Pharmaceutical BIOPROCESSING

Multimodal chromatography: debottlenecking the downstream processing of monoclonal antibodies

The commercial potential of monoclonal antibodies (mAbs) has been continuously increasing during the last years alongside with the number of approved mAb-based drugs and clinical trials. Despite their effectiveness and safety, the general access to this class of biopharmaceuticals is barred by high selling prices. Downstream processing is now considered the bottleneck in the manufacturing of mAbs. Therefore, the design of novel and economic operations and their implementation in the current technology platforms constitutes a pressing need. This review provides an insight into the current state-of-the-art in mAbs purification, focusing on multimodal chromatography as one of the viable options to upgrade the established purification train.

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Purification technologies for monoclonal antibodies

Historical perspective of antibody purification

The dawn of the purification of antibodies, particularly IgG, can be remitted to the plasma fractionation technique, in which ethanol is used to precipitate proteins at their isoelectric points. This relatively simple method was first applied to the extraction of albumin from blood plasma [1], and years later found a homologous application in the purification of IgG for the first intravenous immunoglobulin (IVIG) formulation [2]. The large production scale of intravenous IgG combined with the low manufacturing cost that the plasma fractionation-based process allowed [3], raised the debate about the relevance of borrowing this method for the purification of recombinant mAbs, however new trends started to be framed in a completely different direction, with chromatography playing a major role. Furthermore, it should be taken into account that the impurities profile and the final purity requirements in these applications are totally different (e.g., there is no foreign DNA nor host cell protein impurities in IVIG).

Back in the 1990s, early processes for mAb purification included multiple steps organized in a complex manner, which reflected the lack of process-wise knowledge and the need for improved separation media. Among the multitude of processes employed, one can highlight the use of various filtration media for harvest, the combination of a wide range of different chromatographic steps for protein separation (including Protein A, Protein G, ion exchange, size exclusion and hydrophobic interaction chromatography), the implementation of ultrafiltration and diafiltration at different points of the process, and the choice of methods involving solvents or detergents to accomplish viral inactivation [3].

At the time, as the upstream cell culture titers were very low, there was no need to have media tailored for high binding capacities and the focus was centered on the ability to rapidly process large volumes of feedstock [3]. A change in paradigm took place when increased expression levels and higher cell densities started to be reached upstream [4–6], and also when mAb products evolved from pure murine to fully human protein sequences [7], which required the design of alternative and more versatile worksheets. Currently, almost all marketed mAbs are produced by mammalian cell culture using either Chinese hamster ovary (CHO) or mouse myeloma cells. Advances

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Key terms

Antibodies: Large family of glycoproteins, also known as immunoglobulins (Ig), produced by B lymphocytes in order to identify and neutralize foreign antigens such as bacteria and viruses.

Dynamic binding capacity: Amount of sample that will bind per unit of resin volume under defined conditions. It is highly dependent on the flow rates, and the lower the flow rate, the more it approaches the maximum available capacity.

Host cell proteins: Proteins produced or encoded by the monoclonal antibody-producing cells that are unintended for the recombinant product, and must be removed during the formulation of the final drug substance.

Multimodal chromatography: Emergent chromatographic method (often denoted by mixed-mode chromatography) that explores multiple interactions between the adsorbent and the solutes in a mobile phase.

in molecular biology and protein engineering have led to remarkable improvements in cell culture productivities, with antibody titers now routinely exceeding 10 g/l for the CHO expression system [3].

Changes in the requirements imposed by the regulatory agencies fostered the use of chemically defined components in the production of the biopharmaceutical product, which led to the adoption of serum-free media in upstream processes. In the downstream processes, these requirements led, for example, to the development of mutant Protein A ligands to replace the native bacterial protein ligand [8]. Protein A ligand engineering has also been driven by the desire to have a protein A ligand with improved stability to pH and cleaning solutions, with milder elution pH and with higher dynamic binding capacities [9,10]. The investigation and knowledge gathered over the years, allowed to improve the adsorbent matrices in terms of binding capacity, rigidity and tolerance to higher flow rates, which increased the robustness of the chromatographic steps and simplified their integration and organization in a platform format [11].

Current approaches in mAbs downstream processing

The introduction of biopharmaceutical products in clinical trials has to cope with different challenges, being process development one of the limiting steps. The explosion in the number of mAbs entering clinical trials has created the need for employing a rather standardized approach for process development, in order to reduce the time and the resources required for this task. It is known that a high degree of homology exists among mAbs, however even slight variations in complementarity-determining regions and framework sequences may constitute a specific purification challenge [3], making unfeasible the processing of different mAb products without changes to the operating conditions. Despite these variations, the advantages of employing a generic process with minimal optimization for the different mAb candidates are undeniable and, in fact, this platform strategy has been adopted for the majority of companies working on mAbs downstream processing. In Figure 1, the so-called platform approach is represented, which has been extensively described in the literature [12–15]. This platform approach is based on a common sequence of unit operations that were developed and integrated to allow the maximum speed to clinic, which constitutes one of the major competitive advantages for biotechnology companies.

The first step of the downstream processing train is the initial removal of cells and cell debris from the culture broth using centrifugation, followed by depth filtration to clarify the cell culture supernatant that contains the antibody product. The high cell densities that are now typically achieved increased the burden on this primary recovery, which can be significantly challenging at manufacturing scale and may account for up to 12% of the downstream processing costs, according to a cost of goods analysis from Costioli *et al.* [16].

After harvest, protein A affinity chromatography has been adopted as the capture step of choice by most manufacturers, due to the high selectivity toward mAbs and the extremely high purity that is achieved by directly loading the cell culture supernatants [17]. This affinity capture step relies on the specific interaction of the antibody Fc part with the immobilized protein A, a cell wall protein of *Staphylococcus aureus* (Figure 2). This chromatographic step is also effective in removing host cell proteins (HCP), host DNA, process-related impurities and potential adventitious viral contaminants, while providing a volume reduction of the mAb product [18]. So, the excellent performance parameters that this capture step delivers, alleviate the burden on the subsequent polishing steps, which are nonetheless required to ensure that the product is in compliance with the quality requirements.

Despite all the advantages, a series of limitations can also be pointed out to Protein A, being the most serious one related with the high cost of the resin, which can account for over 50% of the entire downstream processing costs [2]. In addition to the economic-related constraints, Protein A also suffers from leaching problems due to proteases 'action, which may cause the co-elution of ligand fragments along with the target antibody, and from a poor stability to the harsh pH elution and to the sanitization conditions [18]. To address this issue, GE Healthcare developed a Protein A resin (MabSelect SuRe™) that is stable at high pH values. The loading capacity of the resin is the rate-limiting

step [17] in this unit operation, which can be limited to accommodate the increasingly high titers coming from the upstream feedstocks [22].

