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Peptide Affinity Chromatography Applied to Therapeutic Antibodies Purification

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Abstract

The interest in therapeutic monoclonal antibodies (mAbs) has significantly grown in the pharmaceutical industry, exceeding 100 FDA mAbs approved. Although the upstream processing of their industrial production has been significantly improved in the last years, the downstream processing still depends on immobilized protein A affinity chromatography. The high cost, low capacity and short half-life of immobilized protein A chromatography matrices, encouraged the design of alternative short-peptide ligands for mAb purification. Most of these peptides have been obtained by screening combinatorial peptide libraries. These low-cost ligands can be easily produced by solid-phase peptide synthesis and can be immobilized on chromatographic supports, thus obtaining matrices with high capacity and selectivity. Furthermore, matrices with immobilized peptide ligands have longer half-life than those with protein A due to the higher stability of the peptides. In this review the design and synthesis of peptide ligands, their immobilization on chromatographic supports and the evaluation of the affinity supports for their application in mAb purification is described.

Keywords Solid-phase peptide synthesis · Monoclonal antibodies · Biopharmaceuticals · Mass spectrometry

Abbreviations

Ab	Antibody
AC	Affinity chromatography
ADC	Antibody–drug conjugate
API	Active pharmaceutical ingredient
ELISA	Enzyme-linked immunosorbent assay
GMCSF	Granulocyte macrophage-colony stimulating factor
HIC	Hydrophobic interaction chromatography
IEC	Ion exchange chromatography
mAb	Monoclonal antibody
NHS	*N*-Hydroxysuccinimidyl
SEC	Size exclusion chromatography
SLS	Solid–liquid separation
SMPS	Simultaneous multiple peptide synthesis
SPPS	Solid-phase peptide synthesis

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Introduction

Since their development by Köhler and Milstein (1975), the interest in monoclonal antibodies (mAbs) have enormously grown in the pharmaceutical industry. The first mAb with therapeutic applications (Orthoclone OKT3) was approved in 1986. Currently, with those last approved for COVID-19 treatment, therapeutic mAbs outstrip 100 (Fig. 1). Their high specificity for their targets together with their long half-life in plasma makes mAbs and their derivates the biopharmaceuticals of choice in the treatment of many diseases. In fact, most of the biopharmaceuticals approved by the FDA in recent years are mAbs, most of them applied in oncological and autoimmune diseases. Together with canonical mAbs, several antibody–drug conjugates (ADCs) have been recently approved, facilitating the specific delivery of cytotoxic drugs to target tumor cells, increasing their efficacy and reducing chemotherapy adverse effects. As has been recently stated, 9 of the top 20 therapeutic sales are mAbs (Mullard 2021) with a global market of $106.87 billion

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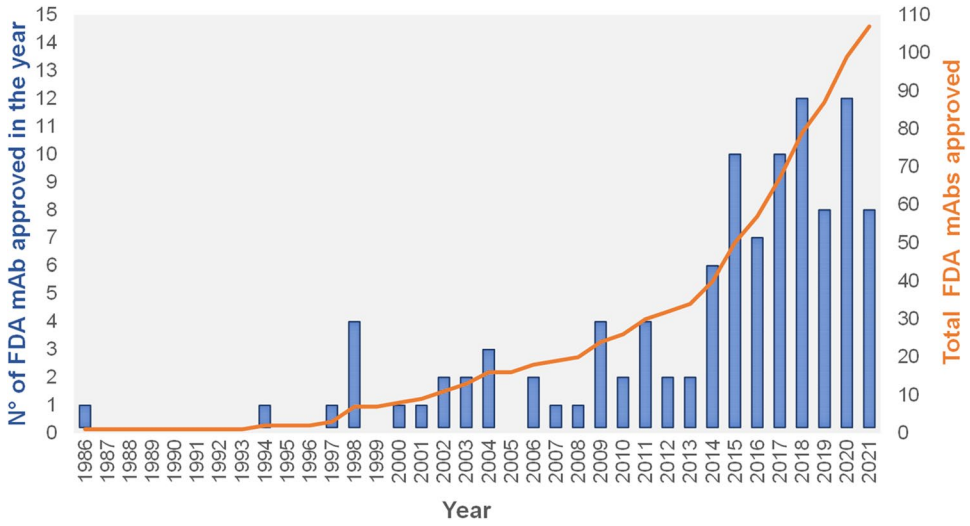

Fig. 1 Number of therapeutic monoclonal antibodies (mAbs) approved by the FDA each year (bars) and total approved (line) (updated until September 30th, 2021)

in 2020 (Global Monoclonal Antibodies (mAbS) Market Report 2021–2030).

Most of the biopharmaceuticals are produced by recombinant living cells such as bacteria, yeasts, or mammalian cells, which express the heterologous protein that constitutes the active pharmaceutical ingredient (API) (Owczarek et al. 2019). During the upstream processing, the cells are grown, and the API is produced in bioreactors. Afterwards, during the downstream processing, the API is purified from the cell culture broth (Jozala et al. 2016).

The high purity level required for their subsequent parenteral administration increases the steps and cost of biopharmaceuticals downstream processing, representing near 70% of the total manufacturing costs (Mehta 2019). As indicated in Fig. 2, the downstream processing involves recovery and chromatographic stages, the latter being the most expensive due to the high-cost chromatographic matrices. The expected purity is obtained with successive ion exchange (IEC), hydrophobic interaction (HIC) and size exclusion chromatography (SEC). Alternatively, affinity chromatography (AC) allows the purification of the target protein from complex mixtures in a single step due to the high selectivity between an immobilized ligand with the target protein, thus increasing the yield and lowering the time and cost of the overall process.

Although mAbs expression in mammalian cells has reached values greater than g/L, their purification methods do not support a scaling up that satisfies their high production level. AC with immobilized *Staphylococcus aureus* protein A is the method of choice for mAbs purification (Bolton and Mehta, 2016). However, protein A is a high-cost ligand. Although protein A can be obtained from *S. aureus* culture, its recombinant production with safe bacterial hosts is preferred. Its recovery and purification from the cell broth requires IEC, HIC and/or SEC steps

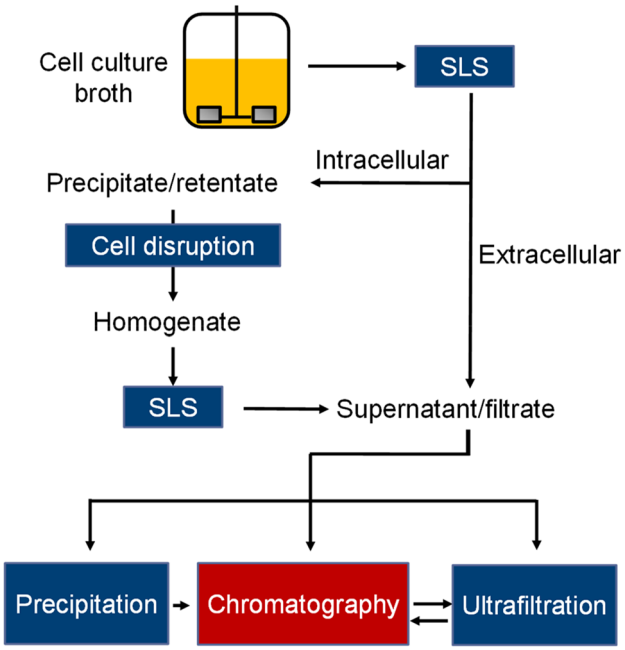

Fig. 2 Biopharmaceuticals recovery and purification steps (downstream processing) (SLS: solid–liquid separation such as filtration or centrifugation)

