs between the environments in the endosomes and the plasma. Therefore, similar to pH dependence, calcium dependence can be used to recycle antibodies from the endosomes. As an example, selections against IL-6R in a calcium-containing buffer, followed by addition of EDTA to chelate Ca^{2+} and thereby elute calcium-dependent antibodies, resulted in the discovery of an antibody with accelerated clearance of the antigen from plasma.[111]

Depending on the final use of the antibodies, enrichment for additional requirements, such as improved stability, or slow off rates, can be achieved during selection. One way to increase the stability is to increase the temperature[112] or add proteases[113] during the selection step for enrichment of antibodies stable to those conditions. For discovery of antibodies with slow off rates, the antigen concentration is typically reduced in consecutive selection rounds,[114] and additional wash steps are added.[115]

In vivo phage display selection

As described previously, using whole cells as antigens for phage display selection is a valid strategy that accommodates many aspects, such as correct folding, post-translational modifications, and functionality of an antigen. However, the complexity and pharmacology of the antigen in a living organism remain lacking. Similar cell types might have completely different expression profiles or post-translational modifications because of variations in tissue microenvironments, both in healthy[116] and diseased tissues.[117]

To fully mimic the *in vivo* profile of the antigen, *in vivo* phage display technology was developed.[118] Here, a phage display library is usually administered intravenously and allowed to circulate, followed by intracardiac perfusion to remove unbound phages. Finally, the phages are rescued from the harvested, homogenized, or lysed target tissues and analyzed by sequencing. If the target of interest is known, the antibodies can be analyzed for binding before sequencing. Enrichment of phages is determined by comparing sequences present in the target tissues