The low pH used during elution from Protein A columns is usually included for viral inactivation, since most of the mAbs can be briefly maintained under low pH conditions without detrimental effects. The viruses can be of endogenous origin, arising from the mammalian cells used in the manufacture of mAbs, or can be adventitiously introduced by occasional infection of the cells during processing. In any case, the kinetics of virus inactivation should be carefully considered [2], in order to define the appropriate time hold for effective pH incubation step.

The following chromatographic polishing steps are aimed at reducing host cell protein levels, host DNA, high molecular weight aggregates and leached Protein A that remain after the capture step [17]. At least two orthogonal chromatographic steps are typically employed, most commonly anion exchange or cation exchange chromatography, although hydrophobic interaction chromatography can also be included as polishing step in a platform downstream processing of mAbs [2,17]. The nature and sequence chosen for the polishing steps is dependent on the nature of the product and the trace impurities to remove, in order to ensure that the final solution is in accordance with the particular formulation to be used [23,24].

For viral clearance purposes, a filtration step is the most suitable choice to ensure the log reductions imposed by safety requirements, since this is a robust operation that is relatively independent of the process parameters, and there is a wide variety of virus filters available for the biotechnology industry [17]. In addition to viral filtration, most chromatographic methods also have some degree of viral clearance. Total viral clearance of the purification process is then calculated by the addition of the log reduction values of all orthogonal purification steps. The completion of the downstream purification process occurs after buffer exchanging the product into the formulation buffer, which is typically accomplished with an ultrafiltration step in diafiltration mode. This final step is of capital importance, since its optimization allows to handle high therapeutic doses in a limited formulation volume.

The need for cost-effective processes seems to start outpacing Protein A capture step as industry standard, despite all its inherent advantages, and has triggered the demand for alternative strategies for mAb purification [25,26]. These should be capable of successfully replacing the affinity capture step and fit in a platform format. The alternatives range from nonchromatographic techniques, like aqueous two-phase separation, membrane filtration, precipitation or crystallization,

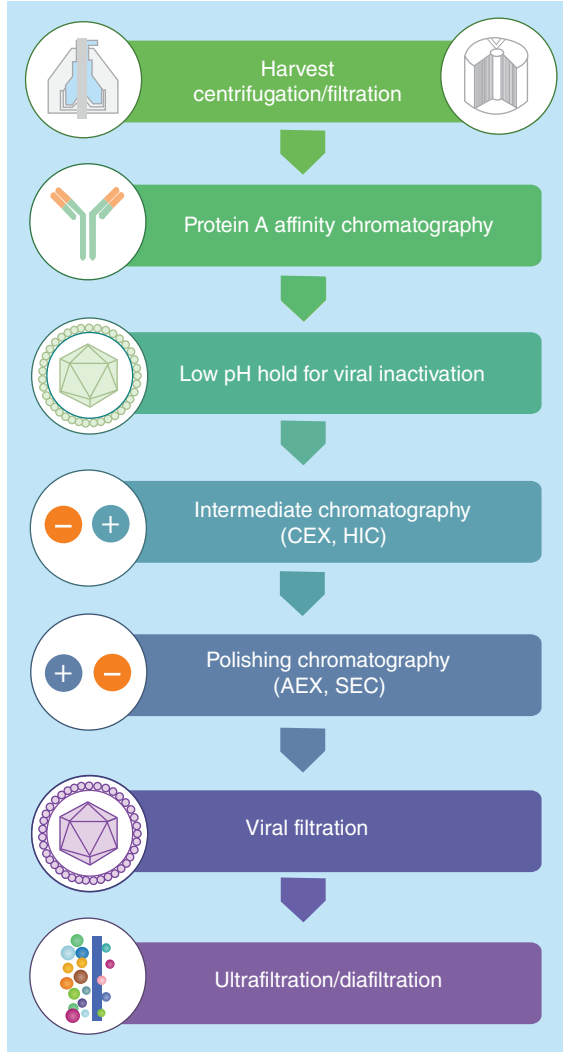


Figure 1. Sequence of unit operations constituting the platform approach employed in the downstream processing of mAbs.
AEX: Anion exchange chromatography; CEX: Cation exchange chromatography; HIC: Hydrophobic interaction chromatography; SEC: Size exclusion chromatography.

to chromatographic steps nonbased in Protein A ligands [27], such as traditional interaction chromatography or emergent modalities like multimodal chromatography (MMC).

MMC has been receiving considerable attention over the last years, with about 152 publications published in ISI-index journals in the last decade (2004–2013). According to Figure 3A, the number of publications per year has been steadily increasing, with a paper-boom starting in 2008. A categorization according to the type of publication can be seen in Figure 3B and accordingly, the majority includes research articles (#101) and patents (#23). Interestingly, from the different research areas that can take advantage of the

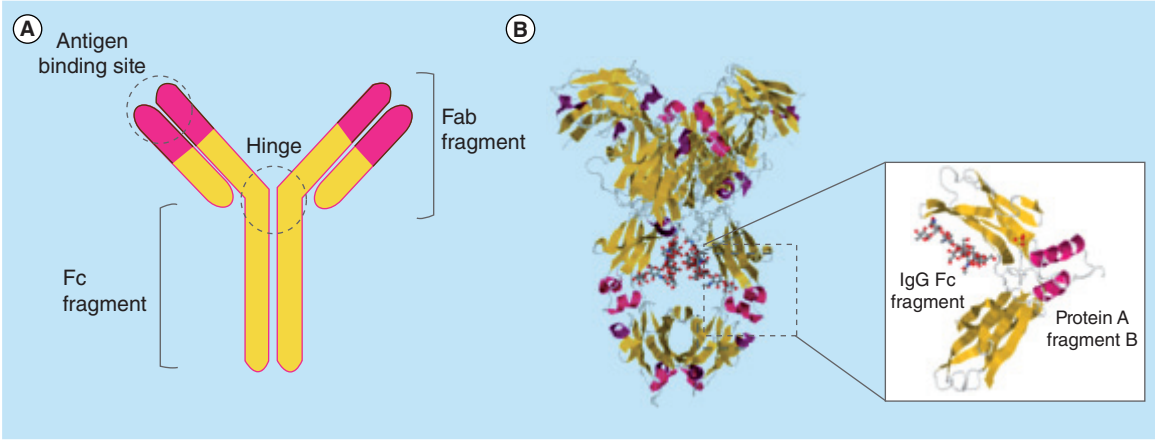


Figure 2. A human IgG monoclonal antibody. (A) Y-shaped structure showing the four polypeptide chains (two heavy chains and two light chains). Variable and constant domains are represented in pink and yellow, respectively. **(B)** Ribbon 3D representation with β-sheets and α-helices represented in yellow and pink, respectively. Zoom in illustrates the binding region of Protein A fragment B (pink) to the Fc fragment. Also represented are the carbohydrate moiety. Structure data files (1HZH [19] and 1FC2 [20]) were downloaded from the Protein Data Bank website [21].

characteristics of this type of chromatography, the purification of mAbs has contributed with an input of 45 publications in the referred time frame. Up to date in 2014, an overall total of 36 publications were already released, which demonstrates that MMC is definitely established in the purification panorama.

