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Affinity chromatography as a tool for antibody purification

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ABSTRACT

The global antibody market has grown exponentially due to increasing applications in research, diagnostics and therapy. Antibodies are present in complex matrices (e.g. serum, milk, egg yolk, fermentation broth or plant-derived extracts). This has led to the need for development of novel platforms for purification of large quantities of antibody with defined clinical and performance requirements. However, the choice of method is strictly limited by the manufacturing cost and the quality of the end product required. Affinity chromatography is one of the most extensively used methods for antibody purification, due to its high selectivity and rapidity. Its effectiveness is largely based on the binding characteristics of the required antibody and the ligand used for antibody capture.

The approaches used for antibody purification are critically examined with the aim of providing the reader with the principles and practical insights required to understand the intricacies of the procedures. Affinity support matrices and ligands for affinity chromatography are discussed, including their relevant underlying principles of use, their potential value and their performance in purifying different types of antibodies, along with a list of commercially available alternatives. Furthermore, the principal factors influencing purification procedures at various stages are highlighted. Practical considerations for development and/or optimizations of efficient antibody-purification protocols are suggested.

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1. Introduction

The production and applications of antibodies have developed very significantly in the last few decades. In particular, the discovery of monoclonal antibodies derived from the development of hybridoma technology and subsequent advancements in molecular biology and genetic engineering methods, leading to miniaturized antibody formats and novel scaffolds [1–4], have been of major significance. The discovery of antibody display platforms, combinatorial chemistry techniques and the proteomics revolution has also resulted in major improvements in antibody generation.

Antibodies now find extensive use in a plethora of *in vivo* and *in vitro* applications. For diagnostics, they are the ideal biological recognition reagents, and, thus, are useful in a range of analytical platforms, e.g. Western blotting (immunoblotting), immunohistochemistry, immunocytochemistry, immunoprecipitation, enzyme-linked immunosorbent assay (ELISA), antibody microarrays, antibody-imaging/immunoscintigraphy, radioimmunoassays, flow cytometric analysis, immunosensors, immuno-polymerase chain reactions (IPCRs) and real-time IPCRs [5–13]. Antibodies are also

used as important tools in immunoaffinity chromatography (a form of chromatography that uses the antigen–antibody interaction for separation of an antigen of interest from a complex mixture of proteins) [14]. Furthermore, antibodies are now increasingly employed in immunoprophylaxis, drug targeting and immunotherapy [15–21]. In addition, antibodies are extensively used in basic research, e.g. for identification and localization of intracellular and extracellular proteins and for mediating and/or modulating different physiological and pathological conditions [7]. Antibodies are also widely utilized for the detection of food-borne pathogens, adulterants, toxins and residues (drug, chemical or pesticide) in food samples and in environmental analysis/monitoring [22–35].

Most of the aforementioned applications necessitate homogeneous antibody preparations. Antibodies are usually isolated from plasma, serum, ascites fluid, cell culture medium, egg yolk, plant extracts or bacterial and yeast cultures. All of these sources contain different proteins in addition to antibodies. Hence, efficient purification of antibodies becomes imperative. For example, purified antibodies are essential for immunodiagnostics using complex clinical samples, for quantitative immunoassays, for drug targeting, immunoprophylaxis and immunotherapy, for proteomics and for immunoaffinity chromatography. Antibody purification is also necessary when chemical modifications such as labeling with

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fluorescent or radioactive probes are needed or when antibody-fragmentation is required for binding or crystallization studies [36].

Antibody purification can be achieved by a range of methodologies based on the specific physical and chemical properties of antibodies, such as size, solubility, charge, hydrophobicity and binding affinity (Fig. 1). Consequently, a large number of methodologies, including precipitation, electrophoretic separations, filtration, liquid and affinity chromatography were applied for antibody purification. However, affinity chromatography-based purification protocols continue to be the most efficient and widely employed [37,38].

2. Antibodies

2.1. Antibody structure

Antibodies or immunoglobulins (Ig), are unique, soluble glycoproteins secreted by B-lymphocytes in response to exposure to a foreign antigen. Antibodies possess the ability to bind specifically to their respective antigen with a high degree of affinity [7,39]. This antibody–antigen interaction forms the basis of the widespread use of antibodies in a vast array of biotechnological and medical applications [40].

Mammalian immunoglobulins are classified into five classes based on their distinct structural and biological properties: IgA, IgD, IgE, IgG and IgM [41,42]. The most abundant mammalian immunoglobulin is the IgG class [43,44]. The rate of synthesis of IgG is higher by comparison to other classes of immunoglobulin and the half-life is longer. IgGs are also smaller than the other immunoglobulin classes and are stable during isolation and purification processes. Hence, IgG is the most widely used immunoglobulin in assay development and antibody-based therapeutics [45]. Immunoglobulin Y (IgY) is the avian equivalent of mammalian IgG [46]. Many advantages are associated with avian IgY including low cross-reactivity with mammalian proteins, high yields (100–150 mg IgY per egg yolk), stability and ease of preparation and no sacrificing or bleeding of the host is required [47,48].

The structure of immunoglobulins varies depending on the isotype. Typically an immunoglobulin is depicted as a Y-shape structure containing two large heavy chains and two smaller light chains, connected by disulfide bonds (Fig. 2). Heavy and light chains are further divided into variable (V) and constant (C) regions.

The amino acid sequence in the variable regions of the antibody varies greatly among different antibodies. Each variable region comprises of three hypervariable (HV) regions interspersed by four framework regions (FR). The FRs provide a backbone structure for