(Rigi et al. 2019). To develop AC resins, recombinant protein A must be immobilized on suitable chromatographic matrices. The high molecular size of protein A requires matrices with low crosslinking degree what decreases its mechanical resistance, thus hampering their industrial scale-up. Furthermore, these matrices have low capacity and short half-life. Protein A leaching from the matrix contaminates the purified mAb and shortens the useful life of the chromatographic support. Moreover, protein A low stability makes column sanitization difficult. Additionally,

the elution of the mAbs from these matrices requires extremely acidic conditions, thus favoring degradation products formation that need to be subsequently separated from the undamaged mAb. Thiophilic (Porath et al. 1985) and mix-mode chromatography (Wang et al. 2013), developed for mAb purification, are based on shorter and more stable ligands. However, they have lower selectivity than protein A.

On the other hand, short peptides can be easily synthesized at large scale on solid phase at lower cost than protein ligands. The solid-phase peptide synthesis (SPPS), consisting of successively coupling α-amino and side chain protected amino acids on a solid support, was first described by Robert Merrifield (1963) and later improved by Carpino and Han (1970), who replaced the cumbersome Boc/Bzl chemistry with the simpler Fmoc/tBu chemistry (Fig. 3). SPPS allows to obtain peptides with high purity and yield. Nowadays, a variety of peptide synthesizers are available in the market. With the introduction of microwave heating in SPPS in the past few years, the yield has been increased even further and synthesis time has been greatly shortened (Pedersen et al., 2012; Singh and Collins 2020). SPPS has been thoroughly reviewed by Jaradat (2018).

Short peptides have been employed as ligands in AC since its introduction by Cuatrecasas in 1968 who purified the enzyme carboxypeptidase A with L-Tyr-D-Trp immobilized on agarose (Cuatrecasas et al. 1968). Their low cost and high stability to a wide variety of adsorption, elution and sterilization conditions makes them ideal ligands for AC. Site-directed immobilization and high ligand density matrices can be easily obtained. Furthermore, peptide libraries screening facilitates affinity ligand design for any given protein of interest.

The design and synthesis of peptide ligands, their immobilization on chromatographic supports and the evaluation of the affinity supports for their application in mAb purification is described in this review.

Peptide Libraries Synthesis

Most ligands for AC have been developed by screening of a large collection of peptides known as peptide libraries. Several peptides can be synthesized in parallel by SPPS using small syringes or columns as reactors. To further increase the molecular diversity and facilitate the discovery of useful peptides for their application as therapeutic drugs or as ligands for AC, many methods have been developed to simultaneously synthesize multiple different peptides. Those strategies have been thoroughly reviewed by numerous authors (Liu et al. 2003; Breitling et al. 2009; Bozovičar and Bratkovič 2019; Madden 2021).

Houghten (1985) developed the tea-bag method suitable for simultaneous multiple peptide synthesis (SMPS) of more than 150 peptides in parallel. In this procedure, polyethylene plastic mesh bags, "tea-bags", are used as reactors in which each peptide is synthesized. Deprotection of the α-amino group and the washing steps are carried out placing all the tea-bags together in the same polyethylene bottle. For each coupling step tea-bags are sorted into separate groups in polyethylene bottles so that each bottle contains all the tea-bags receiving the same amino acids. For final deprotection and cleavage, tea-bags are separated to obtain each peptide in solution.

Geysen et al. (1984) described an alternative SMPS strategy known as multipin method based on multiple peptides synthesis on individual polyethylene sticks mounted on a block in an array that fit into the wells of enzyme-linked

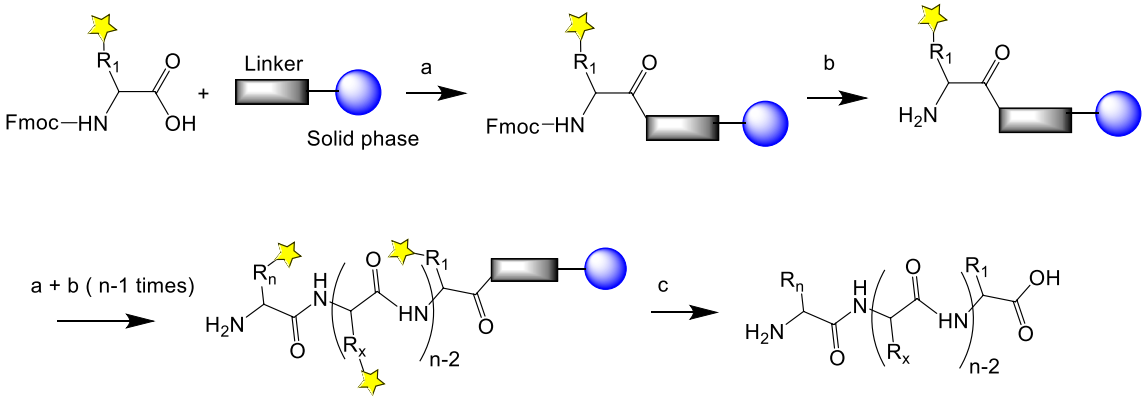

Fig. 3 Solid-phase peptide synthesis: *N*-α-protected (Fmoc in Fmoc/tBu chemistry) and side chain protected amino acid is coupled to a solid phase through a linker. After removing the *N*-α-protected group, the second *N*-α-protected amino acid is coupled. Coupling (a) and deprotection (b) steps are repeated until the desired amino acid sequence has been elongated. Finally, side chain protecting groups (stars) are removed and the peptide is cleaved from the solid support by a global deprotection step (c)

immunosorbent assay (ELISA) plates (8 rows and 12 columns) thus simplifying the subsequent screening by ELISA (Geysen et al. 1987; Tribbick 2002). Afterwards, polyethylene sticks were replaced by SynPhase Lanterns whose shape has been designed to maximize loading surface area (Ede 2002; Parson et al. 2003).

Frank (1988) designed the SMPS spot method which consist in numerous peptides synthesis on a small circular area (spot) of a cellulose membrane in arrays so that the sequence in each position can be spatially addressed (Frank 1992, 2002). To analyze the interaction of the synthesized peptide with the target antibody a dot-blot like analysis is performed (Kurien and Scofield 2015). Afterwards, a glass suitable to prepare peptide array support was developed. The glass was grafted with poly(ethylene glycol) methacrylate (PEGMA) which prevents unspecific protein adsorption during screening (Beyer et al. 2006; Breitling et al. 2009).

Nowadays, different models of automated parallel peptide synthesizer available in the market allow SMPS in columns, plates, or cellulose membranes (Pedersen and Jensen 2013).

To increase even more the diversity, combinatorial peptide libraries have been developed. They are made up of m^n different peptides representing all the possible combinations of "m" different amino acids (building blocks) in peptides of "n" amino acids long (Houghten et al. 1992). The screening of combinatorial peptide libraries has been frequently used to find ligands for AC.

