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Rational design of affinity ligands for bioseparation

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Highlights

- Affinity absorbents are essential tools in protein isolation and purification.
- Rationally designed affinity ligands increase the purification performance for a specific target
- Affinity ligands found by screening combinatorial libraries provide the basis for new adsorbents
- Rational design of affinity ligands can be improved with the support from big data and artificial intelligence tools

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Rational design of affinity ligands for bioseparation

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Abstract

Affinity ligands have been the cornerstone in protein purification. The selective nature of the molecular recognition interactions established between a ligand and its target provide the basis for efficient capture and isolation of purification processes. The plethora of affinity adsorbents available in the market reflects the importance of affinity chromatography in the bioseparation industry. Ligand discovery relies on the implementation of rational design techniques, which provides the foundation for the engineering of novel affinity ligands. The main goal for the design of affinity ligands is to discover or improve functionality, such as increased stability or selectivity. However, the methodologies must adapt to the current needs, namely to the number and diversity of biologicals being developed, and the availability of new tools for big data analysis and artificial intelligence. In this review, we offer an overview on the evolution of rational design techniques, dating back to the years of early discovery up to the current and future trends in the field.

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1 Introduction

Molecular recognition is a key event in biological systems, involving highly specific interactions between two entities leading to important events such as signal transduction, self-assembly, and recognition of cell-surface receptors which are present in nearly all biological systems [1–4]. Examples of molecular recognition events in Nature include the antibody-antigen interaction and enzyme-substrate complex.

Molecular recognition has been explored in affinity chromatography by coupling affinity ligands onto solid supports, with the aim of capturing and isolating target biomolecules. The use of affinity ligands that rely on highly specific and reversible biological interactions, establishes a selective and effective way of capturing the target molecule while depleting impurities (Figure 1) [5]. Extensive research has been done on the development of different immobilization supports, the impact of spacers and ligand density on the performance of purification processes, leading to the establishment of affinity chromatography as we know it today [6–12].

Inspired by Nature, several affinity ligands have been rationally designed with the goal of finding more effective purification media. Affinity ligands can be divided into different categories according to their biological or synthetic origin and structural complexity. Biological ligands (e.g. proteins, peptides, nucleotides, vitamins) can be isolated from natural sources or discovered by *in vitro* selection techniques [13–15]. On the other hand, synthetic ligands are not found in nature and are usually obtained mainly from chemical combinatorial libraries [13–15]. Another important distinction between affinity ligands is whether they have a naturally occurring affinity pair, i.e. natural ligands, or if the observed affinity results from rational design techniques and screening methodologies, i.e. engineered ligands.

The process of designing an affinity ligand capable of establishing interactions with a given target presents a high degree of complexity has been the subject of many developments and improvements throughout the years (Figure 2). Affinity ligands can be coupled to several matrices (e.g. membranes, monoliths, magnetic beads) yielding supports suitable for a diversity of purification techniques. For work regarding non-chromatographic affinity-based bioseparations refer to other articles detailing the advances made in each field [16–22]. In this review we present a comprehensive analysis of the evolution of rational design of affinity ligands and affinity adsorbents for chromatography, providing a historical context, current relevance to the field and future trends of research and development in the biopharmaceutical industry.

2 Natural affinity ligands as tools for bioseparation

The field of affinity ligands such as we know it today, started with the discovery of natural binders during the early 20th century. At the time, techniques as Nuclear Magnetic Resonance (NMR) and X-ray crystallography were in its early days, therefore limiting researchers to the observation of naturally occurring binders.

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One of the early reports of the discovery of natural binders dates back to the 1900's, when Emil Starkenstein studied the influence of chloride salts in the binding of α-amylase to its natural substrate, starch [23]. Insoluble starch placed on a column acted as an affinity ligand and adsorbent, becoming the first reported methodology of isolation of biological compounds (α-amylase in this case) by using a solid support, a process nowadays designated as affinity chromatography.

Other affinity pairs were discovered in the following decades for enzyme purification, namely the use of edestin as an affinity ligand for pepsin purification, elastin for the isolation of porcine elastase, dextran for the isolation of the lectin Concanavalin A, or the use of powdered stearic acid for the enrichment of lipase and heparin [24–29].

In the 1940's boronic acids were found to have innate affinity towards *cis*-diol-containing molecules present in carbohydrates [30]. This feature was first studied as a tool to analyze carbohydrate moieties, and later on used as an approach for the purification of proteins with glycosylation modifications [31]. The antibody purification concept, which is now a multi billion-dollar industry, only started to be sketched with the discovery of *Staphylococcus aureus* Protein A (SpA). The concept dates back to 1940 when a protein from *Staphylococcus aureus* was discovered to be highly reactive towards antibodies present in rabbit serum which caused a precipitation of the immunoglobulins [32]. Despite this interesting discovery, SpA was not used as an affinity ligand for purification of antibodies but rather as a highly reactive compound for immunoglobulins. The full potential of this protein as an affinity ligand was only realized later. Curiously, the first report on antibody purification using affinity chromatography belongs to Campbell's work in 1951 when he isolated anti-bovine serum albumin antibodies from an affinity adsorbent containing diazotized *p*-aminobenzyl-cellulose coupled to bovine serum albumin [33]. This was one of the first works reporting the use of solid supports for the isolation of proteins that were not limited to enzyme-substrate complexes, which was the focus existing at the time. It was not until 1972 that the first reports regarding coupling of Protein A to Sepharose-4B, a chromatographic resin from Pharmacia, for the isolation of immunoglobulin G (IgG) present in human serum was described [34,35]. This affinity adsorbent was optimized employing protein engineering techniques, which ultimately led to the development of what is currently known as Protein A chromatography.

3 Rational design of affinity ligands

The ability to bind a target reversibly but still with high specificity and affinity was until the 1980's limited to natural binders. The natural source of the target biomolecule facilitated extraction, which combined with the innate affinity between the pair justified the lack of ligand development. However, this meant that researchers were limited to affinity ligands offered by nature and that targets with unknown natural ligands could not be purified. Thus, there was a need to improve the available ligands, and to create new ligands for new targets. This was made possible by advances in protein characterization techniques, such as X-ray crystallography, NMR and computer-aided molecular design tools, that allowed the determination of protein's structural features boosting the rational design of affinity ligands. The rational design can be defined as any strategy for the creation of novel affinity ligands with the goal of improving or creating a selective and reversible binding. As Figure 2 shows different strategies can be employed, namely improvement of performance of a natural affinity ligand, ligand discovery through the use of scaffolds and *de novo* ligand design. Table 1 compiles a summary of relevant rationally

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designed affinity ligands employed in bioseparation from 2010 onwards. For a list of previously developed ligands please refer to other review articles [14,36–38].

3.1 Improving the performance of a natural affinity ligand

By using a natural affinity ligand (e.g. protein, peptide) as a template, the residues known to have an important role in binding can be subjected to site-directed mutagenesis in order to acquire variants exhibiting an improved performance (Figure 2). Such methodology has been particularly useful for creating improved versions of enzymes [39–42].