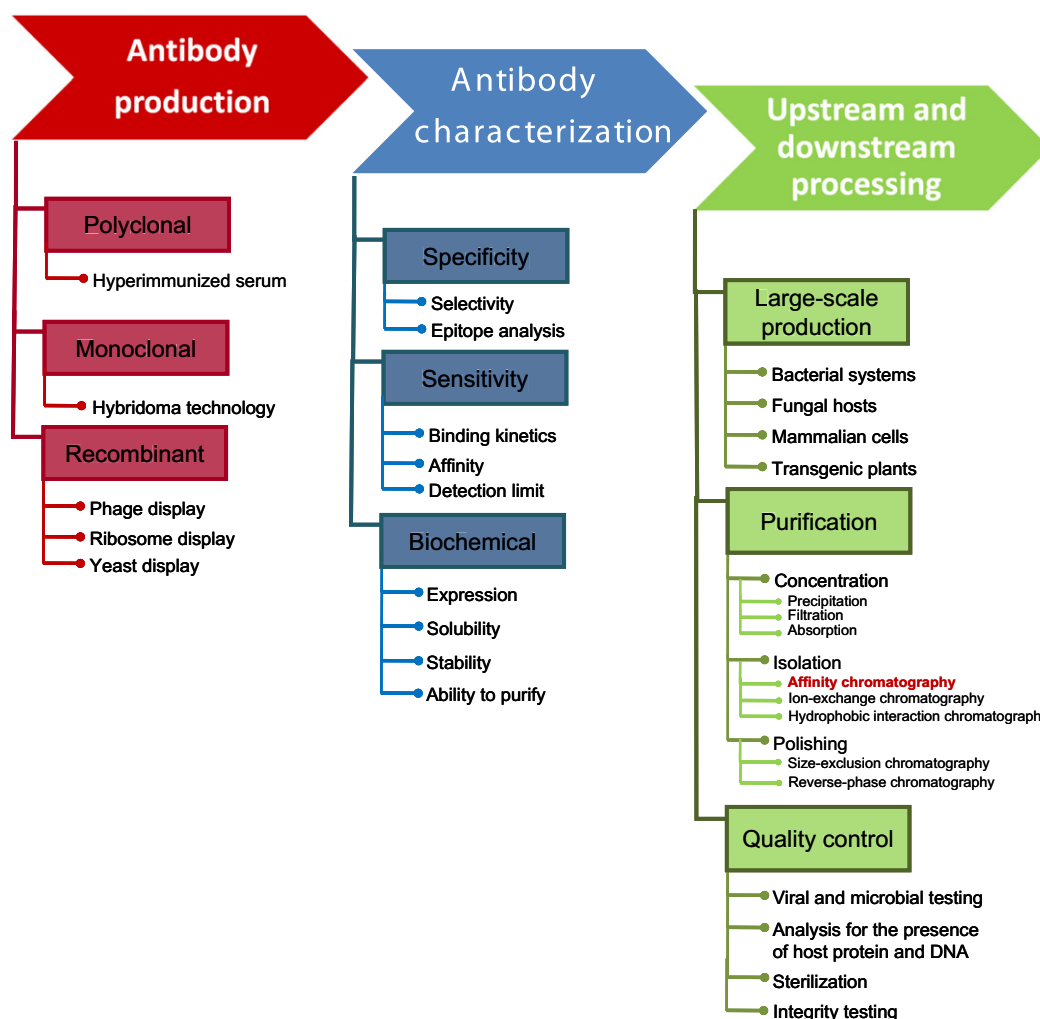


Fig. 1. Production, characterization and purification of antibodies. Antibodies represent a large market sector owing to their application in various fields including diagnostics and therapy. A brief overview of the steps involved in antibody production including scale-up and purification is shown. Careful selection and optimization of each step is necessary depending on the intended use of the product. The quality of the final product is a crucial aspect. Thus, it is regularly checked to ensure compliance with the relevant standards before the product is used.

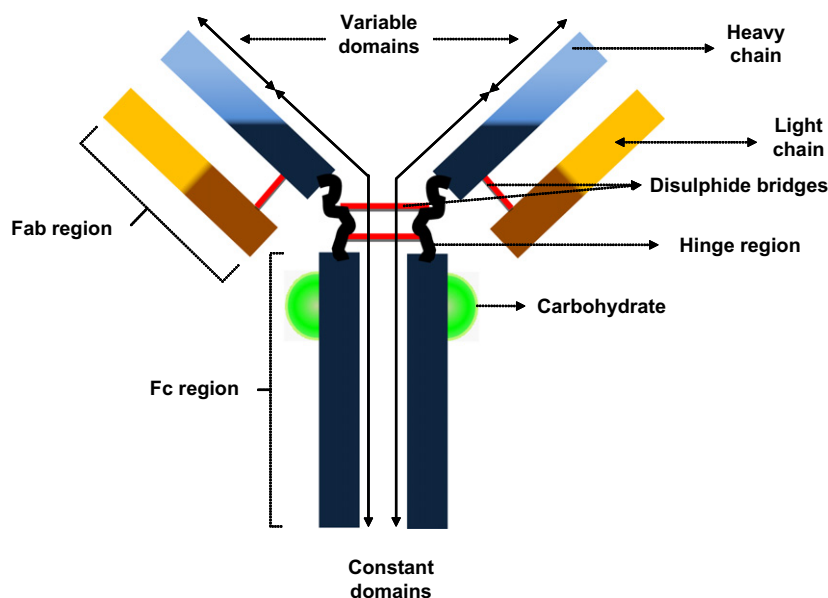


Fig. 2. Basic antibody (IgG) structure. IgGs are molecules of approximately 150–155 kDa in weight, containing two heavy (50 kDa) and two light chains (25 kDa) composed of different domains. The heavy chain consists of a variable domain (V_H) and three constant domains (C_{H1} , C_{H2} and C_{H3}). The two heavy chains are connected by disulfide bonds (SS). The light chain has one variable domain (V_L) and only one constant domain (C_L). There are five different types of heavy chains, α , γ , μ , δ and ϵ , which determine the class of antibody, and only two types of light chains, κ and λ . Each antibody molecule normally contains only one type of heavy chain and one type of light chain.

the antibody and the HV regions confer the ability to identify and bind to a specific epitope on an antigen. The antigen-binding site is formed from six HV region loops, three each from both heavy and light chains. HV regions are also known as “complementary determining regions” (CDRs) (Fig. 2). It is worth noting that FRs can also have an influence on antigenic specificity.

Functionally, an antibody is divided into a Fab region (fragment antigen binding) and an Fc region (fragment crystallizable) (Fig. 2). The Fab region serves as the antigen-binding site and contains the two constant and variable domains of both light and heavy chains. The Fc domain, containing 2–4 heavy chain constant domains (depending on class or species), plays no direct role in antigen binding, but has certain effector functions (e.g. binding complement and binding to cell receptors on macrophages and monocytes), and it also serves to distinguish one class of antibody from another [49].

2.2. Types of antibodies

There are three types of antibody. Antibodies produced from different B-lymphocyte lines are known as polyclonal antibodies (pAbs). pAbs are a heterogeneous mixture of antibodies produced in a host following immunization, with different antibodies recognizing different epitopes on the antigen(s). In contrast, a monoclonal antibody (mAb) is specific to a single epitope on an antigen and is the product of a single B-lymphocyte clone [11]. Recombinant antibodies/antibody fragments (rAbs) are the third type of antibodies. These are antibodies or antibody fragments generated in the laboratory using molecular techniques. rAbs are produced in various formats (Fig. 3), including full-length, small and conjugated antibodies (for detailed information on rAbs see Refs. [21, 50–52]). The most commonly used rAb formats are single-chain fragment variable (scFv) and Fabs [53].