Phage-display combinatorial libraries were first described by George P. Smith (1985). Soon afterwards, Gregory P. Winter optimized the technology to design humanized therapeutic mAbs such as adalimumab, approved in 2002 (Marks et al. 1991). Both were awarded with the Nobel Prize in Chemistry in 2018. Phage-display peptide libraries, with short peptides on their surfaces are usually used to design ligands with high affinity and selectivity for a target protein such as an antibody. The selected ligands are then synthesized by SPPS and immobilized on chromatographic supports. These libraries consist of modified bacteriophages such as M13 displaying different peptides on their surfaces. Random DNA nucleotide sequences encoding the diverse peptides of the combinatorial library are obtained by synthesizing oligodeoxynucleotides with degenerated codons $(KNN)^n$ where K corresponds to a mixture of all four deoxynucleotides and N to a mixture of guanidine and thymidine. Every triplet KNN can code for the 20 amino acids minimizing stop codon. DNAs encoding a library of combinatorial peptides are fused to the coat protein (pIII) gene of M13 phage and cloned into vectors used to transform *E. coli*. Afterwards, *E. coli* is infected with helper-phages to create a combinatorial library in which each phage displays only one peptide entity ("one phage-one peptide"). The screening is performed by amplifying the phage library in an *E. coli* culture. Next, *E. coli* is disrupted and inside phages are purified. Phages are incubated with the target antibody previously immobilized on ELISA plates. While non-interacting phage particles are discarded, the adsorbed particles that contain peptides with affinity for the target antibody are eluted and amplified by infection in *E. coli.* The process of amplification and screening is repeated increasing the elution power of the buffer used. Finally, the peptides displayed in those selected phages are identified by DNA sequencing (Böttger and Böttger, 2009) (Fig. 4). Many ligands for Ab purification have been developed by screening phage displayed libraries (Krook et al. 1998; DeLano et al. 2000; Ehrlich and Bailon 2001; Hatanaka et al. 2012; Yoo and Choi 2015; Khan et al. 2017). Similar biological libraries such as mRNA-display, yeast-display and ribosomal-display technologies has been also developed but most frequently applied in therapeutic drug development (Galán et al. 2016). Only peptides made up of natural amino acids can be tested with these biological libraries.

On the other hand, synthetic combinatorial libraries allowed the incorporation of non-natural amino acids as well as many structural modifications increasing the diversity.

To obtain synthetic combinatorial peptide libraries, a "pre-mix" strategy was developed in which all the building blocks (natural or unnatural protected amino acids) are mixed in a predetermined molar ratio, which compensates for their different kinetics rates (Pinilla et al. 1992, 1994; Ostresh et al. 1994). For their screening and the identification of peptide hits deconvolution methods such as iterative deconvolution (Wilson-Lingardo et al. 1996) and positional scanning (Pinilla et al. 1992) have been developed.

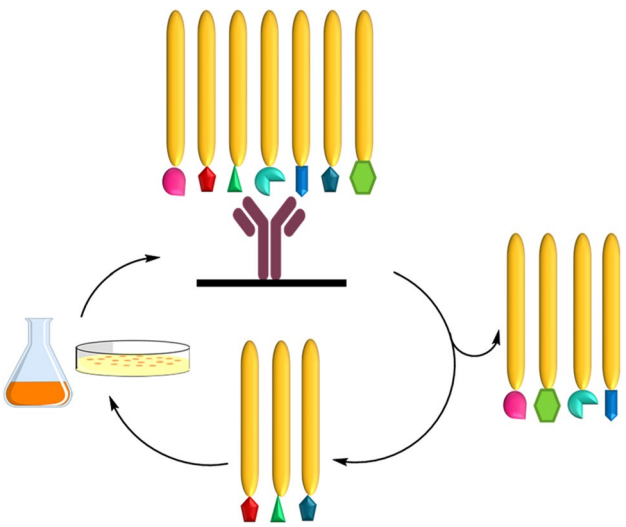

Fig. 4 Phage-display library screening: Phage particles with peptides displayed on their surface are incubated with the immobilized antibody. After washing the non-interacting phages, the ones adsorbed are eluted, isolated, and amplified in *E. coli*. Screening is repeated many times to obtain high affinity ligands

To assure an equimolecular representation of all the library components, Furka et al. 1991 developed the portioning-mixing or split and mix method. The same method was also described by Houghten et al. (1991, 1992) and called it "divide, couple and recombine" (DCR). This approach allows the synthesis of one-bead-one-compound (OBOC) combinatorial libraries frequently used to find affinity ligands (Lam et al. 1991; Lebl et al. 1995). A SPPS is performed using many building blocks. The building blocks can be L or D natural or unnatural amino acids. Each variable position in the combinatorial peptide library is synthesized by dividing the solid support into as many equal portions as the number of amino acids to vary in that position and a different protected amino acid is coupled onto each resin portion. Subsequently, the resin is recombined, the α-amino is deprotected, and the resin is conditioned for the next coupling and divided again. Divide, couple and recombine steps are repeated until the desired length of the peptide is reached. Finally, all side chains are deprotected (Fig. 5). With this method an equimolecular combinatorial library in which each bead displays only one peptide entity is obtained. Depending on the screening method, peptides are cleaved from their solid support and assayed as free peptides (Houghten et al. 1991) or they are left anchored to the resin beads for subsequent solid-phase analysis. (Lam et al. 1991; Lebl et al. 1995). Solid-phase analysis is usually applied to find suitable ligand for AC (Saavedra et al. 2018; Barredo et al. 2021). To assay combinatorial peptide libraries while attached on the resin beads, water-compatible resins such as PEGA, TentaGel or ChemMatrix must be used to allow the on-bead screening in aqueous buffers. To screen these combinatorial libraries, thousands of peptidyl beads are mixed with the target Ab labeled with a reporter group such as biotin or a fluorescent dye. Positive beads with the labeled Ab adsorbed are isolated. Complex Object Parametric Analyzer and Sorter (COPAS™) BIO-BEAD flow sorting equipment (Union Biometrica) is used to isolate fluorescent beads automatically (Kodadek and Bachhawat-Sikder 2006; Marani et al. 2009), thus accelerating the screening process. The peptide on each isolated bead is subsequently sequenced. Even though Edman microsequencing is still used for peptide sequencing, it is an expensive and time-consuming strategy when hundreds of peptides must be analyzed as usually happens after combinatorial peptide library screening. Soft ionization techniques such as electrospray (ESI) or matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) tandem mass spectrometry (MS/MS) are rapid and inexpensive strategies to identify those peptides on positive beads (Fenn et al.1989; Karas and Hillenkamp 1988). A linker between the peptides and the resin is necessary to release them from each bead before MS/MS analysis (Camperi et al. 2005; Martínez-Ceron et al. 2010; Barredo et al. 2021). Numerous ligands for Ab purification have been developed by OBOC libraries (Fassina et al. 1996; Camperi et al. 2003; Verdoliva et al. 2002; Yang et al. 2008).