Multimodal chromatography

Principles & ligand rational design

MMC has been covered by excellent reviews [28,29] highlighting not only the practical potential of this type of chromatography, but also the fundamentals underlying the multimodal phenomenon. In general terms, MMC can be defined as a chromatographic method employing multiple types of interaction between the stationary phase and the mobile phase, in which the different solutes are present. The binding modes that are more frequently employed in multimodal ligands comprise ion exchange, hydrogen bonding and hydrophobic interaction groups [30], although others may be included for specific purposes, and the strength of each individual interaction can be manipulated accordingly.

Selectivities and specificities that differ from those of traditional ligands endow MMC with a versatility that allows to open up a wide range of possibilities to deal with challenging purification problems. However, considering the multitude of interactions that can be promoted with the ligand and all the factors that govern

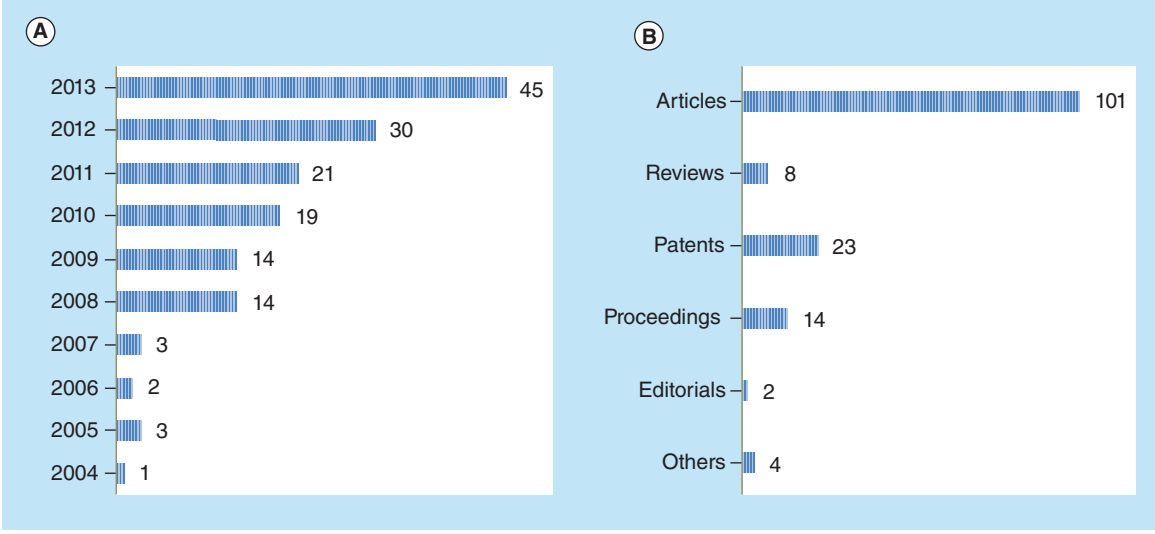


Figure 3. Number of publications focusing multimodal chromatography. (A) Number of publications focusing multimodal chromatography over the last decade, and **(B)** categorized according to the type of publication in the same time frame, according to ISI-web of knowledge. Data retrieved on 14 November 2014.

the different selectivities, the optimization of the conditions to be employed may be a complex process, in which several studies are required. The reported purification studies using these ligands usually start with a design of experiments (DoE) [31,32], to determine the conditions that will allow to take full advantage of the multimodal potential. Monte Carlo simulations also constitute a tool that can be of great importance in improving the process performance [33].

The preparation of MMC media can be accomplished using different approaches. Typically, the ligand carries two or more interactions connected via a chemical scaffold, and additional functionalities can be introduced upon further modifications of the scaffold. However, a simpler approach can also be used, in which the different functionalities are equally, randomly and independently distributed on the matrix. In this last approach, two ligands are effectively present in the resin but in such close proximity that the interaction with the same sample molecule occurs in a complete different manner as if only one of the ligands were present [34]. Thus, instead of having the different functionalities on the same scaffold, these can be provided on separate ligands, which need to be sufficiently close to bind the target in a multimodal fashion. These two different ways of creating multimodal ligands are schematically depicted in Figure 4.

The screening of the appropriate ligand for a separation step is often based on the generation of a diverse library of ligands [34–36], which has been facilitated by increasing knowledge on both protein and ligand structures.

In multimodal ligands, the hydrophobic moiety is typically given by an aliphatic or aromatic group, while the ionic moiety comprises both weak and strong ion exchanger groups, such as amino, carboxyl and sulfonic groups [28]. It should be noted that weak and strong do not refer to binding strength, but rather to the charge behavior at certain pH values. Strong ion exchangers are charged at any practical pH (2–10), whereas the charge of weak ion exchangers depends on the pH. Weak cation exchangers (e.g., carboxyl groups) carry a negative charge at pH values greater than about 5, while weak anion exchangers (e.g., diethylaminoethyl [DEAE]) are positively charged at pH values lower than about 9 [37]. In the architecture of the ligand, it is important to have a well-defined primary functionality for medium control purposes, but attention should also be paid to the additional modes of interaction, particularly to the proportion and location of those groups relative to the primary functionality [38]. The different moieties constituting the multimodal ligand should be wisely chosen, in order to ensure both high capacities and reasonable recoveries.