3. Affinity Chromatography – principles and components

Traditional antibody purification protocols relying on salt precipitation, temperature and pH failed to meet the strict quality

and regulatory standards required for many biopharmaceutical applications. This requirement and the need for highly purified antibodies, eventually led to the development of more selective isolation methods. Affinity purification was introduced, in 1968, by Cuatrecasas and co-workers [54]. Affinity chromatography is a biochemical separation technique that relies on a reversible interaction between a protein and its cognate ligand, e.g. binding of an antigen to its specific antibody. The specificity of binding provided by the ligand is exploited for selective absorption of the target protein from a complex mixture, which can be eluted either by using competitive analogs, denaturing agents or changing factors such as pH, ionic strength or polarity.

Affinity chromatography is undoubtedly the most widely employed method for antibody purification. Over the past few decades considerable efforts were made to streamline the purification process, in terms of specificity, selectivity, reproducibility, economy, product recovery, storage and maintenance. This was achieved by developing novel affinity methodologies linked to the identification and design of novel ligands and matrices for immobilization. In order to obtain high yields and purity it is necessary to consider the type of ligand, the matrix to which it is attached and the purification procedure, which may require optimization depending on the type/class of antibody and its ability to recognize the immobilized ligand.

3.1. Chromatography matrix

A suitable matrix is a prerequisite for affinity chromatography. It acts as a supporting material for the ligand. An ideal matrix should be uniform, macroporous, hydrophilic, chemically and mechanically stable, selective, exhibit minimum non-specific absorption, is insoluble in the solvent used in purification, has ideal flow characteristics and provides a large surface area for ligand attachment. Additionally, a matrix must facilitate chemical activation, thus facilitating the coupling of required ligands.

Cyanogen bromide (CNBr) was the most studied and widely used activation reagent. However, though initially used extensively for coupling reactions for affinity chromatography, it is highly toxic

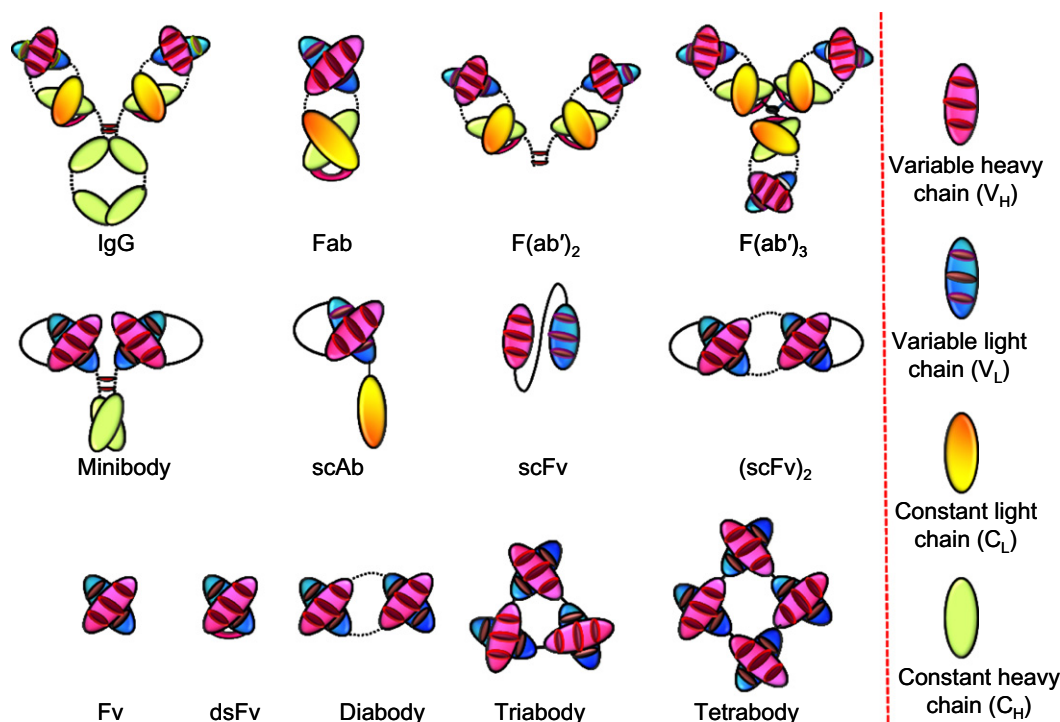


Fig. 3. Schematic representation of different antibody formats. An array of recombinant antibody formats were developed using combinations of antibody domains, joined either with linkers and/or disulfide bonds. Using molecular biology tools, antibody valency and specificity can be tailored into monovalent (fragment variable (Fv), disulfide-stabilized Fv antibody fragment (dsFv), scFv, single-chain antibody fragment (scAb) and Fab), divalent (minibody, diabody, F(ab')₂ and (scFv)₂) and multivalent (tetrabody, triabody and F(ab')₃) formats. Formats with antibody constant domains are highly stable, however, single chain antibody formats are popular due to their small size, expression and tissue penetration capabilities. Linker length can also influence the antibody behavior in terms of stability, flexibility and aggregation.

with attendant safety hazards. Consequently other reagents, such as carbodiimide, tresyl chloride, glutaraldehyde and phosphoryl chloride, can be utilized and are less problematic to use. For details on the selection of activation reagents and methodology the reader is referred to a review by Luong and Scouten [55]. Pre-activated matrices are available commercially (Table 1), and eliminate many of the steps and problems of chemical activation of the matrix prior to use. A wide range of coupling chemistries, involving primary amines, thiols, aldehydes, hydroxyls and carboxylic acids are available for covalently attaching ligands to matrices.

Matrices for use in affinity chromatography can be divided into three groups – natural, synthetic and inorganic. Agarose, dextrose and cellulose beads are commonly employed natural matrices that satisfy the majority of the parameters mentioned earlier. Synthetic supports include acrylamide [56], polystyrene [57] and polymethacrylate derivatives [58], whereas, porous silica [59] and glass [60] are some frequently reported inorganic matrices. For a detailed review on affinity matrices refer to Varilova et al. [61].

Magnetic beads offer an attractive alternative for separation of low abundance proteins from complex mixtures. Affinity separation

based on magnetic bead technology is a simple, affordable, rapid, robust procedure with few pre-treatment steps (centrifugation, column preparation) [62,63]. It also offers the advantages of high throughput automation, suitability for being used in cell separation and capacity for use in immunoassay development, over the conventional resins [64,65]. It is a gentle means of separation exposing the proteins present in high concentration to low shear forces. Magnetic beads are prepared by entrapping magnetite within agarose or other polymeric material, on which the ligand is immobilized. Following the interaction of ligand and protein, under the influence of a magnet, rapid separation can be achieved. The use of magnetic beads has become very popular as indicated by its widespread applications and commercialization.