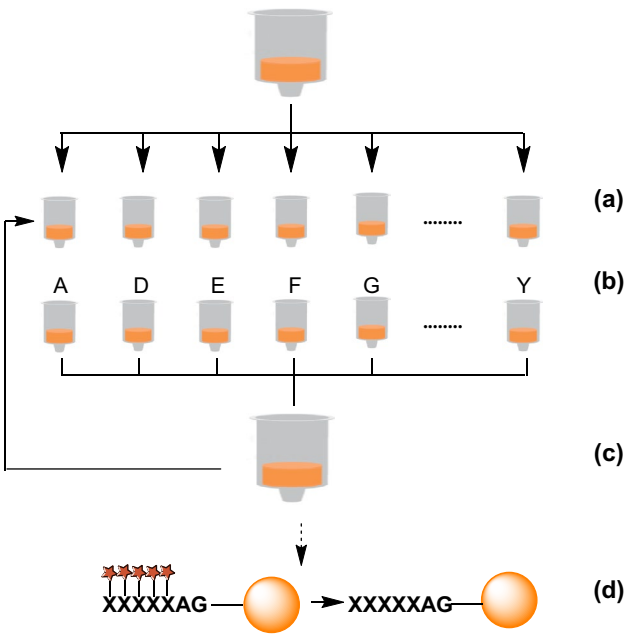

Fig. 5 One bead-one peptide library constructed by split and mix or divide, couple and recombine method. (a) the resin is divided into equal portions; (b) in each portion a different amino acid is coupled (c) after coupling and washing, the resin is recombined. The process is repeated until the desired length of the peptide is reached (X = variable positions). (d) Finally, all side chains are deprotected, leaving the peptides anchored to the resin beads for subsequent solid-phase analysis

In-Silico Design

Nowadays, bioinformatic strategies facilitate peptide ligand development. Current protein-peptide docking and molecular dynamic tools provide very useful information by narrowing down the candidates that must be chemically synthesized and experimentally tested, saving time and money (Salmaso and Moro 2018; Sivakumar et al. 2020; Siebenmorgen and Zacharias 2019). Some protein-peptide docking methods are available on Internet, such as: pepATTRACT (https://bioserv.rpbs.univ-paris-diderot.fr/services/pepATTRACT/); FlexPepDock (http://flexpepdock.furmanlab.cs.huji.ac.il/); pep-SiteFinder (https://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-SiteFinder/); ModPep (https://bio.tools/MODPEP); HPEPDOCK (http://huanglab.phys.hust.edu.cn/hpepdock/), etc. Shi and Sun (2021) reviewed several examples of peptide and pseudo peptide ligands obtained in silico using Protein A as template.

Preliminary Peptide Ligand Evaluation

After library screening and in silico studies, many candidates are selected. Possible candidates are synthesized in small quantities to evaluate their stability and affinity with the target antibody (Fig. 6). Tiny amounts of each candidate peptidyl-resin is mixed with the target Ab labeled with a reporter group such as biotin or a fluorescent dye and incubated in buffer (Fig. 6a1). The color or fluorescence of the resin, depending on the label used, will indicate the adsorption of the antibody. Figure 6a2 shows a fluorescence microscope image of positive fluorescent beads of Ac-PHQGQHIGVSK-ChemMatrix with Texas red-bevacizumab adsorbed (Barredo et al. 2020).

The ligand developed for AC must be stable to proteases present in the crude sample, generally a cell culture broth. Thus, peptide stability must be assessed. Although many methods have been reported for stability evaluation, most of them test the peptide in solution which may differ from the resin-bound peptide behavior (Cavaco et al. 2021). Thereby, immobilized peptide evaluation is preferred. A solid-phase strategy using 4-(hydroxymethyl)benzamide-ChemMatrix (HMBA-CM) resin and mass spectrometry (MS) has been developed to test peptide stability and applied in the selection of ligands for rhEPO, rhFSH, rhGH and bevacizumab purification by AC (Giudicessi et al. 2017; Saavedra et al. 2018; Gurevich Messina et al. 2018; Barredo et al. 2019). The method entitles: (a) solid-phase peptide synthesis on HMBA-CM resin; (b) peptidyl-resin beads incubation in solutions containing proteases or in cell culture broth; (c) whole peptide or C-terminal degradation products detachment from solid support with ammonia vapor; (d) MS sequencing

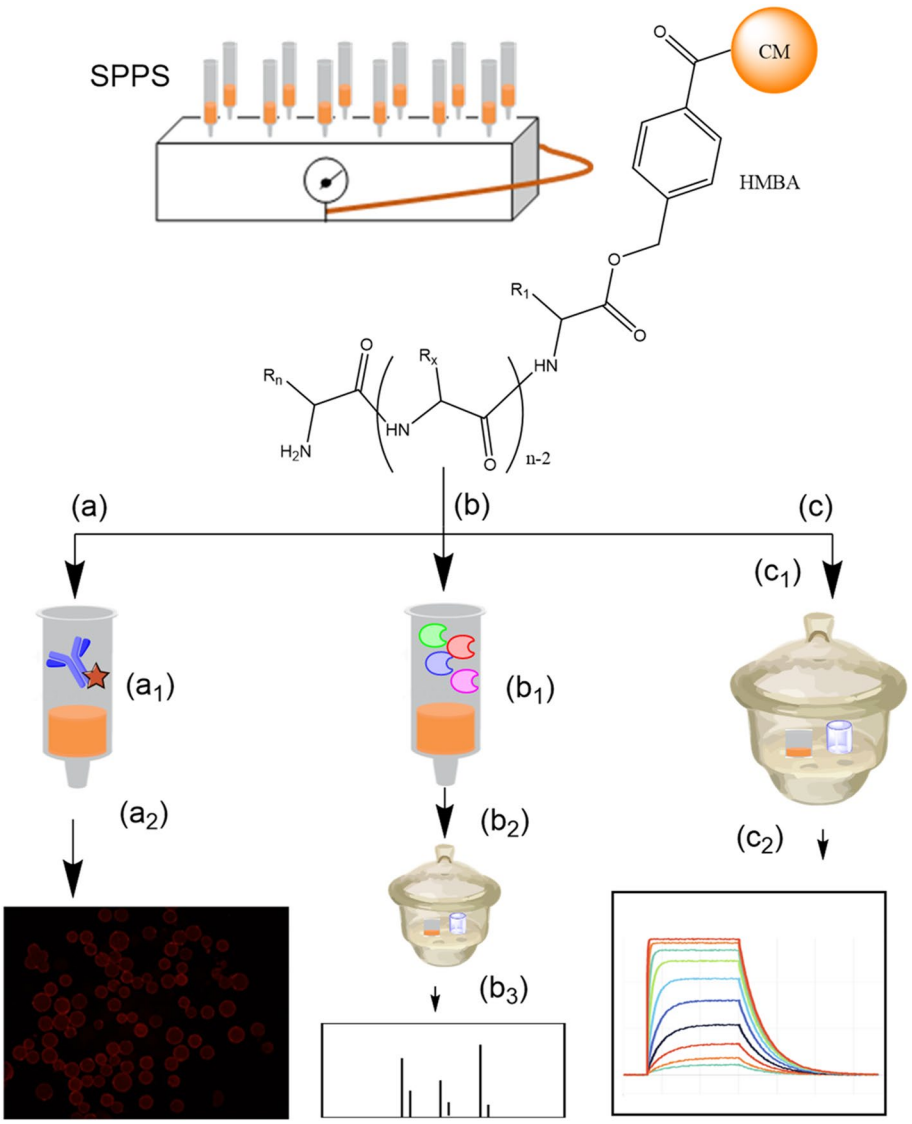

Fig. 6 Parallel synthesis of candidate peptides for their analysis. (a) Evaluation of antibody binding to peptidyl-resin candidates: (a_1) antibody labeled with a reporter group such as a fluorescent dye is incubated with each peptidyl-resin. (a_2) Color fluorescence beads are observed under a microscope. (b) Peptide stability assessment: (b_1) peptidyl-resin beads are incubated in solutions with proteases or in cell culture broth. (b_2) whole peptide or C-terminal degradation products are separated from the solid support with ammonia vapor. (b_3) Peptide and degradation products are analyzed by MS. (c) SPR affinity analysis: (c_1) peptide is separated from the solid support with ammonia vapor. (c_2) interactions between peptides and antibodies can be studied in real time by SPR without labeling the analytes