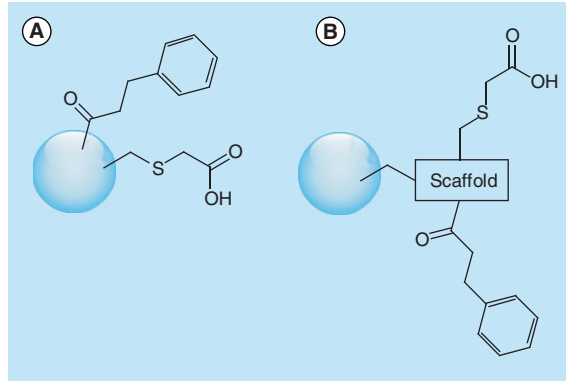


Figure 4. Representation of multimodal media.
Representation of multimodal media created using a random placement approach **(A)** and the connection via a chemical scaffold through a scaffold **(B)**.

In this way, heterocyclic groups represent good hydrophobic patches, which due to their hydrophobicity and dissociation properties, allow adsorption to be performed at moderate or high ionic strengths [39–41]. Regarding the ionic moiety, the pKa is a parameter that deserves careful consideration, since the knowledge of the degree of dissociation of the ionic groups is of capital importance to predict the behavior of the ligand during the purification step, particularly to achieve efficient elution upon decreasing the pH value below the isoelectric point of the protein and the pKa of the ligand.

In addition to these moieties, hydrogen bonding groups are also reported to have influence on the performance of multimodal ligands, through the possibility of hydrogen donation or acceptance, although their impact is frequently subsidiary for selectivity purposes [42]. Thiophilic interactions can also be exploited for ligand integration and can be particularly advantageous in the purification of immunoglobulins, since these biological molecules have a known high affinity toward sulfur-containing ligands [43]. The thiophilic functionality is frequently introduced by means of a reactive site for ligand coupling, where the mercapto groups contribute with sulfur atoms for binding [39].

Regarding the supporting materials, these frequently comprise polysaccharide beads made of agarose or cellulose [42,44], which are biocompatible for protein purification purposes, and at the same time are stable and relatively inexpensive. In analogy to affinity chromatography,

Key term

Design of experiments: Approach in which multivariate data can be fitted to an empirical function, to provide information about the system parameters. In chromatography, it can be used systematically to optimize the performance of the separation by tuning specific factors, such as pH, ionic strength or sample load.

Key term

Hydrophobic charge induction chromatography: Chromatographic technique for separation of biomolecules, in which binding occurs through hydrophobic interaction under near physiological salt conditions, and elution takes place by electrostatic charge repulsion typically by reducing the pH.

a spacer arm should also be introduced in MMC, to ensure adequate accessibility of the proteins to the ligand, and depending on the groups used for the effect, it may occur that the spacer arm contributes itself for protein binding, as reported by Burton and co-workers [45].

In summary, different purposes can be explored within MMC, ranging from capture of specific proteins directly from feedstock at native conductivity, to pH-responsive hydrophobicity for enhanced distinction of structural differences in various protein subpopulations [46,47], which are supported by ever more powerful screening methods allied to a rational design of ligands.

Multimodal chromatography media

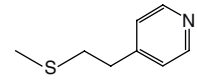
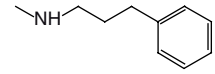
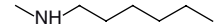
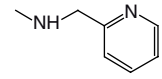
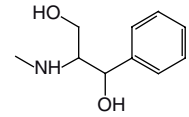
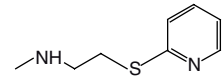
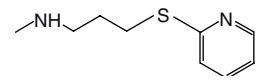
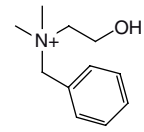
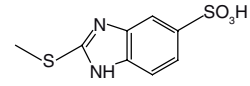
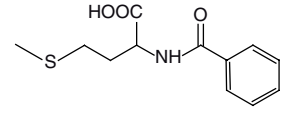
Since the establishment of MMC media as a promising choice for the downstream processing of biological products, there has been a rapidly increasing interest in developing and synthesizing novel ligands for this purpose. Regarding the purification of mAbs, focus has been put on creating ligands whose operating conditions are milder than the ones employed with Protein A affinity chromatography, but also on broadening the selectivity toward different classes of immunoglobulins or antibody-like structures, such as minibodies, since in these cases Protein A lacks the specificity required [48]. Some multimodal ligands that have been routinely reported in the literature are summarized in Table 1, as well the trade name under which some of them are commercialized.

An important family of multimodal ligands is the hydrocarbyl amine family, which comprises the hexyl amine (HEA HyperCel ™), the propyl amine (PPA HyperCel™) and the 2-aminomethylpyridine sorbents. It has been designed to allow completely innovative separation options for protein purification with higher yields and purities [51,52]. This family is one of the most frequently used for this purpose, since the chemistry of the ligands offers hydrophobic and electrostatic interactions. In this family, the site for ligand immobilization is provided by the amine group, which also constitutes the patch for electrostatic interactions. The binding occurs at physiological conditions through a combination of electrostatic interactions and hydrophobic interactions (either aliphatic or aromatic). The elution is achieved through a charge repulsion mechanism, by decreasing the elution buffer pH below to the protein isoelectric point and the ligand pKa, which causes both protein and ligand to become positively charged, thus facilitating protein recovery. This is the basic principle of **hydrophobic charge induction chromatography**, reported by Burton and Harding [49].

Another family of ligands deserving consideration is the Capto™ family, from which the *N*-benzyl-*N*-methyl ethanolamine (Capto™ adhere) and the 2-benzamido-4-mercaptobutanoic acid (Capto™ MMC) are two of the most studied. The development of these ligands derive from the finding that the introduction of hydrogen bonding groups in the proximity of the charged groups would provide high breakthrough capacities at high ionic-strength conditions [34,35]. The Capto™ adhere is a strong anion exchanger with additional possibility of hydrophobic interactions in the phenyl group, and a hydroxyl group for hydrogen bonding. On the other hand, the Capto™ MMC is a weak cation exchanger with a phenyl group as hydrophobic moiety, an amide group for hydrogen bonding, and a thioether group for thiophilic interaction. These ligands are frequently referred to as "salt-tolerant" adsorbents [53], due to their ability to maintain high dynamic binding capacities in a range of different ionic strengths, from moderate to high values. The mechanism of protein elution in these two ligands is complex and difficult to unravel, and it usually requires an increase in both salt concentration and pH value [53], in opposition to the elution by charge repulsion that is mainly driven by changes in the pH value. Several studies have been performed in an attempt to reach optimal elution conditions, including the use of controlled pH gradients [54] and the evaluation of different mobile phase modifiers [55–60], like arginine hydrochloride, ethylene glycol or urea. The work of Charoenrat *et al.* [61] also reports the application of the 2-benzamido-4-mercaptobutanoic acid ligand (also commercialized under the trade name Streamline™ Direct HST I) in expanded bed adsorption chromatography, which revealed to be suitable for the recovery of β-glucosidase directly from the culture broth at high density and high conductivity (15 mS/cm), without requiring prior dilution of the feedstock.