The use of membranes as affinity matrices is widely reported for use in protein purification, due to their simplicity, ease of handling, reduced surface area and lower diffusion limitations compared to gels, resins and beads [66]. Sun and colleagues successfully utilized affinity membranes for the successful purification of a recombinant allophycocyanin-specific antibody [67]. Affinity membranes are adaptable to be used in various sizes/formats. This feature is of great significance in high-throughput applications (proteomics, genomics) allowing multiple analyses to be performed in a short time. Affinity membranes like SwellGel disks (Thermo Scientific Pierce) were used for protein expression and purification analysis [68,69].

3.2. Ligands used in affinity chromatography

Ligands are essential components in affinity chromatography, as they play a major role in the specificity and stability of the system. During the past few decades, an array of ligands were developed and studied to improve protein purification. Detailed coverage of all the ligands used in protein purification is beyond

Table 1
Commercially available activated resins for use in affinity chromatography.

Product name	Functional group specificity
UltraLink Iodoacetyl resin	–SH
CarboLink Coupling resin	–CHO, C=O
Profinity™ Epoxide resin	–NH ₂ , –OH, –SH
Affi-Gel 10 and 15	–NH ₂
Pierce CDI-activated resin	–NH ₂
Epoxy-activated Sepharose™ 6B	–NH ₂ , –OH, –SH
CNBr-activated Sepharose 4 Fast Flow	–NH ₂
EAH Sepharose™ 4B	–COOH, –CHO
Thiopropyl Sepharose™ 6B	–SH
Tresyl chloride-activated agarose	–NH ₂ , –SH

the scope of this review. Therefore, only ligands used extensively for the purpose of antibody purification, are discussed. General characteristics of a ligand, that need to be considered, are its affinity to the target, its specificity, immobilization feasibility, stability in harsh washing and elution conditions and retention of target binding capacity after attachment to the matrix. It is often necessary to direct the orientation of the ligand to avoid steric hindrance of the binding sites in order to achieve maximum binding. In many cases the use of a spacer arm for the attachment of ligand to the matrix may be crucial to avoid these problems, and the length and nature of spacer should be considered [70].

3.2.1. The use of biospecific ligands in affinity chromatography

Biospecific ligands represent a group of naturally derived substances such as antibody binding proteins, bacterially derived receptors, antigens, lectins or anti-antibodies directed to the antibody requiring purification. In the past, high binding affinities of biospecific ligands to immunoglobulin have been reported [37,71].

3.2.1.1. The use of bacterial receptors as biospecific ligands. The emergence of bacterial proteins as a method for the purification of immunoglobulin was initially reported in the 1970s [72]. The high affinity of bacterial proteins towards antibodies has made them powerful tools for use in the detection and purification of antibodies. Staphylococcal protein A (SpA) and streptococcal protein G (SpG), isolated from bacterial cell walls, are the most commonly used ligands for purification of full length antibodies. They allow binding of antibodies from various species, albeit with different affinities based on the various antibody subclasses.

Protein A is composed of five Ig-binding domains (designated E, D, A, B and C) [73]. Crystallographic studies showed that protein A binds to the Fc region of IgG at the junction between its C_H2 and C_H3 domains [74]. It is also found to bind the heavy chain variable region between CDR2 and CDR3 [75,76]. Protein G is isolated from C and G groups of *Streptococcus* and it binds strongly to the Fc region of IgG. It is also reported to possess low affinity towards the C_H1 domain of Fab region, thus, allowing purification of Fab fragments through a b-zipper interaction [77,78]. When comparing both ligands, protein A is often preferred due to the binding of protein G to albumin, α_2 -macroglobulin and kinogen, along with IgG [79], leading to reduced purification efficiency. Protein G also has less binding capacity and reduced stability compared to protein A during harsh elution steps. However, protein A binding is restricted to certain species and antibody subclasses. To overcome the drawbacks associated with native proteins, recombinant and engineered forms of protein A and G were developed. A range of protein A and G products on different support materials are marketed by various manufacturers (Thermo Scientific Pierce, Invitrogen, Merck Millipore and Sigma–Aldrich). A genetically engineered, fusion, protein, combining the IgG binding domains of both protein A and G, and binding to all human IgG subclasses was also reported [80–82] and is available commercially.

The advent of recombinant antibody fragments, such as Fab and scFv, necessitated the identification of new ligands which would recognize the antigen-binding domains of these fragments, as these fragments lack the Fc region, recognized by protein A/G ligands. The identification of protein L, isolated from *Peptostreptococcus magnus*, solved this problem due to its nanomolar affinity towards kappa (κ) light chains of variable region of antibody. It binds specifically to κ 1, κ 3 and κ 4 subclasses of the antibody light chains while not recognizing κ 2 and λ subgroups [37]. When compared with protein A and G, it has an ability to bind antibody from different classes (IgG, IgM, IgY, IgD and IgE) [37].

Most strategies for antibody purification using bacterially derived ligands bind at neutral pHs. Protein A binds most strongly at pH 8.2, protein L at pH 7.5, and protein G at pH 5.0, but protein

G can also bind at pH 7.0–7.5. The concentration of salt in binding buffer can significantly affect antibody binding to the ligand, by impacting the ionic and hydrophobic interactions involved. Acidic elution is the most common method used for elution of bound antibodies from the protein A, G and L resins. Elution buffers with a pH between 2.5 and 3.0 are used for this purpose. However, low pH may adversely affect the integrity of the antibody eluted, resulting in loss of activity. This is avoided by immediately restoring the pH of the sample to neutral or slightly basic conditions [83]. Aggregation is another common problem associated with low pH elution. Additives such as NaCl, Na₂SO₄ or arginine can be used to alleviate such problems [84]. Alternatively, urea or competitive eluants containing histidine and imidazole can be considered for elution [85].

3.2.1.2. Lectins. Lectins are proteins that bind to the carbohydrate moiety of polysaccharides, glycolipids or glycoproteins. Immunoglobulins are glycosylated at various sites depending on the antibody class and species. The most abundant immunoglobulin IgG possesses an N-linked glycosylation site at position Asn-297 [86]. This feature is exploited by lectins for antibody purification. Lectins exhibit varying degrees of specificity based on the sugar content, conformation and bonding, e.g. concanavalin A (ConA), isolated from *Canavalia ensiformis*, recognizes α -D-mannose and α -D-glucose [87,88], whereas, wheat germ agglutinin (WGA) binds to sialic acid and molecules containing N-acetyl-D-glucosamine residue [87]. Similarly, another lectin from mammalian serum, mannan-binding protein (MBPs) has affinity towards mannose and N-acetylglucosamine [89], while a plant lectin, isolated from jackfruit *Artocarpus integrifolia*, jacalin, binds to α -D-galactosyl groups [90]. Lectins are generally considered for IgD, IgA and IgM purifications, which are otherwise difficult to purify using conventional protein A or G ligands. Binding of mAb to lectin target molecules usually occurs at neutral pH. Divalent cations like Ca²⁺ and Mn²⁺ promote binding in the majority of cases [91]. Elution can be carried out at neutral pH using competitive or inhibiting ligands using 0.1–0.5 M sugar concentrations (e.g. D-glucose, α -D-mannose, methyl- α -D-glucoside). Alternatively, the use of increasing temperature and NaCl concentrations, sodium borate-containing buffers, the addition of ethylene glycol and urea can also be considered [91,92].