of the peptide and C-terminal fragments (Fig. 6b). If the ligand is digested by those enzymes, chemical modifications should be introduced in the ligand to increase its stability (Evans et al. 2020). Different strategies have been proposed to increase ligand stability, like the use of some D-amino acids in the peptide or by using the retroinverso analog which has the chirality of the amino acid inverted from L to D and the sequence in reverse direction with respect to the natural peptide. Retroinverso peptides mimic the structure of the original L-peptide, therefore show similar affinity for the target but with increased stability (Giudicessi et al. 2017; Rai 2019). Also, cyclic peptides can be used as ligands due to their reduced conformational flexibility compared to linear peptides which confers strong resistance to proteolytic degradation. Many approaches to prepare and screen cyclic libraries have been described (Manegatti et al. 2013b; Camperi et al. 2016) and reviewed by Martínez-Ceron et al. (2016). Also, peptide stability can be increased by synthesizing dendrimer analogs with a polylysine core typically containing four to eight branches. This technique makes use of the alpha and epsilon amino group of Lys residues to obtain a branched core matrix, which can be used as a scaffold for subsequent peptide synthesis. Branched peptides were widely used by Fassina et al. (1996), who synthesized a tetrameric tripeptide library to search for a protein A mimetic (PAM) peptide, able to recognize the Fc immunoglobulin portion, and usable for AC. This library was prepared by manual solid-phase peptide chemistry, and peptide sequences were identified after three screening cycles. These tetrameric ligands showed high binding capacity and selectivity. Another choice to increase stability is the substitution of some amino acids with more stable organic compounds. Islam et al. (2019) synthesized an analog of the peptide ligand HWRGWV previously developed to purify IgG, replacing the arginine with citrulline. Peptidomimetic and depsipeptide analogs are also good alternatives to increase ligand stability (Knör et al. 2008; Menegatti et al. 2013b).

The interaction between molecules is usually studied in real time by SPR without labeling the analytes (Nguyen et al. 2015; Florinskaya et al. 2018; Martínez-Ceron et al. 2011). Each peptide is attached on the surface of a sensor chip, and then a sample containing the antibody is passed over their surfaces. Otherwise, the antibody is attached to the sensor chip and then samples containing candidate peptides are passed over its surface. During sample injection, binding of molecules to the sensor surface generates a response proportional to the bound mass measured in resonance units (RU) (Fig. 6c).

Selected Ligands Immobilization on Chromatographic Supports

Those selected peptides after preliminary analyses, are synthesized in higher quantities using conventional SPPS protocols (Fig. 2) and subsequently immobilized on previously activated chromatographic matrices. A spacer arm between the ligand and the support is required to extend the molecule away from the matrix surface for a better ligand accessibility and allow steric accommodation between the ligand and the Ab to assure its binding (Hermanson 2013). This spacer arm can be incorporated during matrix activation or synthesized by solid phase together with the peptide ligand. Examples of spacer arms are: Gly, Ala, β-Ala, Lys, α-amino caproic acid, succinic acid, 1,6-diaminohexane, 2-mercaptoethylamine, between many others. To favor site directed immobilization and avoid many peptide orientations on the matrix a Lys is added at the C or N terminus allowing peptide immobilization through the Lys ε-amino group. In those cases where the selected peptide has Lys in its sequence, Cys is added at the C or N terminus, thus allowing peptide site directed immobilization through its sulfhydryl group. Many commercially activated chromatographic supports are available, and most of them contain a spacer arm that terminates in the activated group. *N*-hydroxysuccinimidyl (NHS) activated matrices are frequently used to bind ligands through their amine group with the formation of a stable amide bond, while iodoacetyl activated matrices are useful to bind ligands containing sulfhydryl groups by the formation of a stable thioether bond (Fig. 7) (Hermanson 2013).

Affinity Chromatography Performance

Adsorption isotherms and breakthrough curves can be built to determine the adsorption of target protein to the ligand immobilized on the chromatographic matrix (Chase 1984). Otherwise, adsorption isotherms can be predicted from experimentally measured breakthrough curves (Poursaeid et al. 2019).

To measure equilibrium adsorption isotherms, an equal volume of the chromatographic matrix with the peptide immobilized is added into tubes containing solutions of increasing concentrations of the target protein and equal final volume of adsorption buffer. Those batch systems are stirred at a selected temperature until the equilibrium is reached. Finally, the suspension is centrifuged, or filtrated, and free protein concentration is measured in each tube (c*). Bound protein at the equilibrium (q*) is calculated

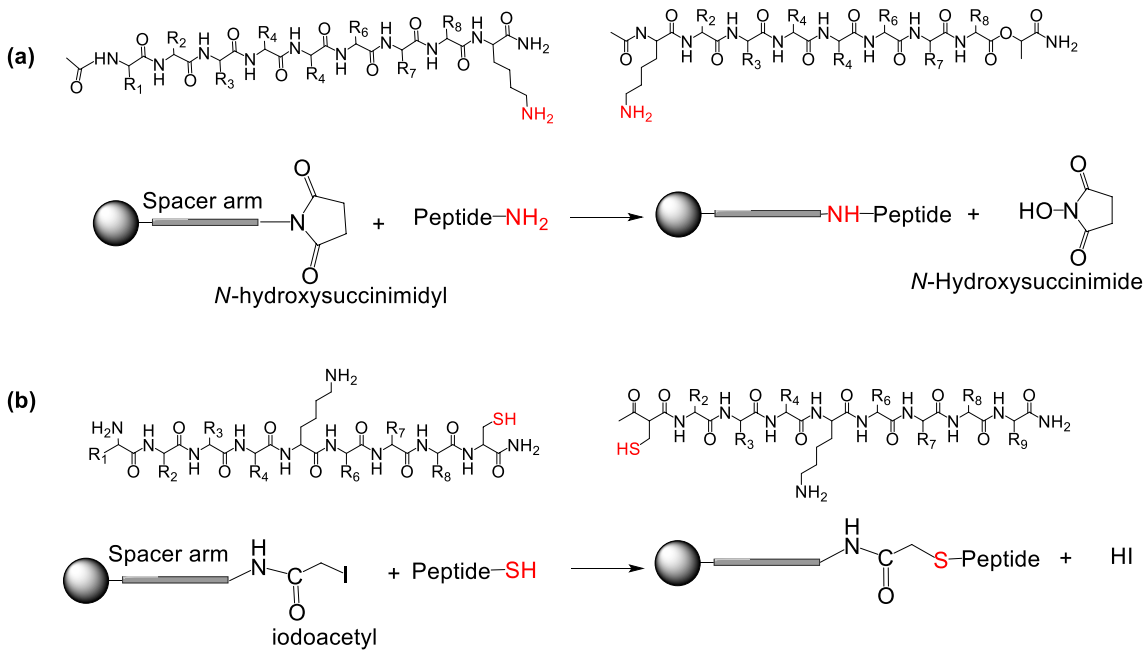

Fig. 7 Peptide ligand site directed immobilization. **a** Lys is added at the C or N terminus allowing peptide immobilization on a *N*-hydroxysuccinimidyl (NHS) activated matrix through the Lys ε-amino group. **b** Cys is added at the C or N terminus allowing peptide site directed immobilization through its sulfhydryl group on an iodoacetyl activated matrix in those cases where the peptide has Lys in its sequence