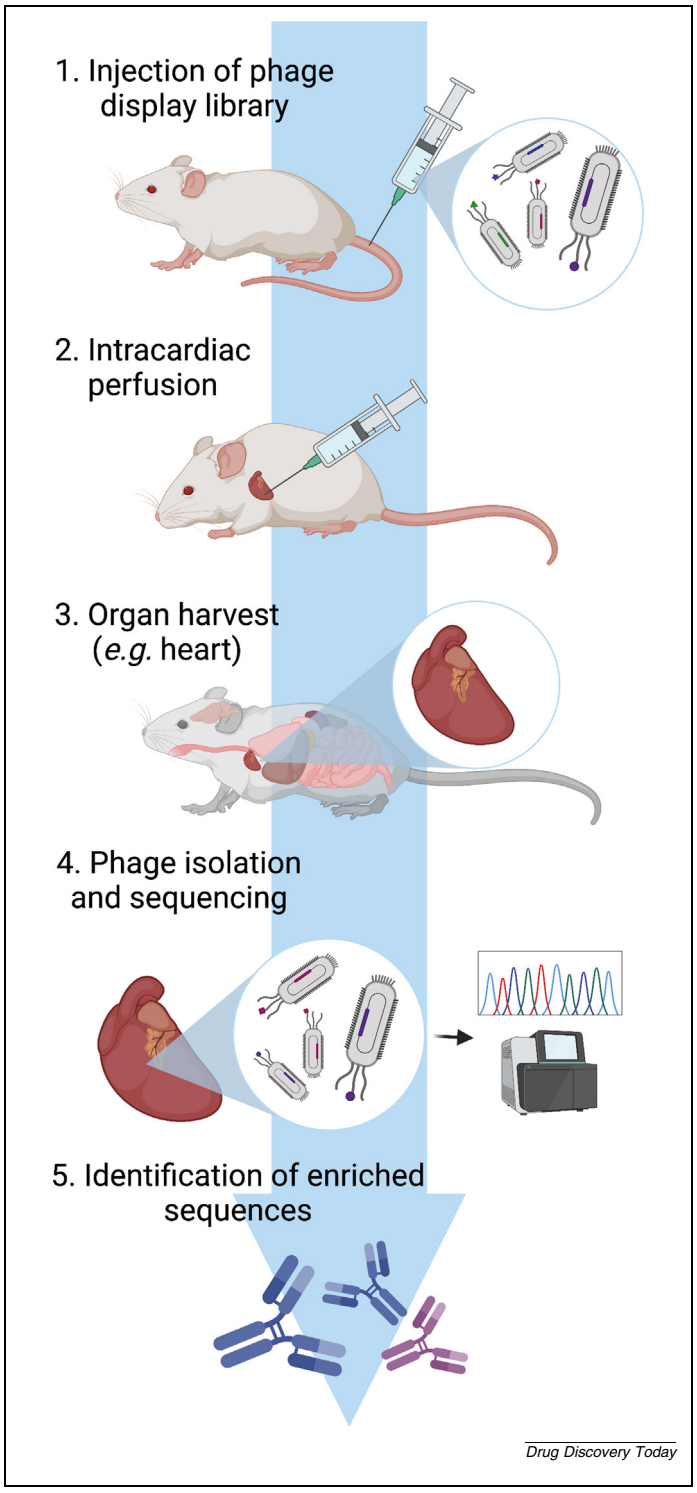

FIG. 6

In vivo phage display selection. (1) Phage display library is injected into the mouse. (2) Intracardiac perfusion is performed before (3) harvesting the organ in question. (4) Phages are isolated from the organ and the DNA is sequenced. (5) Sequences are compared to sequences from phages isolated from other organs or the input to identify enriched sequences.

with sequences present in the input or irrelevant tissues, and enriched sequences are selected for further characterization (Fig. 6).

In the original study describing *in vivo* phage display selection, peptide-based phage display libraries were used for identification of peptides that specifically bound to either brain or kidney blood vessels.[118] Antibodies in the form of scFvs[119,120] and single-domain antibodies (sdAbs)[121,122] have also been discovered using this technology. *In vivo* phage display has mainly been performed using mice or rats, although, a few studies also describe the use of this technology in humans.[123–125] However, it is not possible to perform the intracardiac perfusion step in humans because it leads to the death of the subject. To assign sequences specifically targeting the tissue of interest, phages present in the blood stream are analyzed and used for comparison.

Design of antibody phage display libraries

As described above, selections can be performed using different selection strategies dependent on the final requirements for the desired antibody. In addition to the selection methodologies used, various phage display libraries can also be used to optimize the chances of identifying an antibody with the desired characteristics. Libraries can be based on different antibody formats, on natural or synthetic antibody sequences, and can even be tailored to contain antibodies with specific biophysical or binding characteristics. Different cloning strategies, such as sequential cloning of the light and heavy chain repertoires,[126,127] splicing by overlap extension PCR,[128] or golden gate cloning,[11,129] can be conducted to link V_H and V_L during library construction. Here, we describe some of the general library types, as well as more advanced tailored library designs.

Overall, antibody phage display libraries are divided into two main classes: natural and synthetic libraries, based on the origin of the antibody sequences used for library construction. The sequences are either obtained directly from B cells[73,130,131] or synthetically created using *de novo* synthetic technologies.[131,132]

Natural libraries

Natural libraries capture the antibody repertoire of a donor and can be derived from specifically immunized or non-immunized ('naïve') donors. The immune response following an antigen challenge is accompanied by antibody class-switching from naïve IgM to secreted IgG. Therefore, naïve libraries are typically generated from the IgM repertoire of healthy donors to capture a diverse population of antibodies. By contrast, the IgG repertoire, reflecting the recent immune history of the donor, is used for immune libraries. The immune response is further driven by somatic hypermutations, resulting in improved affinity, expression levels, and antibody specificity.[133] Therefore, on average, antibodies discovered from an immune library have higher affinity than do those directly isolated from naïve libraries. However, antibody-engineering techniques enable optimization of antibodies from naïve libraries to the same or even better performance level compared with antibodies discovered directly from immune libraries.[134] Another difference is that immune libraries are typically smaller in size and can only be effectively used for discovery of antibodies against the antigen used for immunization or closely related antigens, whereas naïve libraries have broader application. For both naïve and immune natural libraries, the diversity of the library goes beyond the natural diversity,[135] because heavy and light chains,[73,127,136] as well as sometimes CDR regions,[137] are combined randomly without consideration for the natural pairing.