One of the most interesting cases for the rational design of affinity reagents is SpA. As mentioned before, it was during the second half of the 20th century that Protein A potential was discovered as a tool for immunoglobulins isolation. Nevertheless, it took a great deal of optimization and protein engineering to improve its performance throughout the years. Protein A is the product of residue-specific modifications of the natural B-domain resulting in a synthetic Z-domain which retains the affinity for the Fc portion of IgG [43]. Most notably, the substitution of the glycine at position 29 for an alanine conferred this synthetic domain resistance to hydroxylamine and cyanogen bromide, which increased the alkaline stability over the wild-type protein domain. Using a destabilized variant of the Z-domain which does not bind the Fc fragment as a template, Hober and co-workers showed that a N23T mutation increased chemical resistance to withstand cleaning-in-place protocols which is a critical parameter for biological affinity reagents [44]. More recently, Xia *et al* showed thatthe addition of six glycines in loop 2 of the Z-domain along with mutations in residues N29T and F30A greatly increased alkaline resistance to sodium hydroxide extending cleaning in place (CIP) up to 2M in 1h and allowed recovery of antibodies at milder pH [45]. The approach that conferred the Z-domain alkaline resistance was tested towards the C-domain of SpA. Interestingly, despite the G29A mutation increased alkaline stability, the results showed that G29W mutation also improved performance compared to the wild type B-domain [46]. The D-domain of Protein A has also been the subject of studies on rational design. Experimental data obtained from site-directed mutagenesis as well as from *in silico* prediction demonstrated the possibility to bind single-domain antibodies (sdAb) indicating which residues played a permissive or non-permissive role for sdAb binding [47].

One of the disadvantages in using Protein A chromatography is the use of harsh elution conditions due to the high K_a observed ($10^{9\text{-}10}M^{-1}$) which may result in partial denaturation or activity loss in antibodies. More recently, several groups used rational design techniques to improve protein A ligand performance to milder elution conditions. Pabst and co-workers showed that two mutants in the Z-domain, Z(H18S)4 and Z(H18S,N28A)4, allowed the recovery of antibodies at milder conditions, increasing approximately 0.5 pH units in elution. Moreover, at pH 4, Z(H18S,N28A)4 presented 30% yield improvement, showing the success of the rational design of protein A [48]. In another example, a calcium-binding loop was grafted between helix two and three of the Z-domain. Here, Hober's group successfully engineered the Z-domain to attain a calcium-dependent elution behavior in the presence of antibodies, therefore drastically reducing the harshness of the elution process [49]. Furthermore, the domain's multimerization was shown to possess significantly high dynamic binding capacities and to reduce host cell proteins and DNA content after purification of trastuzumab from CHO cell culture, when compared to the performance of commercial resins [50]. This multimerization feature of biological ligands in tandem is particularly interesting for affinity reagents because it is shown to improve performance taking advantage of avidity effects [51,52]. The strength of multiple affinity

interactions between target and different monomers of a domain leads to higher yields and recovery values and is a valuable approach when considering to increase the affinity of a given ligand.

The use of rational design techniques has yielded improved variants of proteins as affinity ligands in bioseparation. It is indeed the method of choice when the structural information of the natural affinity pair is available, and when the key residues involved in binding are known *a priori.* However, the strengths of these methodologies are also weaknesses. The use of this approach is infeasible when there is no known natural binder towards a target, limiting researchers to the improvement of affinity ligands previously described. Furthermore, using site-directed mutagenesis may also imply a risk of missing out promising variants. Residues that at first sight are not critical for ligand binding but are important in other functions that can lead to improvement of overall performance may not be considered for mutation and therefore are lost in the design process.

3.2 Ligand discovery through combinatorial libraries

The use of combinatorial libraries for the discovery of novel affinity ligands has been the source of many affinity reagents throughout the years. The affinity ligands can be originated from synthetic or biological scaffolds (Figure 3). In the first case, the ligands are created to mimic the interactions between a natural affinity ligand and its target, hence generating biomimetic ligands. These ligands tend to be minimalist versions of the binder while keeping affinity and selectivity towards the target. In contrast, biological scaffolds are generated through the *in vitro* screening of naïve, immune or *in silico* combinatorial libraries. These libraries are usually very large due to the introduction of distributed mutations along the protein scaffold. The combinatorial libraries are screened towards a target using display techniques to find novel binders.

3.2.1 Biomimetic ligands

The first biomimetic ligands were based on rationally designed chemical combinatorial libraries from synthetic scaffolds. By using multicomponent synthetic reactions as templates and changing the substituents (e.g. R1,R2 in figure 3) used in the synthesis, it was possible to construct and screen multi-dimensional libraries (usually up to 100 variants) in a fast and simple way [10,13,53–55]. The rational for selecting the substituents and library design was inspired in structural information on the critical residues known to play a role in target binding. The chosen substituents will therefore mimic these critical residues and their interactions, hence constituting biased combinatorial libraries. The use of synthetic ligands offers many advantages due to their chemical stability and robustness to sterilization, relative accessibility to reagents, low cost of production and tailor-made structures with high versatility [56,57].

The first biomimetic ligand was Cibacron blue F3G-A, a triazine ligand, initially reported to purify yeast phosphofructokinase and then used to purify a plethora of proteins by affinity chromatography [58–61]. The ligand was particularly interesting as it showed natural propensity towards protein binding sites usually associated with nucleotides and coenzymes [62]. It was later shown through X-ray crystallographic studies that the terminal sulphonate ring analogues could influence the interaction between the dye and the protein binding site showing effectively how the structural information about binding cavities could provide essential insight on specific interactions important for the development of ligands [62,63]. This example was the basis to develop several affinity ligands based on the triazine skeleton and was the pioneer of a scaffold

that originated many novel binders. Most importantly, one of the reasons for the success of the rationally designed combinatorial libraries, is that the synthesis and screening of the library is performed on the chromatographic matrix, therefore selecting affinity adsorbents and not only affinity ligands.

From all the ligands originated from the triazine scaffold, the ligand 22/8 was probably the most extensively studied. Through the study of x-ray crystallographic structures and use of computational tools, the binding site of SpA to antibodies was analyzed. Once the key residues involved were identified, Lowe's group designed a synthetic library to mimic the characteristics and spatial arrangement of the hydrophobic core dipeptide Phe132-Tyr133 [64]. One lead ligand was then improved in a 2nd generation library which found that ligands containing 3-aminophenol and aminonaphthol moieties exhibited high affinity towards IgG. Ligand 22/8 in particular was found to purify antibodies with high yields and purities (>99%) [65]. Furthermore, Mabsorbent A2P, developed by Prometic Bioseparations, was based on a 22/8 derivative showing the implications that the affinity ligand had on the generation of novel bioseparation matrices. More recently, a 22/8 analogue, TPN-BM, was also studied as an affinity ligand coupled to monoliths. The affinity ligand was synthetized according to green chemistry principles, hence reducing processing time, solvents and purification steps. The ligand showed very high binding capacities (160mg IgG/mL resin) and efficient purification of immunoglobulins from mammalian crude extracts (85% yield and 98% purity). [66] The triazine scaffold approach has also been used to the discovery of ligands for various targets, namely human Fab fragment, Human Serum Albumin (HSA), glycoproteins, virus-like particles, and trypsin-like proteases among others [67–74].