A class of diverse *N*-heterocyclic ligands comprising variations in the type and extent of the pyridyl ring substitution has also been explored for protein purification purposes, including antibodies [50,62–63]. These adsorbents are based on a heterocyclic ring (typically a pyridyl ring) from which pend for instance an alkylthiol, alkylamine or hydroxylalkyl nucleophilic group. The pending arm enables an efficient immobilization

Table 1. Examples of ligands that have been synthesized to be employed in multimodal chromatography.[†]

	Name	pKa	Structure
Ligands positively charged	4-mercaptoethylpyridine (MEP HyperCel™[‡])	4.85	
	Phenylpropylamine (PPA HyperCel™[‡])	6.0–7.0	
	Hexylamine (HEA HyperCel™[‡])	10	
	2-aminomethylpyridine [49]	$pKa_1 = 2.2$ $pKa_2 = 8.5$	
	Aminophenylpropanediol [49]	9.0	
	2-(pyridin-2′-ylsulfanyl) ethanamine [50]	–	
	3-(pyridin-2′-ylsulfanyl) propanamine [50]	–	
	N-benzyl-*N*-methyl ethanolamine (Capto™ adhere[§])	–	
Ligands negatively charged	2-mercapto-5-benzimidazole sulfonic acid (MBI HyperCel™[‡])	–	
	2-benzamido-4-mercaptobutanoic acid (Capto™ MMC[§])	3.3	

[†]The commercial names are indicated in brackets.
[‡]Pall Life Sciences.
[§]GE Healthcare.
HEA: Hexylamine; MBI: 2-mercapto-5-benzimidazole sulfonic acid; MEP: 4-mercaptoethylpyridine; MMC: Multimodal chromatography; PPA: Phenylpropylamine.

of the ligand on the support material, while providing a spacer arm liable to be modified in order to alter the hydrophobicity of the ligand. These compounds are characterized by improved aromaticity/hydrophobicity and dissociation properties, which lead to some important performance advantages comparing to their aliphatic or aromatic counterparts [64]. Additional ligand diversity can be introduced by incorporating extra substituents into the heterocyclic ring, and by including analogs with one or more additional aromatic ring structures [50]. One of the most known and used member of the heterocyclic compounds family is the mercapto-ethyl-pyridine (MEP HyperCel™). MEP exhibits a binding mechanism that includes a mild hydrophobic effect, an electrostatic effect caused by the charge on the heterocyclic ring and also a thiophilic effect on the sulfur group [65]. In a physiological pH environment, the binding occurs through the uncharged pyridine ring, in a way similar to the traditional HIC sorbents, and desorption is achieved according to the principles of hydrophobic charge induction chromatography [49]. However, unlike conventional HIC sorbents, these new-generation ligands have enhanced binding capacities and the elution pools do not contain high salt concentrations that compromises the following steps [64]. It is also important to note that the additional affinity toward immunoglobulins provided by MEP, turns it into a candidate to replace Protein A chromatography, as it does not rely on extremely acidic pH values for elution

of the proteins, and other alternative desorption mechanisms, based for instance on using arginine as eluent [62,66], have proved effective for antibody purification.

Multimodal chromatography integration in a purification workflow

MMC has been finding applications in the separation of a wide variety of compounds, such as oligonucleotides [67,68], nucleic acids [69] including plasmid DNA [70], oligosaccharides [71], peptides, phosphopeptides and glycoproteins [72,73], human growth factor [74] and mAb [75,76].

The archetype of MMC is definitely hydroxyapatite (HA) chromatography, with studies in protein purification dating back from the 1950s [77]. Since then, HA has continued to be explored as a step in the purification of different recombinant proteins, particularly mAbs [78]. Hydroxyapatite can be employed as a polishing step in mAbs purification, since it has demonstrated to be effective in removing aggregates [79] or other impurities [80], as well as host cell impurities and leached Protein A [81]. Through the 1980s and 1990s, several new multimodal ligands were developed [30], triggered by the increasing demand of mAbs as biopharmaceuticals, with the main purpose of providing a synthetic alternative to Protein A ligands, but also offering the versatility to be implemented in different parts of the purification train.

MMC is undoubtedly characterized by unique features that can cope with some limitations of conventional media and therefore widen the frame of operating conditions. However, the introduction of this type of chromatography into a purification train requires that some aspects are carefully considered, namely the determination of the functionalities that the ligand should present in order to fulfill the task needs, the optimum conditions that should be applied, the most suitable mode of operation (flowthrough or bind-elute), and the viability of an eventual scale-up. In addition, for a multimodal step to be industrially implemented in mAbs purification, the type and extent of the interactions ligand-antibody and ligand-impurities must be completely unraveled, and the multimodal ligand should be thoroughly characterized in terms of toxicity and tendency to leaching [30].

Key terms

Hydroxyapatite chromatography: Chromatographic technique that contains ceramic crystals of hydroxylated calcium phosphate – $Ca_{10}(PO_4)_6(OH)_2$– in the stationary phase. Hydroxyapatite contains two types of binding sites: positively charged calcium groups that interact mainly through metal coordination; and negatively charged phosphate groups that act as cation exchangers.

High-throughput screening: Methodology employed for evaluating a wide range of operating conditions in a rapid and facilitated manner, by using for instance 96-well plates to perform optimization studies.

Capture applications

The tailored selectivities allied to the cost–effectiveness and resistance to sanitization procedures of multimodal ligands has led to virtually all of them being evaluated as alternatives to Protein A capture step. In addition, the use of Protein A over multiple purification cycles, due to its limited binding capacity and extremely high cost, causes ligand leakage and diminished performance, which can also be addressed by using multimodal ligands. According to the supplier, Mabselect SuRe™ is five-times more expensive than Capto™ MMC (10 l of Capto™ MMC is 31, 400.00 USD and 10 l of Mabselect SuRe™ is 160,237.00 USD) and ten-times more costly than Capto™ adhere (25 ml of Capto™ adhere is 274.00 USD while 25 ml of Mabselect SuRe™ is 2791.00 USD). Another potentially important difference between Protein A and MMC for antibody capture is that Protein A binds almost entirely to the Fc portion of the antibody (Figure 2) while multimodal ligands may have significant binding interactions with the variable regions. In this section, reported applications of MMC as a first capture step in the purification of mAbs will be reviewed, as well as the main conditions applied to achieve adequate performances, and the impact of the particularities of the ligand in each purification design.