Synthetic libraries

Synthetic libraries can be created *de novo* with multiple frameworks and random CDRs[138] or based on natural antibody sequences with synthesis of specific regions of interest in an antibody, typically the CDR loops most likely to be involved in antigen binding.[139] Both synthetic and natural libraries have their pros and cons for use in antibody discovery, some of which will be highlighted below.

Antigen immunogenicity requirements

The creation of an immune library requires immunization with the antigen in question. This requires that the antigen is immunogenic, which is why immune libraries based on human donors cannot be efficiently created against human antigens unless B cells are taken from (naturally) infected patients.[140,141] Therefore, the creation of useful immune libraries against human antigens typically requires the use of orthologous species. For therapeutic purposes, such heterologous antibodies must be 'humanized'[142,143] following discovery. However, humanization can lead to a trade-off with potency, because residues crucial for binding in the original antibody cannot be removed entirely. Alternatively, fully human libraries for self-antigens can now be constructed through immunization of human immunoglobulin transgenic animals.[134]

Naïve libraries, alongside synthetically made libraries, have no antigen immunogenicity requirements and can be used to discover antibodies against all types of antigens, including highly conserved self antigens,[144] as well as those that are toxic to the host.[98]

Library design

Throughout the years, numerous therapeutic antibodies have been discovered through phage display selection campaigns using mainly natural naïve libraries.[145,146] Requirements for a therapeutic antibody include having high stability and a low propensity to aggregate. These traits allow the antibody to be formulated at high concentration, which is often required for administration, and lower the risk of aggregate formation, which is associated with immunogenicity.[147–149] Collectively, biophysical properties of an antibody, that can be used to predict how easy it is to be developed into a therapeutic, are often referred to as the 'developability' of the antibody.[150]

To an extent, the process of B cell maturation eliminates poorly behaved antibodies, because B cell viability is maintained by tonic signaling proportional to the level of surface-expressed B cell receptors.[151] However, the fact that an antibody is from an immune source does not guarantee good developability. Nature does not require individual antibodies to be produced in serum to the level that might be required in antibody drug formulation, where concentrations around 100 mg/ml are typically required. Thus, irrespective of the origin of the antibody, biophysical lia-

bilities sometimes emerge during preclinical/clinical development when higher concentrations are required.[30]

During library construction, developability might be increased through amplification of certain germlines from donor lymphocytes based on the selected primers. However, primer overlap with unfavorable germlines can still occur. In synthetic libraries, developability can be improved by including clinically validated scaffolds and well-paired V_H-V_L germlines.[152] Additionally, germline frameworks not conserved in all humans, such as the VH4b[153], can be omitted in the design.[154]

Finally, removal of sequence liabilities that might influence the antibody homogeneity and downstream manufacturing can be beneficial.[155] Sequence liabilities include NXS glycosylation sites; deamidation NG, NS, and NA motifs; DG isomerization; and M/C oxidation sites.[155] Naturally, the risk that a sequence liability will be important for the binding and functionality of an antibody increases with the number of sequence liabilities that are contained within the antibody paratope. Site-specific control of amino acids within synthetic libraries allow to minimize the occurrence of these motifs relative to natural libraries, which can save time on downstream engineering.

Library diversity

Central to the prospects of discovering a therapeutic antibody against a desired epitope is maximizing the sequence diversity captured within a phage display library. Immune libraries use the natural diversity and affinity maturation process *in vivo* and are enriched for binders specific to the antigen used for immunization, but their use is limited to antibody discovery campaigns directed against only that and closely related antigens. By contrast, unbiased naïve and synthetic libraries can, if they are large enough, be used for discovery of binders against any antigen of interest.[73]

First-generation naïve libraries conventionally approached maximizing diversity by increasing the number of donors used as input into the library. The existence of public clones, antibody sequences that are shared between humans,[156] affects the true diversity of such first-generation naïve libraries. Consequently, the library size, determined by the number of unique sequences, is orders of magnitudes lower than how phage display diversity is measured conventionally (by counting the number of colonies following library transformation).[157] To increase the library size, not only the number of donors used for library construction is of importance, but also simply the amount of genetic starting material used.[158]

When working with synthetic libraries, the researcher has complete control over the input sequences, which means that germlines known to present poorly on phages can be omitted. Furthermore, the use of synthetic technologies can reduce clonal dominance and result in a higher sequence diversity, typically containing > 95% unique clones.[132,154]

Although synthetic libraries are better placed to fill the theoretical sequence space of a phage library with unique clones, it is unknown whether they contain the same level of structural diversity as natural libraries. In particular, the CDR-H3 loop, the most complex and major determinant for antigen specificity,[159] is typically fixed to a narrow loop length in synthetic libraries,[160] which can be a disadvantage for certain targets. Therefore, the theoretical larger size of synthetic libraries might not translate into a more functionally diverse antibody repertoire than those obtained from naïve natural libraries.