The simplicity in operation and the availability of the reagents prompted researchers to study other synthetic scaffolds that could provide a template for the generation of solid support libraries. The Ugi reaction, in particular, attracted some attention due to its one-pot nature which facilitates chemical synthesis in a simple and fast way. This was seen as an advantage over the triazine libraries that are produced by "mix-and-split" methods. It involves a reaction between an amine, an aldehyde or ketone, an isonitrile and a carboxylic acid which results in a peptidomimetic product. The greater diversity allowed in this multi-component reaction enabled the successful application of this scaffold for novel adsorbents towards glycoproteins, erythropoietin (EPO), and affinity tags [75–81]. The Ugi scaffold has been applied to the generation of protein G biomimetic. The ligand A2C11I1 was selected from screening a combinatorial library and contains mimetic compounds of natural residues present in Protein G, namely Asn35 and Trp43 [80]. The affinity adsorbent observed affinity towards the Fc fragment and was able to perform one-step purification of an impressive range of immunoglobulin, ranging from human IgG to camelid Fc fragment with increased performance over their natural counterpart, achieving up to 65% purity. El Khoury *et al* considered a different strategy when designing a combinatorial library to act as a Protein G mimetic but with affinity towards the Fab fragment. The ligand A2C7I1 mimics Tyr38 and Asn42 from Protein G and exhibited a more hydrophilic propensity in binding towards the Fab fragment [77]. It was successfully applied in purification of purified IgG and Fab fragments from crude mammalian and yeast cell cultures.

The ligand A4C7 is another example of an Ugi ligand developed to bind Green Fluorescent Protein (GFP). The affinity ligand showed good results in purifying GFP from crude extracts obtaining 94% yield and purity under very mild elution conditions (0.1M glycine pH 9) [79]. Furthermore, the ability to purify GFP-tagged proteins was demonstrated with a WW domain

tagged to GFP with purities and yields of 92% and 68%, respectively, hence showing the dual applicability of A4C7.

More recently, with the goal of potentiating molecular diversity in synthetic scaffolds, other approaches were explored. One interesting example is the sequential combination of two independent multicomponent reactions, the Petasis and Ugi reactions. The Petasis reaction yields a free carboxylic acid which can be further employed in the Ugi reaction allowing the creation of a 6-dimensional library [82–84]. This combinatorial approach has recently been applied for the generation of a 84 synthetic ligand library targeting the discovery of an affinity ligand towards phosphopeptides [85]. The most promising candidate ligand 8{3,6} composed of Phenylalanine and Tyrosine mimetic, can selectively bind phosphorylated peptides moieties at pH 7.

The use of small peptides as affinity ligands has also been explored. Peptide ligands can be divided into linear, branched and cyclic peptides and are usually composed of 2-9 residues. Peptide affinity ligands have great potential as affinity adsorbents mainly due to their simple structures which retain the desired selectivity for its target while working with a minimal version of larger proteins [86]. Typically, peptide libraries are first screened for binding and eluting the target biomolecule, and afterwards coupled to purification matrices. One of the first examples of biomimetic peptides was the Protein A Mimetic (PAM). PAM was identified from a randomized synthetic tripeptide tetramer library and shown to selectively elute IgG with purities up to 95% [87]. In following studies, PAM was shown to bind in fact a broad class of immunoglobulins ranging from all classes of IgG in humans to IgG present in other animals such as cow, rabbit among others demonstrating a broader applicability than Protein A [88]. Using PAM as a parental molecule, this affinity ligand is still subject of optimizations to this date. This was the origin of D-PAM, a partial inverse analogue of the parental molecule, as well as D-PAM-Φ, which is a variant of D-PAM with small hydrophobic groups in the structure [89–91].

Carbonel's group screened a family of linear hexameric peptides and selected the sequence HWRGWV that exhibited very high affinity and selectivity towards IgG [92]. Interestingly, all 19 ligands obtained from the initial screening results had histidine in the first position. Despite being a nonbiased library, results showed that this amino acid played a key role in antibody binding. Further studies indicated that ligands with the composition of "histidine + aromatic amino acid + positively charged amino acid" had significant higher binding for IgG, such as HYFKFD and HFRRHL. In addition, HWRGWV showed affinity towards all classes of IgG as well as IgA and IgM hence can be regarded as a purification platform for whole antibodies [93–96]. Purification of IgG spiked in complete mammalian cell culture medium (cMEM) was successful, reaching purities and yields of 68% and 65%, respectively. Due to the promising results obtained with this ligand, optimization regarding peptide density, role of ligand spacer and conditions of operation managed to increase the yield up to 95% [11,97]. Purification of monoclonal antibodies from other complex feedstocks such as CHO cell culture, skim milk and whey was also reported [98,99]. Peptoid analogue variants of this ligand have recently been developed by Bordelon *et al*. In this work the researchers were able to translate a protein-binding peptide into a peptoid analogue similar in terms of performance with the parental molecule but with higher robustness [100]. Such peptoids, named as PL-16 and PL-22, are protease-resistant molecules that purify IgG up to 98% with a K_d of 10^{-7}M.

3.2.2 *De novo* ligand design

De novo ligand design is used when there is lack of information about the structure of the complex formed between the natural ligand and the target. Furthermore, it makes use of computational processing power and uses virtual screening methodologies to discover new compounds through the use of molecular docking and molecular dynamics (MD) simulations [138,139]. Usually, it starts with the inspection of the target's three-dimensional conformation to extract structural features then used to design a library by complementarity. The library is then screened by automated molecular docking for its target which allows for a rapid selection of a smaller set of biomimetic molecules. MD simulations can then be performed to resolve solvent effects and flexibility issues [140,141].

Liu *et al* carried out a *de novo* ligand design approach of a peptide ligand towards tissue-type plasminogen activator (t-PA) [142]. After inspection of t-PA's pocket structure, 10 critical residues were identified and a virtual tetrapeptide library was built. The six tetrapeptides with the highest docking scores were coupled with a serine linker and were subjected to further docking. Lead candidate tetrapeptide QDES was selected for MD simulations, which showed a stable conformation. The peptide was synthetized and immobilized onto EAH Sepharose gel showing it was able to purify tPA from crude porcine heart extract.

Aghaee *et al* designed a peptide-based ligand for HSA through computational approaches. First, the binding pocket of HSA was analyzed and a virtual peptide library was created. Through molecular docking it was found that dipeptide Trp-Trp exhibited high score values and it could bind specifically towards HSA [143]. The library was then docked to determine the affinity of the peptides to HSA. The lead candidate was linked to a spacer arm and a chromatographic resin and was subsequently subjected to MD simulations to confirm the docking results. Absorption spectra then confirmed the affinity towards HSA.