The antibody-selective MEP Hypercel™ sorbent has been extensively studied as an eventual alternative to Protein A media, as it provides similar binding capacities at approximately 25% of the cost, and without suffering from ligand contamination or instability. In the study performed by Schwartz *et al.* [82], the isolation of an mAb from a protein-free cell culture supernatant was accomplished with purity values ≥95% and yields ranging from approximately 83 to 98%. Moreover, the ligand proved to be effective in reducing the levels of a model virus (minute virus of mice [MVM]), and also the DNA content, with a large fraction of DNA removed during binding and washing steps. The MEP ligand is preferably operated at a pH near neutrality and at physiological ionic strength, while desorption is easily achieved by changing the pH, rather than by variations in the buffer ionic strength [83]. Equally favorable results were reported by Guerrier *et al.* [80], which were able to directly capture antibodies from mouse ascites fluid and from a cell culture supernatant containing 5% fetal bovine serum (FBS) with purities reaching 83 and 60%, respectively, the latter being

further improved (>98%) by a second step comprising hydroxyapatite chromatography. The elution pH used with MEP, although acidic (pH ~4.0), is much milder than that typically employed with Protein A chromatography (pH 2–3), which reduces the probability of product inactivation or aggregate formation. Even if the difference is only one pH unit, it can be significant especially for some mAbs that are prone to inactivation at acidic conditions.

The less acidic elution that can be achieved with multimodal ligands has motivated the study of other sorbents that also take advantage of this characteristic, including the HEA Hypercel™ and the PPA Hypercel™ [84]. By employing a high-throughput screening strategy, involving microtiter plates [85] and DoE [86], it is possible to optimize the purification conditions maintaining a mild elution pH, and still improving yield and host cell proteins removal, while preventing the formation of mAb aggregates. Toueille *et al.* [31] explored the properties of the HEA Hypercel™ medium, which revealed to be able to strongly capture HCPs and specifically elute the target mAb, contrary to what is observed with Protein A. For this reason, this sorbent may have attractive features to be used with high efficiency for the capture of unstable or aggregation-prone antibodies.

An *a priori* screening of operating conditions allow to investigate how variations in some parameters (pH, conductivity, load concentration) will affect the responses in a system, and then it is possible to compare the model predictions with the actual measurements on columns. The work of Pezzini *et al.* [32] reports this approach for the investigation of different multimodal resins, among which the PPA HyperCel™ was the one demonstrating the highest efficiency in removing HCPs and the highest percentage of mAb recovery. In this study, the authors showed that in spite of having some similarities, the multimodal media tested also have specific particularities that should be taken into account in the optimization of the purification process. For instance, the optimal conditions for the washing step were quite variable in terms of conductivity (7–23 mS/cm), but close in terms of pH (5.5–6.5). Regarding the elution, a pH ranging from 3.8 to 4.0 allowed electrostatic repulsion between the positive ligand and the positive protein surface, at low conductivity (3 mS/cm) to reduce the hydrophobic interaction. There were also some differences with respect to the host cell proteins remaining in the elution fractions, as demonstrated by the profiles determined using MS analysis. Nonetheless, all the resins evaluated performed very adequately for a capture step, after the optimal conditions had been obtained from the model.

Synthetic ligands comprising heterocycles are recurrently described for mAb capture purposes. In the case of the 2-mercapto-5-benzimidazole sulfonic acid ligand (MBI HyperCel), a negatively charged group was also introduced in the ligand construct, in order to repel acidic impurity proteins that would have the same charge at the binding pH. On the other hand, the pH conditions can be managed so that the sulfonic group can also contribute to IgG binding via electrostatic interactions, providing additional alternatives for the binding mechanism [87]. In the work of Girot *et al.* [88], this ligand was successfully evaluated for the capture and separation of antibodies from different feedstocks, including a cell culture supernatant supplemented with FBS. To deal with the presence of albumin, the binding pH was set at slightly acidic (between 5.2 and 5.5) conditions, to achieve complete binding of IgG while avoiding the co-adsorption of albumin. The binding mechanism was found to result from an assembly of electrostatic and hydrophobic interactions, although the presence of a sulfur atom might be considered for enhanced interaction, regardless of the presence of lyotropic salts.

Capto™ MMC, a negatively charged hydrophobic multimodal ligand, has been recently patented for the capture of mAbs directly from cell culture supernatants [89,90]. Joucla and co-workers [91] have conducted a comparative study involving this promising multimodal ligand and a traditional cation exchanger, regarding the capture of an antibody secreted by CHO cells. The binding conditions were optimized in a microplate assay using a pure human immunoglobulin. It was observed that increasing the buffer conductivity showed not to significantly impact the antibody retention by the multimodal adsorbent, while using the traditional cation exchanger the retention was inevitably reduced. The rationale behind such behavior is that a decrease in the electrostatic interaction is counterbalanced by an increase in the hydrophobic interaction, which translates into a salt-tolerant-binding property. Taking advantage of this distinguished property, Kaleas *et al.* [92] performed a process comparison exercise involving the Capto™ MMC and the Protein A affinity chromatography as the initial capture step for the purification of two mAbs directly from harvested cell culture feedstocks. As the elution tends to be a critical task, four different elution strategies were evaluated in the multimodal adsorbent, comprising a pH-gradient, a sodium chloride gradient with and without urea and an L-arginine HCl gradient. Overall, the mAb desorption seemed to be achieved by disrupting the ionic interaction, although the disruption of other eventual interactions may enhance the elution. The performance obtained in terms of antibody yields was comparable for all the strategies employed and for the different feedstocks loaded on both multimodal

and Protein A media, with values ranging from 90 to 100%. The major drawback reported in this case was the lower level of HCPs clearance given by Capto™ MMC, which could probably be enhanced by further optimization of the elution or by introduction of a wash step to selectively remove these proteins.

The increasing interest in using multimodal ligands with different selectivities has motivated the synthesis of homemade ligands exhibiting multimodal ability [50,93–94] to specifically capture mAbs. The pyridine-based ligands reported in the work of Mountford *et al.* [63] were evaluated in both static- and dynamic-binding studies using pure mAb samples, with mAb recoveries of 90% or higher being typically achieved. The PSEA (pyridinylsulfanylethylamine) and PSPA (pyridinylsulfanylpropylamine) series, which differ in the length of the spacer arm between the exocyclic sulfur and the terminal primary amine (Table 1), were further evaluated in the purification of mAbs from crude cell culture supernatants, and while the former appeared to possess a better selectivity, the latter showed higher capacity for protein binding.