3.2.1.3. Antigens as ligands. Antigen-specific antibodies can be obtained by immobilizing a specific antigen on the chromatographic matrix and using it for isolating antibodies from a complex mixture. Whole antigens, or specific peptides representing antigenic epitope(s), can be used for this purpose. Sometimes, when the antigen is expensive or unavailable, ligands mimicking the antigen (in terms of structure or sequence) can be used for this purpose. However, such a method may lead to the selection of antibodies with different affinities [93], which will require further optimization of elution conditions based on the affinity of the antibodies required to be purified.

3.2.1.4. Anti-antibodies as ligands. Anti-antibodies are another attractive approach offering high-specificity options for antibody purification. The C_H and C_L domains of antibodies can be used as potential targets for this process. Recently, single-domain camelid antibodies, possessing single variable heavy chain (V_HH), have become available for this purpose [94]. They are fully functional antibodies with three CDRs offering the advantages of small size (13–15 kDa), high affinity, stability and a three dimensional structure that enables binding to novel epitopes. Single-domain antibodies (sdAb) can be an attractive alternative to conventional ligands as they can be produced on large-scale using microbial systems and can be tailored in terms of specificity and affinity depending on the type of target. The only restraint associated with sdAb is their

monomeric nature which can limit capacity during the purification process. Highly efficient anti-antibody ligands can be generated by selectively screening them for desired features, such as binding affinities and elution characteristics. CaptureSelect® offers an array of affinity reagents which are camelid-derived single domain antibody fragments. They can be used to screen and purify antibodies based on species, subclass, glycoform and many other different antibody characteristics [95,96].

3.2.1.5. Concluding remarks. Despite the popularity of biospecific ligands in protein purification, their drawbacks include high manufacturing and processing costs, instability of the ligand, the potential for ligand to leak from the column, storage, labor, and difficulty with sterilization along with problems associated with low binding capacities. Further disadvantages associated with biospecific ligands include limited life-cycles and low scale-up potential [97]. The majority of biospecific ligands are produced in bacteria from which there is an attendant risk of contaminants, such as viruses, pyrogens and DNA. These shortcomings have led to an ongoing quest for alternative ligands with improved potential to replace biospecific ligands.

3.2.2. Pseudobiospecific ligands

Pseudobiospecific ligands exploit intrinsic properties of the immunoglobulin at the molecular level, e.g. by exploiting its hydrophobic and thiophilic properties. They are promising candidates in the quest for an immunoglobulin binding ligand that combines the advantages of being cheaper, more robust, structurally simple, less toxic and highly stable while also possessing resistance to harsh sanitation or sterilization conditions imposed by good manufacturing practice (GMP) protocols [98]. The development of pseudobiospecific ligands goes some way to addressing these questions. The affinity of such ligands is generally lower compared to the biospecific ligands, however, binding affinity is sufficient to ensure selectivity towards antibody molecules [99]. Commonly used pseudobiospecific ligands include hydrophobic, thiophilic, mixed mode affinity ligands and chelating metal ions [37]. Over the last two decades, studies directed towards developing efficient pseudobiospecific ligands for the purification of native and recombinant antibodies have led to the commercialization of many potential products (e.g. Thiosorb, T-gel, HA-Ultrogel® and MEP HyperCel).

3.2.2.1. Hydrophobic charge-induction chromatography (HCIC). HCIC is based on mild hydrophobic interactions between the ligand and the target, achieved under near physiological conditions [100]. It can be performed due to the pH-dependent behavior of ionizable, dual-mode ligands such as 4-mercapto-ethyl-pyridine (MEP), attached to a hydroxyl-carrying support. At neutral pH, the ligand is uncharged and binds the antibody without any sample pre-treatment steps. Elution is carried out under slightly acidic conditions by reducing the pH of the mobile phase to 4.0–4.5, which imparts a net positive charge on both ligand and target molecule, thus, resulting in desorption of the protein due to electrostatic repulsion [101]. HCIC provides an efficient means of capturing and purifying the antibodies from a broad range of sources, such as animal sera, ascites fluid and cell culture supernatants [102].

3.2.2.2. Thiophilic affinity chromatography (TAC). TAC is another chromatographic method for the purification of proteins, including antibodies, due to their high sulfur and nitrogen content. It uses sulfur-containing ligands attached to a suitable matrix, which binds antibodies in the presence of high concentrations of lyotropic salts (i.e. salts that expose the hydrophobic regions of proteins). Thiophilic gels are the most commonly used absorbents, based

on a reaction between divinylsulfone and 2-mercaptoethanol. The structure of the immobilized ligand can be written as agarose-CH₂CH₂SO₂CH₂CH₂SCH₂CH₂OH [103]. They contain linear ligands with two sulfur atoms which selectively bind to immunoglobulins under high salt concentration. Sulfates and phosphates are the most commonly used salts in thiophilic chromatography. The concentration of salt required to promote the antibody–ligand binding should be determined, so as to avoid the precipitation of protein due to high salt concentration. Elution is carried out by lowering the lyotropic salt concentration. It is necessary to determine the optimum salt concentration required for the elution of different antibody formats so as to achieve maximum recovery of purified protein [104]. A range of thiophilic ligands are reviewed by Boschetti [1].

Thiophilic chromatography is reported to be a highly successful method for purification of IgG from different species [105–108], IgY from egg yolk [109], F(ab')₂ [110], Fab [111] and scFvs [112]. Most thiophilic ligands bind in a salt-dependent manner, exhibiting affinity based on salt concentration. However, a new type of thiophilic ligand, termed a mercaptoheterocyclic ligand is reported to capture IgG with high absorption capability in a salt-independent manner [113]. Recently, an interesting approach was devised by Qian and colleagues [97]. A heterocyclic ligand of 2-mercaptonicotinic acid (MnIC) was used to functionalize magnetic beads, prepared by microsphere polymerization with vinyl acetate (VAc) and divinylbenzene (DVB). The resulting functionalized PVAc–DVB beads were further employed to purify IgG from human serum in a batch-wise mode, under physiological conditions. The purity of the isolated antibody exceeded 94%, retaining more than 99% of functional antibodies.