Specialized antibody phage libraries

The choice of an appropriate library for a phage display selection campaign is one of the most crucial steps for identifying optimal antibody binders. Factors affecting the library choice include application of the end product, nature of the antigen, and the library availability in the laboratory. The emergence of new molecular methods has allowed laboratories to construct their own combinatorial antibody phage libraries to replicate the natural antibody repertoire offered by the immune system. Many of these libraries are designed with structural and sequence diversity with early-stage drug discovery in mind. However, some applications need libraries comprising antibodies with particular structural- or sequence-based characteristics.

Bispecific antibody libraries

Compared with standard human antibodies, in which both binding sites are directed against the same epitope, bispecific antibodies (bsAbs) are engineered with two binding sites directed to different epitopes. These two binding sites can be directed to the same antigen (biparatopic bsAbs) or two different antigens. In the latter, one paratope could be used to target a specific region or cell, whereas the other one could bind the antigen of interest. The archetypical application of bsAb is T cell redirection in cancer immunotherapy[161] (i.e., the redirection of the cytotoxic activity of effector T cells to specifically eliminate tumor cells), but other disease areas outside cancer are also being explored, such as inflammatory disorders, diabetes, viral and bacterial infections, and Alzheimer's disease.[162]

A huge variety of bispecific antibody formats exists, including bispecific IgGs (bsIgGs), heterodivalent V_HH dimeric constructs, and BiTEs.[162–165] However, some of these antibody formats are not simple to manufacture, such as bsIgGs, which are challenging to produce in a single host cell because co-expression of the heavy and light chains from two different antibodies results in random pairing of the chains and a complex mixture of IgG molecules. Ultimately, this reduces the overall yield of the bsIgG of interest, and the relatively low concentration of the bsIgG among the byproducts (including the incorrectly chain-paired IgGs) results in the need for elaborate purification techniques. To simplify the expression and purification processes, several strategies for chain pairing have been developed, including specialized phage display libraries with common light or heavy chains.[166] This approach allows for the concomitant expression of three different chains (instead of four) in the same cell and results in a mixture containing only two monoclonal antibodies (mAbs) and one bsAb (instead of ten different molecules) (Fig. 7a–c). Moreover, libraries with common light or heavy chains could also be used for the discovery of binding domains that can be used as building blocks to create new antibody formats.[163,167]

Chain pairing with common heavy chains

ScFv-based phage display libraries with a common heavy chain (V_H) and different repertoires of light-chain variable genes (V_L)