As the window of operations is enlarged with the multitude of options provided by these ligands, it is also important to bear in mind that high-throughput screening is likely to be required for the rapid development of processes based on MMC as capture step, since adjustments will be needed on a case-to-case basis. However, the gains in terms of versatility and economy seem to compensate any time-related constraints for process optimization. In Table 2 are summarized the purification conditions and the corresponding performance parameters obtained for some of the ligands used in the capture of mAbs from different types of feedstock covered in this section.

Charged/hydrophobic multimodal resins with strong cation exchange functionality appear as feasible options to fit in a capture step, unlike their anion exchanger counterparts, which strongly interact with phospholipids and DNA, thus reducing IgG capacity in either flowthrough or elution modes. For this reason, this class of negatively charged multimodal ligands has been explored as a valuable option to include in the polishing of mAbs, even in purification sequences comprising a Protein A affinity chromatography as first step.

Polishing applications

In most IgG downstream processes, the Protein A affinity chromatography is followed by two polishing steps in order to fulfill the final specifications required by regulatory agencies, in terms of host cell proteins, DNA, viruses and aggregate content. The ineffective removal of these compounds during purification can be detrimental for the safety of the therapeutic formulation, since multiple side effects can be unpredictably triggered. The chromatography steps that are typically employed in polishing stages involve cation and anion exchange resins, hydrophobic interaction resins and also ceramic hydroxyapatite adsorbents, which have already been reviewed in the beginning of this section.

Given the potential of multimodal ligands, attempts are currently being made in order to cut one of the polishing steps, and to evaluate the feasibility of processes combining a highly selective first capture step with only one multimodal anion exchange chromatography polishing step. Capto™ adhere, which has been recently launched by GE Healthcare, was specifically designed for the polishing of mAbs following Protein A chromatography, and it is the most widely reported ligand in studies for this purpose. The application of this multimodal ligand has been however limited by poor understanding of its chromatography behavior and attributes. Nevertheless, several research studies have been performed in order to evaluate its performance in flowthrough mode, particularly for aggregate removal, and at a commercial manufacturing scale [96]. Overall, Capto™ adhere is reported to perform better than conventional anion exchange resins, exhibiting higher binding capacity for aggregates, while mAbs pass through the medium without being retained. Chen *et al.* [97] described the advantage of loading the neutralized Protein A elution fraction directly on Capto™ adhere, in order to achieve a better reduction of dimer aggregates, and thus eliminating the need for preconditioning the pool. Similar finding was reported by Gao *et al.* [94], which were able to reach a purity of 97.4% in terms of aggregate removal, with the rationale that mAb dimers would bind more strongly to the ligand than the monomeric forms, due to the combination of hydrophobic and electrostatic interactions.

In another study, Eriksson *et al.* [98] proposed a platform step to follow Protein A also based on this multimodal anion exchanger. The conditions were optimized using DoE for the operation in flowthrough mode. The authors have observed that not only the amount of dimers/aggregates was considerably reduced (<0.1%), but that it was possible to retain key contaminants, including host cell proteins, DNA, leached Protein A and viruses. Regarding viral clearance, two model viruses (minute virus of mice [MVM] and murine leukemia virus [MuLV]) were successfully removed at both high and low ionic strength, which would not be expected to occur in a conventional anion exchange.

Although Capto™ adhere is usually operated in flowthrough mode, according to the suggestions

Table 2. Typical purification conditions and corresponding performance parameters for some of the multimodal resins frequently reported for the capture or polishing steps in mAbs downstream processing.

Ligand	Type of feedstock	Purification buffers	Performance parameters	Ref.
MEP HyperCel™	Cell culture supernatant containing FBS	A: 25 mM phosphate + 25 mM NaCl, pH 7.2	Yield = 76%	[80]
		W: A + 25 mM sodium caprylate	Purity = 69%	
		E: 50 mM acetate, pH 4.0	PF = 40	
	Protein-free cell culture supernatant	A: 50 mM Tris-HCl, pH 8.0	Yield ~ 83%–98%	[82]
		E: 50 mM acetate, pH 4.0	Purity ™ 95%	
HEA HyperCel™	Cell culture supernatant	A: PBS	Yield = 92%	[31]
		W: 5 mM sodium phosphate, pH 7.4	HCPs = 730 ppm	
		E: 50 mM sodium acetate, pH 5.5	Aggregates < 0.5%	
PPA HyperCel™	CHO cell culture supernatant	A: pH 7.3; 13 mS/cm	Yield = 93% HCPs = 430 ppm	[32]
		W: pH 5.5; 23 mS/cm		
		E: pH 3.8; 3 mS/cm		
MBI HyperCel™	Cell culture supernatant containing FBS	A: 50 mM acetate + 0.14 M NaCl, pH 5.2 E: 50 mM carbonate + 0.14 NaCl, pH 9.0–9.5	Yield: no antibodies were found in the flowthrough fraction Purity >90%	[88]
Capto™ MMC	CHO cell culture supernatant	A: 0.1 M sodium citrate, pH 5.0 E: 0.1 M sodium phosphate, pH 7.5	Yield = 92%–93% Purity = 95/96%	[91]
	CHO cell culture supernatant	A: Variable to match the pH of the feedstock E1: pH gradient from 7.0 to 10.0 E2: salt gradient from 0 to 0.3 M NaCl, pH 7.0 E3: 2 M urea + gradient of 0.3 M NaCl, pH 7.0 E4: L-arginine HCl gradient from 0 to 0.3 M, pH 7.0	Yield = 90%–91% Monomer = 96.8%–98.5% HCPs = 500–2600 ng/mg	[92]
2-PSEA	Pure mAb sample	A: 600 mM sodium sulfate + 25 mM Tris, pH 9.0	Yield = 85%–96%	[63]
		E: 25 mM HEPES, pH 7.0		
2-PSPA	Pure mAb sample	A: 600 mM sodium sulfate + 25 mM Tris, pH 9.0	Yield = 84%–91%	[63]
		E: 25 mM HEPES, pH 7.0		
Capto™adhere	Elution pool from Protein A capture	A: 50 mM phosphate, pH 7.5 E: 50 mM citrate, pH 2.6	Yield = 80.1% HCPs =14.5 ppm Aggregates = 2.6%	[94]
	Elution pool from a MCSGP capture	A: 10 mM phosphate, pH 8.0 E: 10 mM phosphate + 10 mM citrate, pH 4.0	Purity >99.7% HCPs <3 ppm	[95]