as the total amount of protein present at the beginning of the experiment less the amount still in the soluble phase at equilibrium. Usually, maximum capacity (q_m) and dissociation constant (K_d) are calculated using the Langmuir binding model (Chase 1984) in which the adsorption describes the hyperbola: $q^* = (q_m \text{ x } c^*)/(K_d + c^*)$ (Fig. 8a). However, protein adsorption on the affinity matrix can be described by many adsorption isotherms (Wang and Guo 2020). Different adsorption buffers and temperatures are usually assessed in order to select optimum conditions.

To measure breakthrough curves, crude sample containing the target Ab is fed continuously through the affinity adsorbent packed column while the concentration of the Ab in the column outlet is measured. The graphic of the Ab concentration at the outlet of the column (C) divided by its concentration in the sample (C_0) as a function of volume or time describes a sigmoidal curve with a breakthrough describing that column capacity has been exhausted (Fig. 8b). The dynamic capacity is defined as the mass of Ab adsorbed when the value of C/C_0 reaches 0.1. Different flow rates

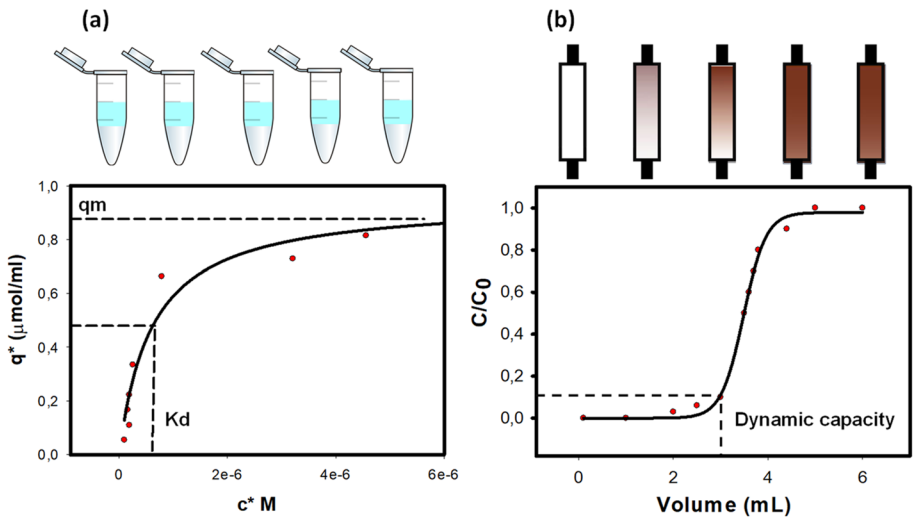

Fig. 8 Affinity chromatographic performance evaluation. **a** Equilibrium adsorption isotherm measurement. **b** Breakthrough curve measurement

or Ab concentration are usually evaluated to obtain sharper curves and therefore increase the dynamic capacity (Chase 1984; Barredo et al. 2021).

Examples of Peptide Ligands Designed for Antibodies Purification

In Table 1, several ligands with affinity for different immunoglobulins are shown. All the ligands were obtained by screening of peptide libraries. Some of them have been afterwards modified to increase their stability. When reported by the authors, specifications about the resin used, the elution buffer, affinity or binding capacity, recovery shield, and purity are shown.

In 1991, Tribbick et al., using a multipin library screened by ELISA, found linear epitopes which were used as ligands for AC fractionation of polyclonal Abs from human serum in order to increase their specificity.

Later, Fassina et al. (1996), using a library synthesized by the split and mix method, designed a tetrameric peptide called PAM (Protein A mimetic or TG19318, for its ability of mimicking protein A) and used to purified IgG by AC. The purity obtained was close to 95%. According to Fassina's group, the ligand was stable to a vast variety of sanitization agents including ethanol and 0.1 M sodium hydroxide. The column selectivity for IgG from rabbit, goat, sheep and mouse was similar to protein A columns. Further studies showed that this ligand specificity was even broader than protein A: it can interact with IgGs from human, pig, rat, cow, horse, mouse, rabbit, goat and sheep. It can also interact with IgY from egg yolk, IgM, IgA, and IgE. Maximum column capacity was 25 mg IgG/mL (Fassina et al., 1998). Afterwards, to increase the stability against proteases, Verdoliva et al. (2002), synthesized the retroinverso of PAM (D-PAM). The ligand was immobilized on a chromatographic support and IgG was directly captured from the serum in a single chromatographic step, with a recovery yield ranging from 60 to 90%, a purity degree higher than 90%, and with a full recovery of antibody activity. When determining column capacity, differences were found according to the IgG source but in average it was about 50 mg IgG/mL of resin. They also found that their peptide was very stable to protease activity maintaining its binding capacity even after prolonged incubation with mouse serum (D'Agostino et al. 2008).

Dinon et al. (2011), using the previously designed D-PAM peptide and with the help of dynamic simulations, designed and synthesized a phenylacetyl-D-PAM (D-PAM-Φ) by introducing small hydrophobic groups (phenylacetyl) at the α amino of the N terminal Arg of D-PAM. D-PAM-Φ was immobilized on an activated solid support and compared with the parent D-PAM affinity matrix. The D-PAM-Φ affinity sorbent selectively capture human IgG from cell culture supernatant. Its dynamic binding capacity was 10 mg/mL with a purity higher than 90%.

Krook et al. (1998), using a phage-display decapeptide library with 4×10^8 phages, found two peptides that interact with high affinity with the Fc portion of the human IgG and with less affinity with IgG from rabbit, chicken, donkey, swine, mouse, and sheep.

Ehrlich and Bailon (2001), using phage display, found several peptides with affinity for IgG. Some of them were synthesized and used as affinity ligands in AC. The peptide EPIHRSTLTALL was selected as the best one considering its 42% sequence homology alignment with the Fc binding domain of protein A.

DeLano et al. (2000), using a cyclic phage-display library made several rounds of selection and found two peptides Fc-I and Fc-II. Then after certain mutations in Fc-II gene another peptide Fc-III was found. This peptide could inhibit the binding of protein A to the Fc with a K_i of 25 nM. Finally, after docking analyses they realized that there was a "consensus" site on the Fc of IgG where the peptide ligands and protein A and G interact. The Fc-III peptide obtained by the disulfide bridged phage-display library were subsequently improved by Dias et al. (2006), who designed a backbone cyclic β-hairpin peptidomimetic with 80-fold higher affinity for the Fc domain. The peptidomimetics designed called Fc binding particles were synthesized by SPPS, immobilized on a solid support for AC and used for mAb purification (Kang et al., 2016). Gong et al. (2016), also synthesized an analog of the Fc-III, the Fc-III-4C, a double cyclic peptide ligand with 4 Cys and 2 disulfide bonds, generating a double cyclic structure. This peptide showed a higher binding affinity than that of IgG with Protein A.