Pina *et al* designed a new tailor-made affinity pair for recombinant protein purification. In this work, a RKRKRK affinity tag was rationalized, and by screening a 64-membered Ugi scaffold a complementary affinity ligand was found [78]. The ligand A7C1 was shown to recover RKRKRK-tagged proteins with an affinity constant of 4×10^{-6} M, which is within the range of other affinity ligands.

In order to avoid the demanding task of selecting affinity binders for virus-like particles experimentally, Fernandes *et al* designed *in silico* two synthetic triazine ligands to bind the surface of virus-like particles amphotropic murine leukemia virus envelope (VLP-AMPHO) [144]. Homology modelling on the target protein was first performed, as no structural data was available. After determination of a plausible structure for the target, complementary ligands were designed to bind to the surface. MD simulations showed two lead ligands with highest energy of binding which were validated experimentally, A5A10 and A10A11. The ligands recovered the target protein at mild elution conditions between 80-100%, thus showing the success of the strategy.

3.2.3 Biological combinatorial libraries

The process selection of binders based on the screening of biological combinatorial libraries starts with the identification of a protein domain that can serve as a scaffold. The ideal characteristics for a scaffold are a small size (ideally less than 20kDa) with defined flexible

regions that allow for randomization, lack of cysteines, and no post-translational modifications to facilitate biological production [101,102]. The flexible regions are not critical for folding, hence they are amenable for mutations. These regions can then be randomized, either partially or totally, while other regions are maintained unaltered due to their role in preserving conformation (Figure 3). In order to increase diversity within the mutated region, it is common to use degenerated codons that can codify for the incorporation of any aminoacid residue. However, special attention is required in order to select degenerated codons that avoid introduction of stop codons within the sequence eventually leading to non-functional binders. While unrestricted randomization can be performed, which is suitable for discovery of novel function, if the aim is to create a scaffold to guide the evolution towards a well-defined goal, randomization of specific residues known to play a key role in the desired function can help to obtain higher quality libraries. The libraries with sizes ranging from 10^7-10^{15} members can then be screened for novel binders hitherto unknown through *in vitro* selection techniques such as phage, mRNA, yeast and ribosome display [103–107]. The use of these techniques became popular as it is possible to obtain a high degree of diversity within the scaffolds and lead ligands exhibiting high selectivity and affinity. It was applied for the generation of affinity reagents (Table 1), therapeutic antibodies, vaccines among others [74,103,108–115].

The creation of a *de novo* protein or peptide scaffold is cumbersome as it is not easy to generate novel proteins with robust, predictable and stable 3-dimensional structures. The main difficulty is how to create new structures, with high melting temperatures and how to fit sequences that that are able to fold into particular backbones [116]. In general, the strategies have rather focused on repurposing naturally occurring proteins in order to find new functionality or improved functional features [117,118]. Despite the difficulties in finding low free energy states for these structures, the use of softwares, i.e. RosettaDesign, that perform fixed backbone design, sequence optimization and structure prediction has enabled the *de novo* design of peptide scaffolds, most notably the first computationally designed scaffold, Top7, a 93-residue α/β protein with a topology that did not exist at the time [119].

When repurposing a protein domain to create a functional scaffold, previous reports describing affinity for a specific target is not essential, which means the scaffold has the possibility of acquiring novel functions. However, engineering a previously described domain known to bind a certain target will have higher margin for improved performance due to the past track record that the protein domain carries [120].

Some of the developed scaffolds include Affibody, Nanofitin and Repebody. A more in-depth analysis concerning affinity ligands for bioseparation generated from protein scaffolds and is available elsewhere [101]. The Affibody scaffold is derived from the previously mentioned synthetic Z-domain, which was generated from the B-domain of SpA. It is a 6kDa three-helix bundle Z protein, without cysteines and with a rapid and independent folding [121]. The technology is currently being explored by a biotech company under the same brand name, and has been successful in the generation of affinity ligands for bioseparation, namely for isolation of Transferrin, Human Serum Albumin and IgG [122–124].

Nanofitins are artificial proteins derived from the Sac7d scaffold of extremophile *Sulfolobus acidocaldarius.* These proteins are small (~7kDa), extremely stable when exposed to high temperatures and pH and are easily expressed in *E. coli* [125]. They are under Affilogic development and provided promising binders for the isolation of IgG, chicken egg lysozyme and bacterial PulD [126–128].

Repebody is a non-immunoglobulin scaffold which uses 3-6 leucine rich repeat modules derived from variable lymphocyte receptors. Each module has 20-29 residues in length and a β-strand-turn-α-helix structure. This approach enables the modulation of affinity by increasing or decreasing the number of modules, thereby also influencing the avidity of the system.[129] The scaffold has generated high-affinity binders to IgG through interaction with the Fc-region with high purities and recovery yields, 94.6% and 95.7% respectively [130].

The albumin binding domain (ABD) derived from streptococcal protein G was improved in order to obtain dual affinity. By randomizing positions away from the HSA binding site, Tove Alm and co-workers used ABD as a scaffold, hence creating a library with variants that retained the HSA binding but could exhibit affinity towards new targets [131,132]. This strategy was applied to see if ABD scaffold could provide an affinity tag with high selectivity towards both HSA and SpA. ABDz1 exhibited dual specificity for these biomolecules and results showed it could bind both HSA Sepharose and MabSelectSure. Furthermore, the fusion of ABDz1 onto three different proteins demonstrated it could act as an affinity tag for protein purification that could use either affinity matrix.

Non-proteinaceous affinity ligands have also been explored as alternatives to conventional affinity reagents. Systematic evolution of ligands by exponential enrichment (SELEX) is an iterative selection process that uses aptamers composed of oligonucleotides as the building blocks of the combinatorial libraries. Unlike previous techniques, the discovered ligands are selected from fully in vitro processes, independently of living systems and are uniquely composed of short single-stranded DNA or RNA oligonucleotides which can fold into various 3-dimensional structures such as G-quartets providing them unique features such as high shelf-life, chemical stability and low immunogenicity [133–135]. This strategy provides an oligonucleotide alternative to other display techniques and it has been successfully applied for the discovery of ligands for affinity chromatography. Kuehne *et al* recently used LD201mΔ1, a high affinity DNA-aptamer towards human L-selectin for a single-step purification from cell culture supernatants which resulted in a 3.6-fold higher protein yield [136]. On another example, Forier *et al* reported the use of three different DNA-aptamers for the purification of three plasma-related proteins from different sources: Factor VII, Factor H, Factor IX.[137] The aptamers exhibited a high degree of selectivity and were highly stable under harsh regeneration conditions (100h in NaOH).

The creation of biological combinatorial libraries is an effective way of discovering novel ligands through its high-throughput screening methodologies. Despite that the dimension of the library seems attractive at first sight, it often leads only to a small set containing binders which is a disadvantage of this approach. The random nature of the mutations within the scaffold can lead to non-binders, hence decreasing the “functional library” size.