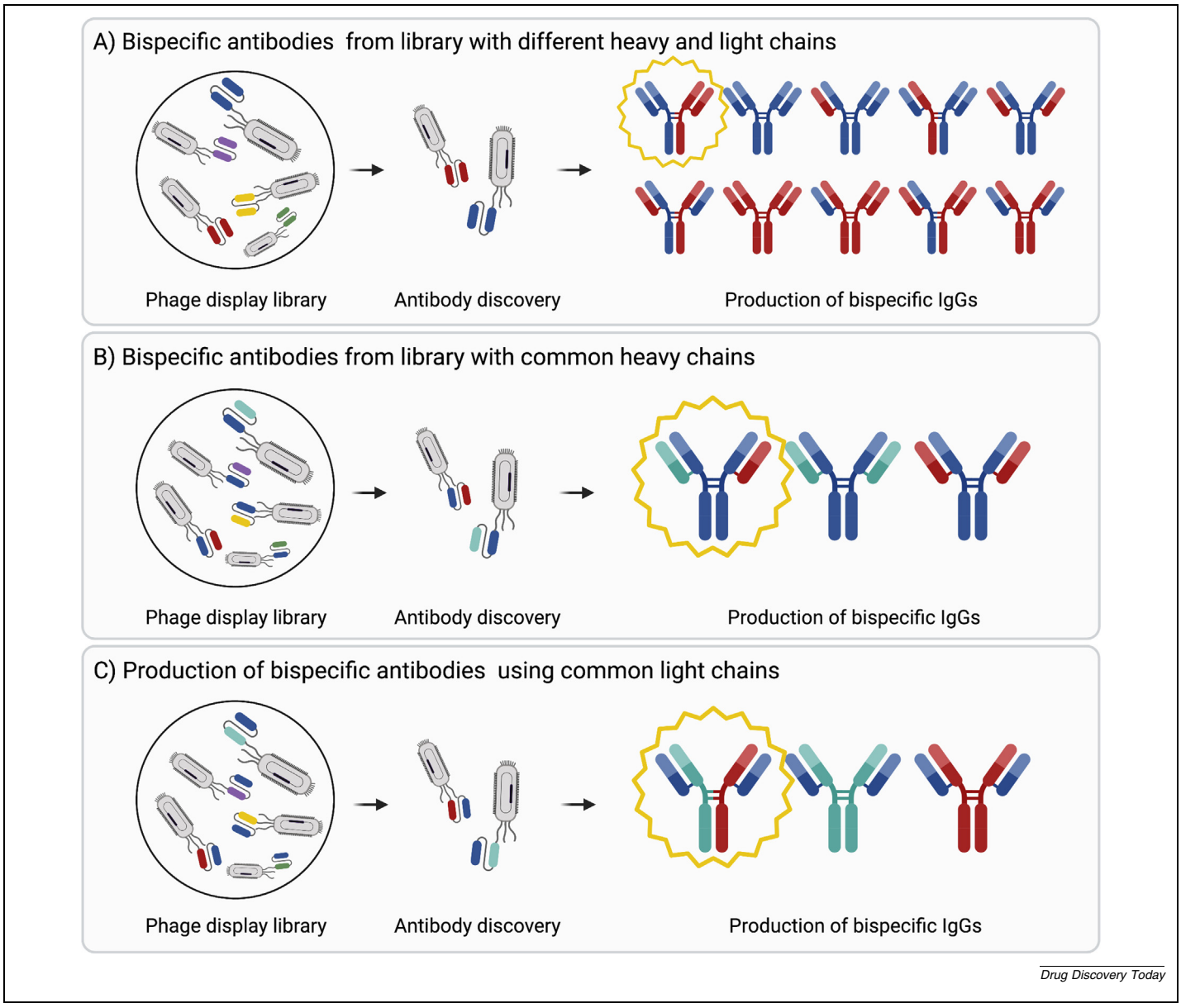

FIG. 7

Production of bispecific antibodies. **(a)** From libraries with different heavy and light chains, resulting in one bispecific antibody and nine incorrect antibody species. **(b)** From libraries with common heavy chains, resulting in one bispecific and only two incorrect antibody species. **(c)** From libraries with common heavy chains, resulting in one bispecific and only two incorrect antibody species.

can be created and used to select antibodies against two different antigens. The fixed V_H could be chosen for its favorable properties for *in vitro* display technologies, its occurrence in natural human antibody repertoires, or intrinsic stability. Alternatively, the V_H could be derived from an already existing mAb. The V_L sequences could either be isolated from circulating B cells from healthy individuals or patients, or diversity could be generated *in vitro* using advanced mutagenesis strategies.[168] This allows for the isolation of candidates with different target specificities that share the same heavy chain but carry either κ or λ light chains. Based on its structure, such a fully human bsIgG format, carrying both a κ and a λ light chain, is referred to as a κλ-body.[168] These specialized libraries have been successfully developed and used against several soluble and cell surface human antigens, resulting in the discovery of high-affinity IgGκ and IgGλ. This confirms that the light chain can be sufficient to drive antibody specificity and has enabled the isolation of high-affinity antibodies that can be used for construction of functional bsIgG for several antigen combinations.[168]