3.2.2.3. Hydroxyapatite chromatography (HAC). HAC has regularly been applied as a tool for the purification of antibodies due to its high selectivity and ease of use as it can be performed under neutral pH conditions [114–119]. Hydroxyapatite crystals (Ca₁₀(PO₄)₆(OH)₂) generate a mixed-mode resin, binding proteins via two mechanisms, (i) phosphate groups (p-sites) of the hydroxyapatite crystals interact electrostatically with amines or other positively charged amino acid residues on proteins via cation exchange, and (ii) calcium ions (c-sites) on the surface of hydroxyapatite crystals bind to either carboxyl clusters or phosphoryl groups on proteins in a metal-affinity type of mechanism [120–123]. Elution of antibodies can be performed using a phosphate or NaCl gradient as desorption properties will vary depending on the amino acid content [124]. Taishiro and colleagues analyzed the retention times of 37 mAbs using HAC and concluded that elution was dependent on structures of both the constant and variable regions, amino acid content and their accessibility to the ligand [125]. HAC is also reported for use in removal of antibody aggregates, which is an important polishing step in large-scale purification procedure for industrial applications. The principle of method development, elution strategies, strengths and weaknesses of HAC, are described in a recent review by Gagnon and Beam [126].

3.2.2.4. Immobilized metal affinity chromatography (IMAC). IMAC or metal chelate chromatography is a widely used method for purification of antibodies. Biomolecules with exposed His, Cys, Ser, Glu, Asp and Trp have affinity towards metal ions [127–129]. This feature is exploited in designing ligands for IMAC, which are attached to a matrix via a covalently linked chelating compound and spacer group [130]. Refer to references [131,132] for details on IMAC. Commonly used chelating compounds are iminodiacetate (IDA) and nitrilotriacetate (NTA) which are classified as tri and tetradentate ligands based on the number of coordination sites that are available to each transition metal ion used in IMAC such as Ni²⁺, Cu²⁺, Co²⁺, Zn²⁺, Fe³⁺ and Ga³⁺ [133]. The number of electron pair

donor atoms (e.g. nitrogen, oxygen and sulfur) on the chelating compound determines the strength and the binding stability of the metal-chelate complex.

Sandra and colleagues compared four metal ions and buffer systems for purification of IgG from human plasma and showed high purity absorption of IgG for all metals irrespective of the buffer system used [134]. IMAC is reported to be used for purification of antibodies from different species and subclasses [134–143]. The region of antibody involved in binding depends on exposure of amino acid residues possessing affinity for the metal ion. In a study by Todorova and colleagues, metal ions (Ni^{2+} , Cu^{2+} , Co^{2+} and Zn^{2+}) demonstrated high affinity towards Fc region of the IgG rather than the Fab regions, which was purified efficiently in a single step IMAC procedure [144].

IMAC is one of the most popular methods employed for purification of proteins expressed using recombinant systems. Several vector systems are commercially available which express proteins containing a stretch of 5–6 histidines in the final product. This imparts millimolar affinity towards metal ions to the His-tagged proteins, and, as a result, they can be separated from host proteins [145]. Binding takes place around neutral pH and elution can be carried out by reducing pH or by displacement methods using competitors (e.g. imidazole).

IMAC is widely reported for successful purification of scFv antibody fragments [12,146]. It is a versatile technique as it can be used under denaturing conditions in the presence of detergents and chaotropic agents. During the purification of proteins from inclusion bodies, which are nuclear or cytoplasmic aggregates of proteins, high concentrations of chaotropic agents such as guanidinium chloride and urea are regularly used. To purify proteins under these conditions, IMAC can be used. The purified product can be later refolded using a combination of various reagents depending on the individual protein [147]. An on-column refolding system was reported by Guo and co-workers for IP10 scFv-fusion protein resulting in successful recovery of functional proteins from inclusion bodies [148]. Mild conditions (salt, pH) required for elution of protein along with the robustness of IMAC matrices are considered to be beneficial over traditional protein A or G chromatography [136]. Many IMAC resins, columns and beads are commercially available (e.g. from Bio-Rad Laboratories, Pall Life Sciences, Thermo Scientific Pierce, GE Healthcare, QIAGEN, Merck Millipore and Sigma–Aldrich).

3.2.2.5. Concluding remarks. It is thought that pseudobiospecific ligands may have the potential to replace biospecific ligands as the ligand of choice for antibody purification. They may feature as a cost-effective and robust alternative to Protein A/G purification methods. However, pseudobiospecific ligands have their own limitations. They are not as specific as biospecific ligands, and, as a result, significant process optimization is required for each individual protein to attain high selectivity. The use of pseudobiospecific ligands is preferred in combination with other purification methods. However, pseudobiospecific ligands are reported to attain high levels of purity in a single step in various studies, but they still have to go some way to achieve widespread acceptability by proving their reliability as sole ligands in downstream processing applications.

3.2.3. Synthetic ligands

Development of specific antibody-based affinity ligands with increasingly improved binding properties has been enhanced by the emergence of combinatorial techniques, sophisticated molecular modeling approaches, *in silico* designing, high throughput lead generation and screening programs combined with high resolution methods (X-ray crystallography and nuclear magnetic resonance (NMR)). Synthetic ligands represent a new genre of compounds

with low molecular weight that circumvent most of the shortcomings associated with biospecific ligands. They also have certain benefits over pseudobiospecific ligands, such as increased recycling options and greater control over the purification procedure. They can be constructed by (i) rational design based on the structural and functional aspects of the template, (ii) combinatorial methods, using libraries of synthetic ligands, or (iii) a combined method using both (i) and (ii) [149]. Once the ligand is selected it can be further tailored for specificity and affinity and tuned depending on the target, to suit the purification procedure. A range of peptidyl and non-peptidyl affinity chromatography ligands were generated using this approach.

3.2.3.1. Peptidyl ligands. A group of short linear peptides composed of histidine on the N-terminus followed by aromatic amino acid(s) and positively charged amino acid(s), was shown to possess high binding affinity towards IgG [150]. Among these, peptide His-Trp-Arg-Gly-Trp-Val showed a broad affinity spectrum by binding human IgG subclasses along with IgGs from cow, mouse, goat and rabbit. Another peptide sequence (Ala-Pro-Ala-Arg) was isolated from a synthetic tetrapeptide library for specific purification of anti-granulocyte macrophage-colony stimulating factor (GM-CSF) monoclonal antibody (mAb) from mouse ascitic fluid, yielding 95% pure protein [151].