4 Future of rational design techniques

So far, we have detailed how rational design techniques were used for the discovery of affinity ligands in the context of bioseparation. In general, researchers take advantage of the datasets already available (e.g. structural insights from x-ray crystallography and NMR, protein sequence and inherent function correlation extracted from multi-omics databases), as the basis to develop a strategy for the discovery of affinity ligands. The data gathered allows to understand the active site of a target and provide key findings about the molecular recognition pattern of the

respective ligand, which can then be incorporated into the features of the ligand-design methodology.

However, we are now living in a revolutionizing digital globalized era where information has never been so readily available to everyone around the world. This has an impact in the biopharmaceutical industry where evolution and innovation are ceaseless. With the increasing complexity and diversity of biological drugs in the pipeline along with the emergence of computer-based technologies it is crucial to envision the future directions the industry is taking, and in particular, how it will affect ligand design methodologies (Figure 4).

4.1 Shifting paradigm of the industry towards new needs and sustainability

At the beginning of this review article we showed Emil Starkenstein's early discoveries in 1910, where he observed the affinity of α-amylase to starch witnessing *in loco* the first steps of molecular recognition in bioseparation. Soon after Starkenstein's findings, other naturally occurring affinity pairs were discovered such as the elastin-elastase and Protein A-antibody affinity pair. The first reports on biological ligands resulted thus from empirical evidence rather than based on any rationale behind the experiments. It was only when complementary techniques for the elucidation of three-dimensional structures of proteins and their binding sites became available, that it was possible to study whether certain residues or interactions played an important role in ligand binding. From there, a rational strategy was developed for the *in silico* design of novel binders which made significant contributions to the development of a variety of affinity ligands, ranging from DNA aptamers to mixed-mode ligands.

Most of the rational strategies are designed towards a single target that often leads to the need of an extensive repertoire of affinity adsorbents that are functional only for a certain given molecule. On the contrary, the current efforts of the biopharmaceutical industry are in streamlining, better integrating unit operations to decrease costs and increase overall efficiency and sustainability of the processes, preferably in a continuous fashion [145,146]. There is a need to develop adsorbents that can act as platforms and provide multi-target separation for molecules that share common characteristics. The industry is slowly realizing that in order to save resources, both financial and human, there must be a drive for a shifting paradigm from a one ligand one target approach to a one ligand multi-target strategy. This principle of broad ligand design is already being applied in the therapeutic field to solve complex therapeutic problems such as Alzheimer's, Parkinson's and Trypanosomatid disease [147–150]. Despite initial beliefs of adverse effects caused by molecules interacting with several targets, it is now clear that single-target drugs are not effective in treating complex pathologies [147,150]. The solution may rely on multi-target drugs which have proven to be successful and can provide a safer profile in achieving a therapeutic effect [148,149].

This example of multi-target drugs could inspire the implementation of these platform technologies in the downstream processing field and be greatly beneficial for diverse areas. Some of the benefits of using platform technologies include the application to a larger number of targets; validation of the molecular recognition due to its platform characteristics; the creation of robust processes that can be better transferred from process development to manufacturing and the overall lower cost and increased efficiency [151]. The purification schemes must adapt through the integration of novel affinity adsorbents that behave as platform-like purification systems for novel molecular entities. Optimizations in the upstream processing of the targets

have shifted the bottleneck towards the purification pipeline. The seemingly increasing titers of expression hosts triggered the development of new solutions that can address faster and fewer purification cycles. One of the challenges the industry is currently facing is how to increase efficiency in the capture chromatographic step and how to distinguish between closely related isoforms, such as full-length antibodies and antibody fragments. The use of platform-like affinity ligands that can handle multiple targets, but at the same time remain selective for specific compounds, with higher binding capacities can help to achieve this goal.

The most well-known example of a platform-like system is Protein A chromatography for the downstream processing of monoclonal antibodies (mAbs). Despite it is widely used in the biopharmaceutical industry as an initial capture step, the use of this system cannot handle the purification of antibody fragments that do not have an Fc fragment. To address this issue GE Healthcare developed LambdaFabSelect and KappaSelect that are alternative strategies for the purification of Fab fragments, single chain antibodies and engineered nanobodies. Despite reducing the number of possible affinity adsorbents to be used these cannot be considered as true purification platform-like systems as they rely on purification of either the lambda or the kappa chain of antibodies which are mutually exclusive if considering a broader applicability.

Biosimilars is a fast-growing field that would greatly benefit from platform-like technologies. These biomolecules are variants of an existing drug with an expired patent that behave similarly to the original molecule and have comparable structure, biological activity, efficacy, safety and immunogenicity profile [152–154]. Currently, 60 biosimilars have been granted approval by the European Medicines Agency (EMA), including a variety of active substances such as somatropin (Omnitrope), insulin glargine (Glarzia) and infliximab (Remsima). Despite the similarities between biosimilar and its original drug the manufacturing process of both molecules is quite different, hence pharmaceutical companies must produce and purify the biomolecule taking into account inherent changes that occur during production of such complex biomolecules [155]. The use of an affinity adsorbent, immobilized with group-specific ligands, transversal to a class of biosimilars would be greatly beneficial for reducing costs and increasing productivities in the biopharmaceutical industry.

Isolation of stem cells is a research area which has also garnered increased attention in recent years in the field of regenerative medicine. Due to its clinical purposes, stem cells need to be produced and purified in high doses with high purities while maintaining critical quality parameters such as cell viability and functionality. The conventional isolation strategies rely on physicochemical and biophysical methods rather than on affinity-based strategies, namely fluorescence-activated cell sorter (FACS) and magnetic-activated cell sorter (MACS) which require cell labelling and are not as scalable as affinity chromatography [156]. There is a need for bioseparation technologies to ensure that cell populations that share similar features are separated and that the technologies are scalable [157]. Indeed, the strategies for designing affinity ligands are compatible with stem cells. *De novo* ligand design can be performed when there is structural information available about the target, hence creating a complementary ligand. When there is structural information available between the target and its natural receptor, biomimetic ligands can be designed to mimic the interactions. Screening of combinatorial libraries employing affinity ligands discovered through cell-SELEX have been reported and could provide a reliable alternative for isolation of this biopharmaceutical [158,159].