Chain pairing with common light chains

The most common strategy of chain pairing relies on the use of common light chains and distinct diversified heavy chains. This takes advantage of the high diversity in CDR-H3, which tends to dominate binding interactions in many cases.[169,170] Although the first scFv-based phage display libraries with common light chains appeared during the 1990 s,[171,172] subsequent use in bispecifics is more complicated than the corresponding heavy-

light chain pairing with a common heavy chain. Indeed, modification of the heavy chain in the Fc region is necessary to force the heterodimerization of two different heavy chains (e.g., 'knobs-into-holes' technology,[156,172,173] use of opposite electrostatic charges,[174–176] or grafting a heterodimeric interface onto the homodimeric interface of the IgG[177]). However, this strategy has been successfully applied with scFv and Fab libraries, with common light chains extracted from naïve or immune repertoires, as well as from existing therapeutic antibodies.[172,178,179] More recently, complexity has further increased with the generation of bispecific antibodies with histidine-enriched common light chains, allowing the antibody to bind its targets in a pH-dependent manner,[180] as further described below.

Libraries focused on CDR-H3

The CDR3 of the immunoglobulin heavy and light chains are the most important regions involved in antigen contacts in antibody–antigen complexes. In particular, CDR-H3 shows the largest diversity of the CDRs, both in terms of sequence and length,[169] and this diversity is often sufficient for driving the specificity of an antibody.[169,170,181] Therefore, small libraries with relatively large structural diversity can be created by focusing on diversity in the CDR-H3 residues alone.[182]

Researchers have shed light on structural differences in CDR-H3s between antibodies from different species. For example, it was found that the CDR-H3s in antibodies from galline, camelid, and bovine species are longer than the corresponding loop of human antibodies, although examples of long CDR-H3s in humans exist.[183] Galline antibodies contain a high proportion of small amino acids that are associated with flexibility,[184] and high-affinity binding galline antibodies typically have an increased cysteine content, which creates long loops with complex, disulfide-constrained structures. Using yeast display technology,[185] or immunization followed by sorting and NGS of antigen binding B cells,[186] bovine antibodies with CDR-H3s specific against epidermal growth factor receptor (EGFR) and complement component C5, respectively, have been discovered. This highlights the likelihood that long loop binders can also be discovered from bovine antibody libraries using phage display technology. In camelids, the V_HHs tend to bind with protruding loops into concave cavities on the surface of the antigen, whereas, in bovines, ultralong CDR-H3 regions form a 'stalk and knob' independently folding mini domain, similar to a knottin domain, which projects far out from the surface of the antibody and is diverse in both its sequence and disulfide pattern.[187] This 'minifold' has a general shape and dimension similar to several small disulfide-bonded protein families, including protease inhibitors, ion-channel blockers, venom toxins, and G-protein-coupled receptor ligands.[188–190] Thus, these atypical paratopes could provide the ability to interact with different epitopes, particularly recessed or concave surfaces, as exist in many enzymes, pores, and channels, compared with traditional antibodies.[191,192] As a proof of concept, an antibody format, 'KnotBodies', which are similar to the peculiar bovine antibodies and display knottin domains in place of the CDR2 loops,[193] was recently developed. These knottins, which are difficult to engineer and have short *in vivo* half-lives on their own,[194] benefit from the increased stability and extended half-life of the antibody scaffold. Both the knottin and the antibody loop sequences could be engineered and used in phage display selections to optimize binding selectivity and, as an example, the blocking potency of an antibody against an ion-channel.

Altogether, elucidation of other structural features of antibodies from other species has revealed eccentricities that can be used to bind new types of difficult-to-target epitopes, and new specialized phage display libraries with these features are coming to light.[195–198].

Side-and-loop libraries

SdAbs and antibody mimics typically bind antigen clefts via their CDR loops.[199,200] As a consequence, when the antigen epitope is different (i.e., rather convex), it is less likely that an sdAb with a convex paratope will be able to bind. Hence, researchers have generated a new recognition surface on single immunoglobulin-like scaffolds by tailoring the location of amino acid diversity to residues outside the conventional loop positions.