A group of ligands termed biomimetics were generated to mimic natural ligands for protein purification. Protein A mimetic (PAM, Peptide TG19318) is a novel peptide-based synthetic ligand with a chemical formula $(\text{Arg-Thr-Tyr})_4\text{-Lys}_2\text{-Lys-Gly}$. It is composed of four identical peptide chains assembled together, capable of mimicking protein A in the recognition of the Fc portion of immunoglobulin [152]. It was isolated by synthesizing a multi-meric peptide library and its potential to bind immunoglobulins was determined by screening its ability to competitively inhibit the interaction between immunoglobulin and protein A. It is an extensively characterized synthetic ligand shown to have an affinity of 0.3 μM towards the immunoglobulin constant domain and a broad range specificity towards antibodies from different sources (human, cow, horse, pig, mouse, rat, rabbit, goat, sheep and chicken) and classes (IgG, IgY, IgM, IgA and IgE) yielding approximately 95% of pure protein under optimized conditions [47,153–156]. Optimum binding of PAM to antibody occurs at room temperature in neutral buffers with low ionic strength. High salt concentration and chloride ions reduce binding efficiency. Efficient elution can be achieved by adopting acid or alkaline elution (pH 3.0–9.0). In comparison to its natural counterpart (protein A), PAM is stable under harsh sanitizing conditions. A derivative of PAM, called D-PAM, synthesized by replacing all amino acids with the corresponding D derivatives, is reported to be immune to protease degradation [56], thus, providing additional stability while being employed for antibody purification.

3.2.3.2. Non-peptidyl ligands. Among non-peptidyl synthetic ligands textile dyes, particularly Cibacron Blue FG-3A, has been used for almost 40 years for the purification of proteins. Dye-based ligands rely on the ability of reactive dyes to bind proteins in a selective and reversible manner [157,158]. Reactive dyes consist of a sulfonate containing chromophore, to impart color, and a reactive system that acts as a scaffold for immobilization of the chromophore and matrix [159]. Usually azo, anthraquinone and phthalocyanin are used as chromophores, whereas an achlorotriazine ring is used as the reactive system. Previously, either monochloro-triazine or dichloro-triazine compounds were used, however, today cyanuric chloride, a chlorinated derivative of 1,3,5-triazine is a very commonly used precursor for such synthesis. The presence of three electronegative atoms (chlorine) makes the carbon atom electropositive, thus, making it susceptible to nucleophilic substitution [157]. Some examples

of commercial dyes are Reactive Green 5 (RG-5), Reactive Red 120 (RR 120) and Reactive Brown 10 (RB 10). Recently, Wongchuphan and co-workers studied the application of dye-ligands as absorbents for affinity capture of rabbit IgG and concluded high binding absorption occurs at pH 7.0 [160]. Details on dye-affinity ligands are available [161–164].

Increasing demands for enhanced performance led to the selection of simple textile dyes as biomimetic ligands [165] tailored for specific needs, which later inspired the *de novo* synthesis of ligands [166]. New generation biomimetics were synthesized by *de novo* design of a lead compound, constructed by studying the interaction of natural ligands with their associated targets and mapping crucial residues, followed by further optimization by combinatorial methods. A family of small ligands was developed using the aforementioned approaches, adding to the family of triazine ligands. One good example of such ligand is 22/8 synthesized from artificial protein A (ApA). ApA is a non-peptidic, triazine-based fully synthetic ligand, generated by *de novo* design [167]. It was synthesized using the interaction between SpA and IgG as a template [74]. This allowed the identification of a dipeptide motif (Phe132–Tyr133), as crucial for binding. ApA was synthesized by coupling this Phe and Tyr motif to a triazine scaffold, such as 1,3,5-trichloro-sym-triazine or cyanuric chloride [71]. The properties of ApA were further enhanced by a lead optimization process which involved the construction of an IgG-binding ligand library using cyanuric chloride by a “mix-and-split” procedure. The 88-member ligand library consisted of cyanuric chloride analogues, with the first highly reactive chlorine displaced with amino-derivatized agarose and the second and third chlorines substituted with different aliphatic, aromatic, bicyclic and tricyclic structures [168]. On screening the library against IgG, a bifunctional ligand, termed ligand 22/8, bearing 3-aminophenol and 4-amino-1 naphthol on the triazine ring, was found to be very effective, displaying a high binding affinity ($K_a = 1.4 \times 10^5 \text{ M}^{-1}$) and recovering highly pure (98–99%) IgG [169]. Ligand 22/8 possesses high stability (indicated by its ability to withstand 1 M NaOH over a period of 140 h) along with broad range specificity. It efficiently purified antibodies from different species (chicken, cow, rabbit, pig, horse, rat, goat, sheep and mouse), classes (IgA and IgM) and IgG subclasses [169]. This work led to the commercialization of two novel synthetic protein A mimetic ligands called MAbsorbent A1P and A2P from ProMetic BioSciences (Cambridge, UK).

A P. *magnus* protein L (PpL) mimic, ligand 8/7, was synthesized in a similar fashion to ligand 22/8. Initially, the interaction between PpL and Fab was mapped and key contact residues were identified. It was noted that hydrogen bonds and salt bridges play an important role in the binding. A total of 11 different hydrophilic amino acid residues (Ala, Asp, Gln, Glu, Gly, Ile, Leu, Lys, Phe, Thr and Tyr) of the PpL domain were found to play a key role in promoting hydrogen bonds or salt bridges, necessary for the interaction. Thirteen structurally similar chemical compounds were selected for the synthesis of a library [170]. A 169-membered, triazine scaffolded combinatorial library of ligands was constructed using “mix-and-split” procedure and was screened for binding to human IgG and Fab [170]. Ligand 8/7 was selected as the lead ligand and further characterized for binding specificities and affinity. Ligand 8/7 recognizes both κ and λ light chains of IgG as opposed to its natural counterpart, which recognizes only $\kappa 1$, $\kappa 3$ and $\kappa 4$. It also binds immunoglobulin from different classes and sources, with an estimated ability to achieve purification levels of up to 7-fold with approximately 95% purity [170]. Commercially, Fabsorbent™ F1P HF from ProMetic BioSciences (Cambridge, UK), is the only available non-peptidyl protein L biomimetic.

With a growing impetus in triazine-based affinity reagents, some novel ligands were synthesized in recent years. One such ligand is ligand 8–6, synthesized for affinity-based purification of

IgY. It is reported to be highly efficient for achieving purification of chicken, duck and pigeon IgY [171], recovering up to 78.2% of IgY with a purity of 92.1%. Other ligands that can be considered for antibody purification are artificial lectin ligands 8/10 and 11/11 with binding affinity towards sugar residues [172,173]. Considering the advantages of plant-based systems on upstream production of antibody therapeutics [174–176], certain ligands were specifically developed to purify antibodies from plant extracts. In this case, the plants were genetically engineered and bred to facilitate antibody production. Phe-Trz-Asp LAK-mimetic and 4E10lig ligand are two such ligands, used for purification of anti-HIV mAb 2F5 and 4.E10 from corn and tobacco extract [177,178].