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Another area that could benefit from platform technologies is vaccines development. Vaccines have proven beyond doubt their benefits, however there is still a lack of distribution of vaccine availability throughout the world, especially in the developing countries. The high investment required for vaccine development is usually an obstacle to affordable and sustainable distribution worldwide [146]. This problem has been addressed by the United Nations in the 2030 agenda, encouraging the research for the development of newer and more cost-effective vaccines for the developing countries [160]. As in any bioprocess, the major costs of production focus on the downstream processing step. The lack of selective adsorbents and standardized purification technologies for vaccines, combined with a great deal of diversity of antigens, represent the bottleneck of process development [161]. The standard method for purification of viruses relies on ion-exchange and hydrophobic interaction techniques rather than affinity-based ones. Furthermore, the few cases of development of vaccines using affinity methodologies, such as lectin and heparin chromatography, rely on naturally existing ligands that have never been designed and engineered for a specific goal [146]. In order to address this need, the rational design of affinity ligands should guide the screening towards common and shared characteristics among these diverse antigens. The success of this approach will be greatly dependent on having structural information about the different targets and targeting the few shared traits among them to increase the odds of finding ligands that promote selectivity at a multi-target level exhibiting a platform-like behavior. The recent effort of companies in developing novel affinity adsorbents with immobilized mixed-mode ligands is, in part, a response to the growing need of integration of multiple interactions into single molecules. Mixed-mode chromatographic adsorbents provide applications translatable to diverse fields and can be important in achieving sustainability in a long-term by integrating many different chromatographic principles into one adsorbent.

4.2 Big data and Artificial Intelligence

During the past decades high-throughput techniques for the development of affinity ligands have become routine in the search for potential binders. We are living in an era that is constantly creating large amounts of information, that we will only have the means to analyze it in the future through the contribution of algorithmic approaches using big data, and artificial intelligence (AI) [162]. The success of every drug approved by the regulatory agencies will depend on how biopharmaceutical industries adapt to this new challenge and realize the need to have scientists skilled in the area of big data analysis. There are three essential steps for using these tools: i) data integration; ii) data exploration; iii) exploitation of that information [163–168].

In the context of biotechnology data integration and data exploration tools can be used to optimize process development, to predict promising candidates for drug discovery, epitope mapping among others. One of the focus of improvement in the biopharmaceutical industry is the development of new affinity reagents that can act as purification platforms. Affinity ligands bind to their targets due to specific molecular recognition events is often the result of improvement and engineering of ligands over generations. This knowledge gained from decades of work on the generation and enhancement of affinity reagents could benefit from the processing power of AI tools to discover potential binders, not detectable otherwise. As Figure 4 shows, big data could prove to be particularly useful to extract information from databases and based on that information suggest a set of molecules for experimental testing. Furthermore, the obtained results can then be fed back to the algorithm to predict a new set of molecules to be tested and start a new cycle. Ultimately, the use of this approach could lead to the discovery of

new affinity ligands possibly leading to improved performance and overall efficiency of purification processes.

Recently, Tallorin *et al* applied this concept to the discovery of active, orthogonal peptide substrates for different classes of PPTases (4′-phosphopantetheinyl transferase). Using an iterative POOL approach (Peptide Optimization with Optimal Learning) they were able to identify short highly selective peptides that work as substrates for this class of enzymes [169]. The process comprised: i) a training set with an initial set of peptides that are natural substrates for PPTase; ii) based on the algorithm a set of peptides was recommended for screening; iii) the set of peptides was immobilized onto a membrane and enzymatically selectively labelled; iv) the characteristics of the peptide lead candidates were fed back to the algorithm to repeat the iterative process [169].

A similar approach has been used by Yoshida *et al* which combined an evolutionary algorithm with machine learning prediction and experimental validation to discover a new antimicrobial peptide towards *Escherichia coli* [170]. First, two generations of peptide libraries were generated from a natural antimicrobial peptide. The peptides were synthetized, and their performance evaluated *in vitro* in terms of their IC_{50}. The results were then used to predict additional amino acid substitutions, hence repeating the cycle. At the end, a lead peptide was identified with a 162-fold more antimicrobial activity that the original molecule, with 20-fold lower IC_{50} values.

Sarkar *et al* used machine learning algorithms to make predictions of linear peptide sequences that can bind to SH3, WW and PDZ domains [171]. Through support vector machine-based prediction models and using reported binding peptides for the training set of each domain, an accuracy of prediction of more than 92% was obtained. This resulted in the creation of LMDIPred, a publicly online available tool that can provide a set of peptides predicted to bind the chosen domain.

AI tools could provide essential insight into the discovery of novel affinity ligands in challenging fields. As mentioned before the diversity of antigens present in the surface of viruses is a bottleneck in the development of affinity reagents, which delays vaccine development. AI tools could address this issue by focusing on structural similarities between the different antigens in order to find common interactions that could help in designing new platform-like affinity ligands. The use of these tools is a good example of how molecule discovery can shift from the screening of very large randomly mutated libraries which often lead to a few promising binders, to the use of a more rational approach using smaller, but higher quality set of data which yields novel high-affinity molecules.

5. Concluding remarks

The first period on the use of affinity ligands in bioseparation was the discovery of natural binders as adsorbents, which dates to the early 20th century. At the time, the discovery of affinity ligands was not based on any experimental design but rather due to observation of natural events.

Once some techniques started to be more powerful, namely X-ray crystallography, NMR, spectroscopy and computational tools, another period began. Scientists started looking at structural information generated from existing databases or from high-throughput data extracted from multi-omics methodologies and applied rational techniques with the goal of finding ligands

that could interact with a desired target. This was accomplished by site-directed mutagenesis of single residues, known to have a crucial role in ligand binding, or by biased combinatorial ligand libraries based on designed scaffolds screened for binding to a particular target.

The challenge now relies on the adaptation of rational design techniques in an era of digital technology in the biopharmaceutical industry. One of the drawbacks regarding vast application of affinity purification is the lack of versatility of the affinity reagents. Because most affinity ligands are developed to bind a single target, researchers often ended up with an adsorbent with very high affinity and selectivity for a single target but not translatable to other applications. Another related problem in the biopharmaceutical industry is the lack of sustainability in their processes. Both problems can be solved, at least partially, with the development of more efficient affinity reagents that exhibit a platform-like behavior for multiple biomolecules. The industry could benefit from their transversal properties and shift the paradigm towards a single ligand multi target approach to attain sustainability in the long-term.

The recent developments in the areas of big data, artificial intelligence and machine learning, clearly highlighted they will be essential to any biotech enterprise in order to avoid getting surpassed by competitors in the near future. There must be an effort by the biotech industry to realize the potential of these tools and to have qualified personnel in these scientific areas. The use of AI can be particularly interesting and greatly benefit discovery of novel binders through the perfect combination of the processing and analyzing power of machine learning with the information generated from decades of work on affinity ligands.