Camperi et al. (2003), synthesized a combinatorial tetrapeptide library by the split and mix method. After screening the library, they selected the peptide APAR with high affinity for the anti-granulocyte macrophage-colony stimulating factor (GMCSF) mAb. The peptide was immobilized on agarose with a ligand density of 0.5 µmol/mL, and the adsorption isotherm showed a K_d value of 0.015 mg/mL (9.4×10^{-8} M) and a maximum capacity of 9.1 mg of mAb/mL. The mAb was purified with high effectiveness by AC with a dynamic capacity of 3.9 mg of mAb/mL and recovering 95% of the purified mAb in a single step.

Verdoliva et al. (2005), developed a disulfide-bridged cyclic peptide library with the formula $(NH_2\text{-Cys-X-X-X})_2\text{-Lys-Gly-OH}$ where X represents the variable positions. They found a peptide called Fc-RM with the formula $(NH_2\text{-Cys-Phe-His-His})_2\text{-Lys-Gly-OH}$ which could purify both monoclonal and polyclonal IgG from biological fluids with a recovery yield up to 90%. The peptide was capable to bind both Fab and Fc. The dissociation

Table 1 Examples of peptide ligands used to purify antibodies by affinity chromatography

Target protein	Peptides	Method	Solid support	Elution buffer	Dissociation constant and binding capacity	Yield	Purity	Reference
Seric Ab	SGNEDAGK	Multipin	Polystyrene	low pH	–	–	–	Tribbick et al. (1991)
	SGKEKEGD		plates		–	–	–	
	SGKEKEGD				–	–	–	
human IgG Fc	PAM (TG19318)	One bead-one peptide	Eupergit C30N	0.1 M acetic acid	–	–	Approx	Fassina et al. (1996)
			Emphaze				95%	
IgG from other sources, IgM, IgA, IgE and IgY from egg	PAM (TG19318)	One bead-one peptide	CH-Sepharose 4B	0.1 M sodium bicarbonate (pH 8.5)	$K_d = 0.3$ µM	80–90%		Fassina et al. (1998)
			Protein-Pak		(Human IgG)			
			Epoxy-HyperD		$q_m = 25$ mg/mL			
human IgG Fc	TWKTSRISIF	Phage display	–	SpA or low pH	–	–	–	Krook et al. (1998)
	FGRLVSSIRY		–		–	–	–	
IgG Fc	Fc-III:	Phage display	–	–	$K_i = 25$ nM	–	–	DeLano et al. (2000)
	DCAWHLGELVWCT	cyclic library	–	–	(of SpA)	–	–	
IgG Fc	EPIHRSTLTALL	Phage display	Amino-NuGele™	0.2 M NaCl 0.2 M HOAc	$q_m = 320$ µg/g	–	–	Ehrlich and Bailon (2001)
IgG Fc	D-PAM	One bead-one peptide	Emphaze™	0.1 M acetic acid (pH 3.5)	$q_m = 50$ mg/mL (average)	60–90%	> 90%	Verdoliva et al. (2002)
antiGM-CSF mAb	APAR	One bead-one peptide	Agarose	5 M LiCl	$q_m = 9.1$ mg/mL $K_d = 94$ nM	95%	–	Camperi et al. (2003)
IgG Fab and Fc	Fc-RM: $(CFHH)_2$-KG	Disulfide-bridged peptide library	Emphaze™	100 mM acetic acid (pH 2.7)	$K_d = 20$ µM	67–90%	> 90%	Verdoliva et al. (2005)
IgG Fc	FcBP-1	Computer mimetic design	–	–		–	–	Dias et al. (2006)
	FcBP-2		–	–	$K_d = 2.2$ nM	–	–	
human, goat bovine mouse and rabbit IgG Fc	HWRGWV	One bead-one peptide	Toyopearl AF Amino 650 M resin	2% acetic acid or other pH 4 buffer	–	60%	–	Yang et al. (2008)
	YYWLHH				–	60%	–	
	HYFKFD/HFRRHL				–	30–40%	–	
	HVHYYW							
	HHLWYY							
	HHLYYW							
human IgG Fc	HIW/KGE				–	–	–	
IgG Fc	D_2AAG	One bead-one peptide	SepharoseFF BW-resin	10 mM sodium formate (pH 3.6)	$K_d = 10$ µM	88%	> 93%	Lund et al. (2012)

Table 1 (continued)

Target protein	Peptides	Method	Solid support	Elution buffer	Dissociation constant and binding capacity	Yield	Purity	Reference
					$DBC_{10\%}$ = 15 mg/mL			
	DAAG			100 mM sodium chloride (pH 3.6)	$DBC_{10\%}$ = 48 mg/mL	> 85%	> 93%	
IgG Fc	D-PAM-Φ	Dynamic simulation	Sepharose CH 4B	0.1 M acetate buffer (pH 4)	DBC = 10 mg/mL	–	> 90%	Dinon et al. (2011)
IgA	Opt-1	Phage display	HiTrap NHS-activated HP column	0.1 M glycine-HCl (pH 2.5)	K_d = 33 nM	–	–	Hatanaka et al. (2012)
	Opt-2	Mutations			K_d = 16 nM	–	–	
	Opt-3	in residues			K_d = 72 nM	–	–	
mouse or human IgG Fc	NARKFYKG	Spot-synthesized	Toyopearl AF Amino 650 M resin	0.1 M acetate buffer (pH 4)	K_d = 6.5 µM	88.70%	68%	Sugita et al. (2013)
	NKFRGKYK	peptide array			Kd = 8.9 µM	81.60%	83%	
human IgG	cyclo[Link-M-WFRHYK]	mRNA display	Toyopearl AF Amino 650 M resin	0.2 M sodium acetate (pH 4)	K_d = 7.6 µM	96%	93%	Menegatti et al. (2013a; b)
		library			q_m = 19.7 mg/mL			
IgG	FYWHCLDE	Dynamic simulation	Sepharose gel	0.5 M NaCl	K_d = 1.5 µM	87%	90%	Zhao et al. (2014)
				50 mM citrate buffer (pH 3)	q_m = 56.1 mg/g			
	FYTHCAKE				–	–	–	
Mouse IgG_{2a} Fc	RRGW	Molecular docking studies	biosensor	–	K_d = 0.56 nM	–	–	Tsai et al. (2014)
rabbit IgG Fc	ABP1: KHRFNKD	Phage display	biosensor	–	K_d = 20 nM	–	–	Yoo and Choi (2015)
human IgG Fc	FC-III-4C:	Derived from FcBP2 and Fc-III	NHS-activated	–	K_d = 8.2 nM	–	–	Gong et al. (2016)
mammalian	Double Cyclic		Sepharose		K_d < 30 nM	–	–	
IgG Fc	Peptide Ligand				DBC = 28.9 mg/mL			
IgY	Y4-4	Phage display	HiTrap™	0.1 M glycine-HCl	K_d = 7.3 µM	approx	93%	Khan et al. (2017)
	Y5-55		Streptavidin HP		K_d = 4.4 µM	70%		
	Y5-14		column	0.25 M NaCl	–	–	–	
human IgG	FYEILH	Biomimetic approach	Sepharose	0.5 mol/L NaCl	q_m = 49.7 mg/mL	73%	94.02%	Wang et al. (2019)
					K_d = 1.8 µM			