Koide *et al*. observed that an FN3 monobody (an antibody mimic selected from a diversified library of the tenth FN3 domain of human fibronectin), was forming a binding surface via the longest loop and the face of a β-sheet.[201] Based on this observation, they created a monobody 'side-and-loop' library,[201] in which the longest loop and the adjacent β-sheet were carrying diversity. This corresponds, by structural homology, to the CDR3 loop and the β-sheet of an immunoglobulin that mediates heterodimerization between the variable domains of the heavy and light chains. After a few rounds of phage and yeast display selection, using a 'side-and-loop' and a 'loop only' library in parallel, it was demonstrated that the two libraries performed differently against different targets. Indeed, for one target (GFP), the side library clones had higher affinity than the counterparts from the loop library, whereas, for another target (hSUMO1), the trend was the opposite. This demonstrated that alternative library designs focused on the side-and-loop surface could be more effective than conventional loop-based strategies in recognizing epitopes with distinct topography.[202–205]

Histidine-enriched libraries

The pH dependence of antibody–antigen interactions has an effect on subcellular trafficking dynamics and antibody recycling,[206] as described above. The literature has numerous examples of effective engineered antibodies with pH-sensitive binding derived from existing antibodies.[107,207–210] The principle is relatively simple and relies on the incorporation of histidine residues in the binding interface, which are ionizable at pH lower than 6. Upon protonation of these residues in the acidic endosome, structural transitions, caused by altered electrostatic interactions, account for a loss of binding to the antigen. Moreover, the total number of ionizable histidine residues involved in antigen binding impacts the degree of pH sensitivity.[208]

Whereas most examples of engineered antibodies with pH-sensitive binding used histidine scanning alone or combinatorial histidine scanning libraries derived from existing antibodies, there are only a few attempts of *de novo* isolation of pH-dependent antibodies from naïve libraries. The first example took advantage of a synthetic scFv-based phage display library

enriched in histidine residues to find pH-dependent binders to the human chemokine CXCL10.[110] The library was constructed to be histidine enriched by alternating YAT and NHT codons in 8–15 amino acid positions in the CDR-H3. However, after three rounds of selection, the pH dependency of the best clone was too low, and new libraries enriched for histidine in all the CDRs from the light and heavy chains were created. These new scFv-based libraries led to a final reformatted IgG clone with a low nanomolar affinity at pH 7.2 and a 22-fold faster dissociation rate at pH 6.0. Another recent study used a histidine-enriched and CDR3-diversified V_{NAR} domain yeast display library against EpCAM, in which only one pH-dependent binder was found.[211]

The upfront selection of optimal conformations or sequences rather than the re-engineering of an antibody that was not initially selected for pH-dependent binding properties appears an attractive idea. Indeed, it is understandable that already existing antibodies are not always amenable to transformation into pH-dependent antibodies. However, the only two examples of *de novo* isolation of pH-sensitive antibodies might be an indication of the process difficulty, including the need to generate additional sublibraries for pH dependence and affinity maturation. Furthermore, the histidine-mediated pH-dependent binding restricts the number of suitable epitopes because they need to have positively charged, or proton donating residues. In other words, a negatively charged or proton-acceptor epitope is theoretically a difficult target for a pH-dependent binding antibody.

Concluding remarks

In this review, four major parameters that can be altered to tailor an antibody discovery campaign using phage display selections have been presented: antibody format, antigen presentation, selection strategy, and design of phage display library. The information provided in this review can be used individually or in combination for designing an antibody discovery campaign, dependent on the requirements of the desired antibodies.

Declaration of interests

The authors declare that they have no competing interests.

CRediT authorship contribution statement

Line Ledsgaard: Conceptualization, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Anne Ljungars:** Conceptualization, Investigation, Writing – original draft, Writing – review & editing. **Charlotte Rimbault:** Investigation, Writing – original draft, Writing – review & editing. **Christoffer V. Sørensen:** Investigation, Writing – original draft, Writing – review & editing. **Tulika Tulika:** Investigation, Writing – original draft, Writing – review & editing. **Jack Wade:** Investigation, Writing – original draft, Writing – review & editing. **Yessica Wouters:** Investigation, Writing – original draft, Writing – review & editing. **John McCafferty:** Investigation, Writing – review & editing. **Andreas H. Laustsen:** Conceptualization, Investigation, Writing – review & editing.

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