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Figure legends

Figure 1

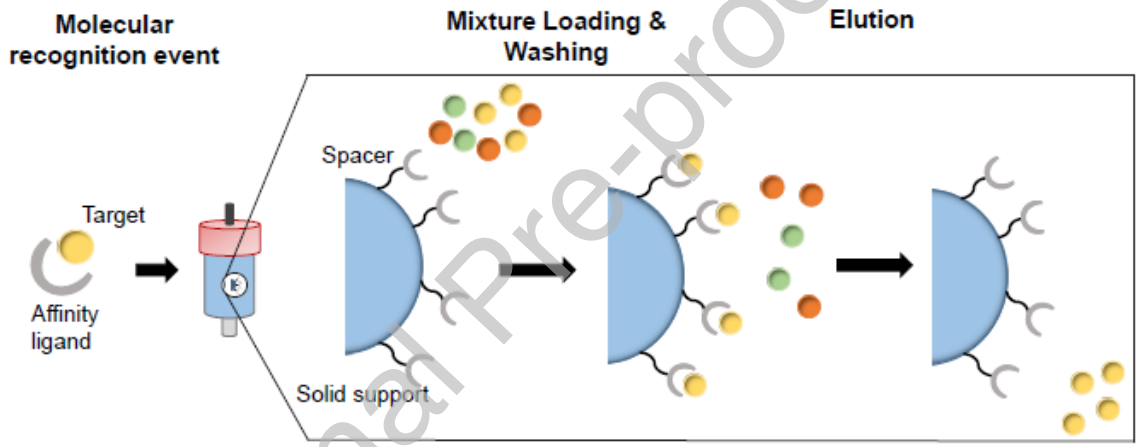

Figure 1- Overview of the affinity chromatography process. Molecular recognition events between affinity ligand and target, laid the foundation for coupling biomolecules onto solid supports. Upon application of a mixture of biomolecules, the target is retained in the column. Upon change of conditions, e.g. pH, conductivity or addition of competing agent, the target is eluted.

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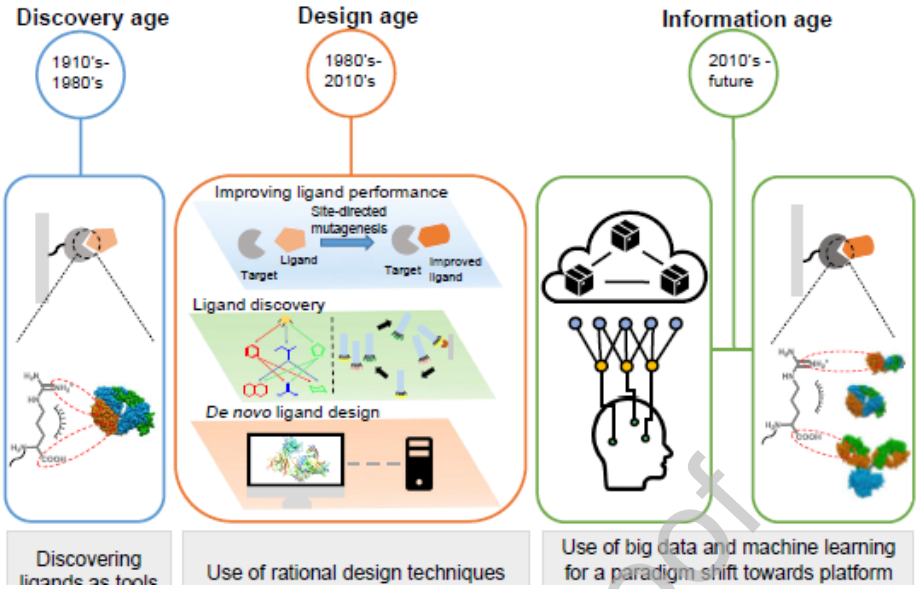

Figure 2- Overview of the evolution of the design techniques of affinity ligands throughout the years: Discovery age; design age and information age.

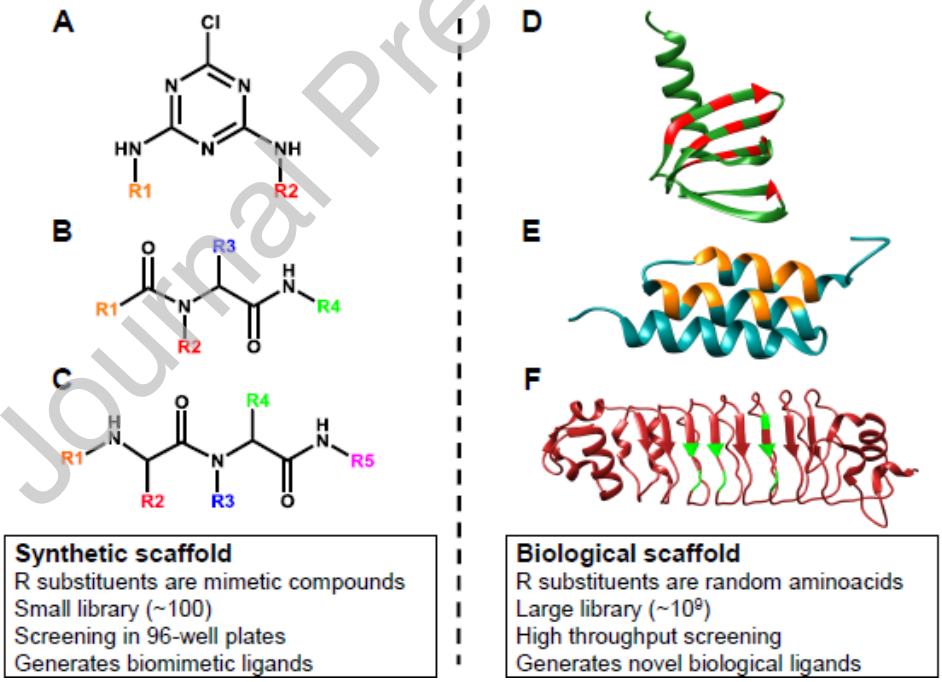

Figure 3- Comparison of synthetic and biologic scaffolds. In both cases, variation is introduced through substitutions of components (R) in the scaffold.In the synthetic scaffold the R represent the chemical compounds at each position, whereas in the biologic scaffolds the mutated residues are represented in a different colour. (A) Triazine scaffold; (B) Ugi scaffold; (C) Petasis-Ugi scaffold; (D) Nanofitin scaffold (PDB ID: 2XIW); (E) Affibody scaffold (PDB ID: 1Q2N); (F) Repebody scaffold (PDB ID: 3RFS).