Table 1 (continued)

Target protein	Peptides	Method	Solid support	Elution buffer	Dissociation constant and binding capacity	Yield	Purity	Reference
murine IgG	peptoid PL16: HWRGWV	One bead-one peptide	Workbeads™	0.1 M acetate buffer (pH 4)	–	47%	94%	Reese et al. (2020)
rabbit IgG						66.50%	91.70%	
caprine IgG						63%	91–95%	
donkey and llama IgG						93%	97%	
IgY					-	42%	92%	
IgG bevacizumab	PHQGQHIGVSK	Biomimetic approach	Pierce NHS dry agarose	Phosphate buffer (pH 7)	K_d = 0.22 µM q_m = 38 mg/mL	94%	98%	Barredo et al. (2020)

SpA staphylococcal protein A; K_d dissociation constant; q_m maximum binding capacity; *DBC* dynamic binding capacity

constant for this peptide was 20 µM and its recovery yield was between 67 and 90%.

Yang et al. (2008), synthesized a hexapeptide combinatorial library and, using a three-step screening, found seven peptides with affinity for the Fc region of human IgG and capable of purifying it from complex mixtures. One of these peptides, HWRGWV, was useful to purify all subclasses of IgG from bovine, mouse, goat and rabbit sources. Among other hexapeptides also described by the same authors, HWRGWV exhibited the best performance and almost 60% recovery yield. YYWLHH peptide could also bind almost 60% of IgG but it was not as specific as HWRGWV. Unlike protein A, HWRGWV could bind all subclasses of human IgG including IgG_3. Afterwards, Reese et al. (2020), used a peptoid variant of the peptide HWRGWV to bind IgG from several mammalians as well as chicken immunoglobulin IgY.

Lund et al. (2012), reported two novel peptide ligands, D_2AAG and DAAG, both containing Arg, Gly and a synthetic aromatic acid: 2,6-di-*t*-butyl-4-hydroxybenzyl acrylate (DBHBA) which were identified by screening a mix and split combinatorial library. The resins, with the peptides immobilized with a binding affinity of ~10^5 M and a dynamic binding capacity up to 48 mg IgG/mL, were used to purify the IgG from a cell culture broth with a yield higher than 93%.

Hatanaka et al. (2012), using a phage-display library, found a novel peptide for human IgA purification with a K_d of 1.3 µM. After improving this peptide with a partially randomized phage-display library, they found the peptide Opt-1 with an increased affinity (K_d = 33 nM). However, it does not specifically bind IgG and was recovered with many contaminants. They afterwards modified the peptide obtaining Opt-2 and Opt-3. The last peptide exhibited higher specificity and binding affinity for IgA with a K_d = 72 nM.

Sugita et al. (2013), used a spot-synthesized peptide array to find two octamers (NKFRGKYK and NARKFYKG) against mouse IgG which were derived from outer membrane sequences of IgG-Fc receptors. They also found that they have capability for recognizing human IgG and the K_d of both were 0.11 µM and 0.15 µM, respectively. When purity was measured after purification from a cell culture medium, the values were 83% for NKFRGKYK and 68% for NARKFYKG and the yields obtained were 81.6% and 88.7%, respectively.

Menegatti et al. (2013a), designed a cyclic peptide library and found the peptide cyclo[Link-M-WFRHYK] that binds to the Fc moiety of human IgG but not to its Fab fragment. As a cyclic peptide, it has resistance to harsh basic conditions when cleaning and regenerating the column. After immobilizing the peptide on a chromatographic resin, they developed the adsorption isotherms, obtaining a K_d of 7.6 µM and a q_m of 19.7 mg/mL. They

purified IgG by AC and obtained a yield of 96% and a purity of 93%.

Zhao et al. (2014), designed a virtual peptide library which was screened using semi-flexible molecular docking and found the peptides FYWHCLDE and FYTHCAKE with affinity for IgG. These were used in a chromatographic column and the first one was found to have the best performance, having an adsorption capacity of 56.1 mg/g of drained wet beads and a dissociation constant of 0.22 mg/mL (1.5×10^{-6} M). FYWHCLDE could purify IgG from a serum sample in a single step with 90% purity and 87% of recovery yield.

Tsai et al. (2014), using molecular docking analysis of the Fc region of IgG, found a novel peptide ligand (RRGW) with affinity for mouse IgG_{2a} with a K_d of 5.56×10^{-10} M.

Yoo and Choi (2015), using a phage-display library found the peptide KHRFNKD with affinity for rabbit IgG. By immobilizing it in a quartz crystal microbalance (QCM) biosensor they found it binds the IgG with a K_d around 2×10^{-8} M.

Khan et al. (2017), screening cyclic phage-display libraries, developed peptide ligands useful to purify chicken egg yolk immunoglobulin (IgY) by AC with a yield of 70%. However, oligomers of IgY were produced due to the low pH of the elution buffer.

Wang et al. (2019) used the saturation transfer difference (STD) nuclear magnetic resonance (NMR) spectroscopy to study the Fc-Protein A interaction and found the minimal region of the protein A that interact with the Fc domain. STD NMR has been used to find the binding region (epitope) of a ligand when bound to its receptor protein (Wagstaff et al. 2013). By considering the structure of the protein A and previous studies that described the region that interacts with the Fc domain, they found the minimal fragment binding domain of protein A and they designed the hexapeptide FYEILH. The peptide was immobilized on Sepharose resin, and the adsorption isotherms showed a K_d of 1.8 µM and a q_m of 49.7 mg/mL of wet drained beads. With that peptidyl-resin human IgG was purified by AC with a purity of 94% and a yield of 73.0%.

Barredo et al. (2020), using the structure of the endothelial growth factor (VEGF) which is the target of the IgG bevacizumab, reported that the peptide Ac-PHQGQHIGVSK contained in the VEGF binds the antibody. The peptide was immobilized on agarose resin, and the adsorption isotherms for bevacizumab binding to Ac-PHQGQHIGVSK-agarose showed a K_d of 0.22 µM and a q_m of 38 mg/mL. Samples of CHO cell culture filtrates containing bevacizumab were purified with that peptidyl-agarose resin with a recovery yield of 94% and a purity of 98%. This novel peptide has the advantage of allowing elution under mild conditions with 20 mM sodium phosphate, pH 7.0, which are ideal for conserving the integrity of bevacizumab while most of the other ligands needed an acidic pH for eluting the mAb.

Conclusions

The growing interest in the development of alternative ligands to protein A for the purification of therapeutic mAbs encourages the development of short peptide ligands by means of combinatorial libraries and in silico strategies. Although today AC with immobilized protein A is currently in use for large scale mAbs purification, alternative short peptides affinity ligands will allow the development of AC more economic matrices with higher resistance, stability, and capacity ideal to lower the high cost of therapeutic mAbs production.

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