CHO: Chinese hamster ovary cells; FBS: Fetal bovine serum; HCP: Host cell proteins; HEA: Hexylamine; MBI: 2-mercapto-5-benzimidazole sulfonic acid; MCSGP: Multicolumn countercurrent solvent gradient purification; MEP: 4-mercaptoethylpyridine; MMC: Multimodal chromatography; PBS: Phosphate buffered saline; PF: Purification factor; PPA: Phenylpropylamine.

of the supplier, there are some reported cases in which the operation in bind-elute mode was found to be advantageous in the polishing stage. Voitl and co-workers [99] explored the possibility of employing Capto™ adhere to separate an antibody (pI = 8.3–8.6) from lysozyme (pI = 11.35), by binding the antibody at neutral pH, while the weakly bound impurity flowed through the sorbent. Depending on the isoelectric point of the mAb and the impurity protein, this may not be achieved in a pure anion exchanger, making an apparently simple separation between two different proteins a completely impossible task. Other studies mention newly developed processes comprising a polishing step with Capto™ adhere in bind-elute mode to consistently obtain a product within the specifications limits in terms of HCP content, which failed in the operation in flowthrough mode [95].

In summary, the application of multimodal resins as stationary phases for the polishing of mAbs holds great promise for shortening the number of steps required to achieve the final product specifications. Although the primary mode of interaction relies typically on anion exchange groups, the different groups included in the multimodal ligand endow it with the versatility required to operate either in flowthrough or in bind-elute mode, depending on the intended purpose. Table 2 also includes information regarding the purification conditions used with Capto™ adhere in the different operating modes, as well as the corresponding performance parameters.

Conclusion & future perspective

The ability to produce antibodies with predefined specificity [100] marked the dawn of the modern therapeutic antibody industry, and since then an unprecedented attention has been paid to these promising molecules [101]. Their versatility and robustness made them key compounds in research, diagnostic and therapeutic applications, further reinforcing their potential in a market ascending to the billion dollars [102]. However, this remarkable success inevitably requires a balance in terms of the manufacturing costs, in order to make these recombinant proteins more affordable and accessible to the general population. With manufacturing costs shifting from the upstream to the downstream processes, the focus is now on designing, optimizing and intensifying the purification steps, particularly the capture of mAbs, which is the major responsible for cost-related constraints.

Among the different possibilities that have been proposed to address these issues, MMC has been in the front line of the discussion, since the properties of the ligands can be tuned in order to allow an efficient capture and a significant purification in a single step, which is frequently not possible using single-mode adsorbents, even when employed sequentially. Nevertheless, the multimodal adsorbents currently available for the purification of mAbs are still not widely adopted for process-scale applications. This may stem from the generalized assumption that they are not thoroughly understood, and so extensive studies would be required to predict ligand behavior and to achieve chromatographic separation. Future inroads are expected to rely on model-based approaches [103] combined with spectroscopy techniques [104–106] to provide further insights on the structural basis of protein interactions with multimodal ligands. A more profound comprehension of the predominant regions involved in protein binding and the identification of synergetic interactions may be of utmost importance to demonstrate the competitiveness, and even superiority, of multimodal chromatography over conventional methods.

The evolution of the ligands themselves may also be a path for the progress of MMC, and for that, advances in computational and experimental methods may be the key for allowing the tailoring of ligands for the capture and polishing of virtually any protein. Furthermore, to fully debottleneck the downstream processing of recombinant proteins, the development of resins with higher ligand densities would be required to achieve much higher binding capacities.

Multimodal ligands are also amenable to be used in different supports, such as monoliths or membrane adsorbers, which may be a distinctive advantage since the future of packed-bed chromatography for biomolecules purification is now under questioning [107]. Notwithstanding, the industry gold standard for purification is unlikely to change, and particularly regarding the Protein A affinity step, the incredibly high performance that is achieved will probably never find a parallel with any other non-affinity method. For this reason, to shift the current paradigm it is important to evaluate the overall performance of multimodal ligands integrated in a complete process, rather than focusing on their achievements *per se*, ensuring that the downstream bottlenecks do not cancel out the upstream gains.

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Executive summary

Historical perspective of antibody purification

- The plasma fractionation technique can be considered the basis for the purification of antibodies for therapeutic applications.
- Early processes for mAb purification included several stages organized in a complex manner, including multiple chromatographic steps for protein separation.
- With advances in the upstream production, and changes in the requirements imposed by the regulatory agencies, improved adsorbent matrices started to be used to increase the purification performance.

Current approaches in mAbs downstream processing

- The downstream processing of mAbs currently follows a standardized platform approach, in order to minimize the time and resources required for process development.
- Protein A chromatography is the capture step of choice, but it is the major responsible for the high manufacturing costs of mAbs, in addition to other inherent limitations.
- The need for cost-effective processes triggered the demand for alternative strategies, among which multimodal chromatography (MMC) appears as a promising option.

Principles & ligand rational design

- In MMC, a plurality of interactions can be simultaneously promoted, most frequently electrostatic interaction, hydrogen bonding and hydrophobic interaction.
- The ligands can be created to provide enhanced selectivities toward challenging feedstocks, allowing to capture specific proteins at native conductivity or to display pH-responsive hydrophobicity.

MMC media

- Several new ligands have been synthesized aiming at replacing Protein A affinity chromatography, which are advantageous in its salt tolerance property and mild elution conditions.
- Different ligand families, including hydrocarbyl amines, hydrophobic cation/anion exchangers and *N*-heterocyclic ligands, are among the most extensively explored for mAb purification, and the majority are already commercially available.

Integration in a purification workflow

- MMC finds applications in the separation of a wide variety of biological compounds, namely nucleic acids, peptides, glycoproteins, and mAbs.
- Charged/hydrophobic multimodal ligands with strong cation exchange functionality are being successfully evaluated for the capture of mAbs, allowing recoveries and purities comparable to those of Protein A affinity chromatography, in some cases.
- The anion exchanger counterparts are unlikely to evolve as capture steps, due to their strong interaction with phospholipids and DNA, however good results are being obtained with their application as polishing steps, to remove HCPs or aggregates.

Future perspective

- The adoption of MMC for process-scale applications will require further understanding of the mechanisms underlying the separation of proteins.
- Model-based approaches are starting to be used to predict the interactions that are promoted upon certain conditions, and design-of-experiments strategies are frequently required for process optimization.
- The gains that MMC introduces in terms of versatility and operating costs seem to surpass the time-related limitations with optimization, and future paths may depend on this type of chromatography to debottleneck the downstream processing of mAbs.

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