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Figure 4

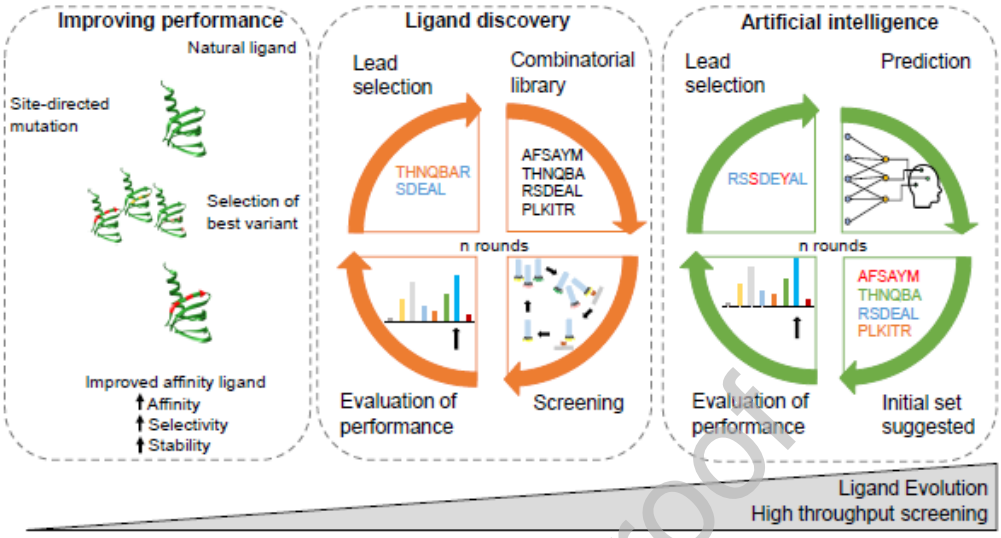

Figure 4- Comparison of the methodology applied for different rational design techniques. When looking for improving performance, structural insight drives the rational design to make single-point mutations of key residues to select a lead candidate based on desired functionality (PDB 2XIW used for visual representation). In ligand discovery the combinatorial library is screened towards the target and based on their performance, the lead candidates are selected. Lead candidates can be subjected to affinity maturation (in case of biological scaffolds) or second generation libraries (in the case of synthetic libraries) to further improve performance. In future design, machine learning algorithms will provide an initial set of molecules to be tested experimentally. Based on the results they will be ranked and the algorithm will predict and provide feedback on a new set of molecules that can perform better than the previous generation.

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Table 1- Summary of designed affinity ligands employed for bioseparation. Comparison between different properties of the ligands namely target, affinity constant, binding capacity, purities and recoveries obtained for the given example. Displayed results only from 2010 onwards.

Strategy	Ligand	Target	K_d (M)	Binding capacity (mg/mL)	Purity (%)	Recovery (%)	Ref.
Improvement of performance	D-domain SpA	sdAb	0.6-11 x 10^{-6}	-	-	-	[47]
	Z-domain Ca2+	IgG	4.4 x 10^{-7}	-	-	-	[49]
	D-domain SpA	IgM	6.02 x 10^{-9}	-	-	-	[172]
	5 unit repetitions Z-domain (N23T, F30A)	IgG	-	0.196	100	100	[45]
	C-domain G29A	IgG	1 x 10^{-8}	-	-	-	[46]
	Z(H18S,N28A)4	IgG	-	43-52	-	70-98	[48]
	D-PAM-Φ	IgG	-	10	-	-	[91]
	E141A/H164A OmCl	Complement component C5	4.1 x 10^{-9}	-	-	-	[173]
	SpyCatcher	SpyTag	7.3 x 10^{-8}	4-13	98.9	-	[174, 175]
Biomimetic ligand	A6A5	HSA	-	-	98	100	[73]
	PL-16 PL-22	IgG	0.57 x 10^{-6} 0.78 x 10^{-6}	56.76 47.94	95 93	83 80	[100]
	A9C10I8	Human EPO	-	-	80	15	[81]
	A2C7I1	IgG and Fab	5.34 x 10^{-5}	17	93	99	[77]
	A13C24I8	Glycoproteins	1.45 x 10^{-6}	16.7	92	69	[76]
	A4C7	GFP	4.2 x 10^{-6}	1	94	94	[79]
	A2C11I1	Mammalian Immunoglobulins	4.78 x 10^{-6}	24.6	65	54	[80]
	Cyclic FSLLSH	Human EPO	4.6 x 10^{-7}	5.21	95	90	[176, 177]
	SJ047 SJ055	Ferritin-based influenza antigens	2 x 10^{-7} 1.91 x 10^{-8}	0.25 0.1	85 87.5	97 95.5	[178]
	D_2AAG DAAG	IgG	1.7 x 10^{-6} 3.85 x 10^{-7}	17 48	>90 >90	90 90	[179]

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	A21C11I8	Glucose oxidase (GOx)	9.99 x 10^{-4}	24.5	98	100	[180]
	NKFRGKYK NARKFYKG	IgG	1.12 x 10^{-7} 1.54 x 10^{-7}	4.9 5	83 68	69 80	[181]
	B4	GOx	-	-	97	100	[182]
	8 {3,6}	Phosphorylated peptides	-	0.24	100	-	[85]
	TPN-BM	IgG	2.2-2.9 x 10^{-5}	160	98	85	[66]
	AbSep	IgG	5.13 x 10^{-6}	78	90	85	[183]
	FYWHCLDE	IgG	1.5 x 10^{-6}	56.1	95	95	[184]
	Cyclic-M9	GK14-P	1.37 x 10^{-3} (K_A)	0.54	97	61	[56]
	Ac-FYHE	IgG	-	87.9	99	94.4	[185]
	Ac-$HW_{Met}CitGW_{Met}$	IgG	1.08 x 10^{-5}	72	92	94	[186]
	Ac-YFRH	IgG	-	21	98	89	[187]
***De novo* ligand design**	DWDLRLLY	Murine polyomavirus VLP	-	-	70.1	-	[188]
	A5A10 A10A11	VLP-AMPHO	0.38 mL/µg 0.06 mL/µg	0.013 0.041	- -	100 80	[107]
	A2C2 A3C1	NWNWNW WFWFWF	9 x 10^{-7} 7.9 x 10^{-7}	0.079 0.019	45 -	- -	[189]
	Dipeptide Trp-Trp	HSA	3.32 x 10^{-8}	-	-	-	[106]
	A7C1	RKRKRK	4 x 10^{-6}	0.5	50	30	[78]
Biological combinatorial ligand	Armadillo repeat	Peptide neurotensin	7 x 10^{-6}	-	-	-	[190]
	Anti-HSA Affibody	HSA	10 x 10^{-9}	-	-	-	[129]
	Anti-HER2 Affibody	Z_{HER2}	90-283 x 10^{-12}	-	-	-	[191]
	Anti-IgG Nanofitin	IgG	1 x 10^{-6}	165	95	95	[133]
	Anti-IgG Repebody	IgG	3.6 x 10^{-8}	25.9	94.6	95.7	[137]
	ABDz1	HSA and Z_2-domain	4 x 10^{-7} (Z_2-domain) 4.8 x 10^{-8}(HSA)	-	-	-	[138, 139]
	WIPNSEFEHERTK	Kappa Fab fragment	2 x 10^{-6}	-	90	84	[116]
	CAAALAKPHTENHLLT	VLP-AMPHO	2.76mL/µg	2.77	-	90-100	[74]
	GVKCTWSSIVDWVCVDM	Chicken IgY	7.3 x 10^{-6}	-	70	90	[118]
	LD201mΔ1	Human L-selectin	12 x 10^{-9}	0.32	-	62	[143]
	Mapt 2.2CS	Factor VII	-	7.6	100	-	[144]

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	Mapt H1.1CSO Nonapta5.1	Factor H Factor IX	- 1.2×10^{-9}	- -	99 98	- -	
	Nt ssDNA	Concanavalin A	-	-	90	66	[192]
	H_3T	His_6 tag	1.22×10^{-5}	-	90	-	[193]
	Cyclo[Link-M-WFRHY-K]	IgG	7.6×10^{-6}	19.7	93	96	[194]

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