Although triazine scaffolds are well-defined and popular, recently ligands (e.g. A3C1) were developed using an Ugi reaction scaffold for purification of Fab and IgG [179]. It involves a four component reaction using a derivatized chromatographic matrix support as one of the components. The product is directly formed within the macroporous matrix, thus, reducing the number of steps involved. Ugi reaction scaffolds are promising for the generation of synthetic ligands, based on ‘one-pot’ reaction and multicomponent chemistry, and show great potential for diversity generation and other applications [179].

3.2.3.3. Concluding remarks. Synthetic ligands provide additional scope for expanding the capacity of purification strategies offering a reliable, cheap, scalable and stable means of purification. Rational designing of ligands and the, so-called, systemic evolution of ligands by exponential enrichment technology (SELEX) has provided an intelligent tool for generating ligands with desired characteristics. In antibody purification, specificity and affinity are essential paradigms, as is the elution procedure. Synthetic ligands seem to balance all these aspects. This might explain the reason for the substantial growth witnessed in this field in the last two decades, leading to commercialization of products isolated from this technology (MAbsorbent A1P, MAbsorbent A2P and Fabsorbent™ F1P HF). This approach is constantly expanding, and seems to have additional scope with Ugi-based ligands and aptamers [179,180].

3.2.4. Affinity tags as alternative ligands for recombinant antibody purification

Affinity tags offer an attractive approach for rAb purification. Affinity tags can be short polypeptide sequences or whole proteins, co-expressed as fusion partners with the target proteins. Ligands specific for the tags are used for the purification of the protein-tag hybrid, enabling single-step purification of the target protein. Apart from facilitating purification, fusion tags are also advantageous, in certain cases, in increasing the expression and solubility of recombinant proteins [181]. Affinity tags ensure proper orientation of the antibody, thus, making the functional domains accessible for interaction [182]. They also provide a system for immobilization, quantitation and detection, which is exploited in a number of immunoassay platforms (ELISA, Western blot and microarrays) to probe protein–protein interactions.

An ideal affinity tag is one that can be used with a number of proteins without disturbing structure and function whilst allowing one-step purification. It should be easily removable from the final product, if required. A range of different types of affinity tags are reported, each having its own advantages and disadvantages [181–184]. The use of polyhistidine tags is unquestionably one of the preferred choices. Tags may be classified based on the nature of the interaction with their cognate ligand such as protein–protein, enzyme–substrate, epitope–antibody or polyaminoacid–metal chelate [182]. The choice of affinity tag depends on the intended use of protein. Table 2 provides an overview of the commonly used affinity tags used in antibody purification. Several studies compared the efficacy of different types of affinity tags

[185–187]. Lichty and co-workers [188] compared eight affinity tags applied their purification capacity and found epitope tags to be more effective. However, the efficiency of the purification will vary depending on the protein expressed and the methodology used.

Combinatorial tagging, involving more than one tag, is another approach that can be used for achieving maximum benefits, e.g. a combination of a MBP tag with small peptide tags is widely used for protein solubilization purpose [184]. Similarly, recombinant antibody expression vectors, using a combination of affinity tags, are available to facilitate purification and provide alternate detection systems (Table 2). In certain cases, e.g. applications in the biopharmaceutical industry, it is absolutely necessary to remove the tag before final application. A review by Arnau and colleagues discusses different approaches used for tag removal [189]. Effective usage of affinity tags for downstream purification purposes will require careful consideration of the tag design, its selective capability, compatible expression systems and strategies available for its removal.

4. Affinity chromatography – methods and considerations

Isolation of antibodies from crude and complex samples has always been a challenge. The issue was further complicated after the development of variable antibody formats (Fv, scFv, dsFv and Fab), which require special considerations. Purification technologists strive to find solutions to these problems. Apart from the concerns related to yield and recovery of the protein, there are stringent regulations that require the quality of the protein products to comply with purity requirements (>99%), in order to allow their usage as drugs for disease treatment. Most of the protocols are targeted to achieve purification in one step, however, the current available techniques and the reagents used, are inadequate to deliver high quantity and quality purification, thus, requiring additional purification to improve the procedure.

Affinity chromatography is the preferred method for primary purification of antibody due to its unique interaction characteristics, allowing the reliable separation of antibodies from crude mixtures. Since the emergence of affinity chromatography, various factors such as pH, ionic strength, temperature, presence of competing species and flow characteristics, etc. have been exploited to improve the purification procedure. Basically, affinity chromatography relies on four essential steps – preparation of absorption media, sample adsorption, washing and elution (Fig. 4) to achieve maximum yield and purity, and each step can be optimized.

4.1. Preparation of absorption media

The choice of absorption media is important for a purification procedure. Absorption media is prepared by immobilizing the

ligand of choice to the affinity matrix. (Ideal properties for both ligand and the matrix were mentioned earlier). A ligand with an affinity range, 10^{-6} – 10^{-10} M, for the target is preferred, however, moderate affinity ligands with an affinity of 10^{-4} [190] can also be used to avoid harsh elution conditions that might be detrimental for the protein. General considerations for the preparation of absorption media are as follows:

- Activation chemistry should be stable over a wide range of pH, buffer conditions and temperature resulting in negligible leaching of ligands.
- Choice of coupling method should be made so as to avoid improper orientation, multisite attachment or steric hindrance of the ligand, which may cause masking of the binding sites and, subsequently, lead to loss of activity. Site-directed attachment, spacers or secondary ligands can be considered for immobilizing the ligand onto the matrix [191].
- Based on the reaction involved, coupling buffer should be carefully selected to avoid competition of certain ions for the activated surfaces.
- Choice of ligand depends on the source of antibody and its associated format.
- Purity of ligand to be immobilized is of utmost importance, as it will influence the quality of purification.
- Absorption throughput, i.e. ligand density per volume of matrix needs careful optimization so as to promote target accessibility and binding [192,193].
- Once immobilization is complete, free-activated groups need to be blocked using blocking reagents such as ethanolamine or ethylene diamine, to avoid non-specific absorption of targets.
- Choice of spacer can impact the interaction between the ligand and the target antibody [194,195] and, thus, it should be selected carefully.

4.2. Sample preparation and binding

Before initiating the purification of antibodies, it is important to assess the properties of the sample. Antibody may be solubly expressed in cell culture supernatants or may be present in an intracellular compartment. Depending on the site of antibody expression, extraction procedures such as osmotic shock, French press or sonication may be required to release the antibodies into the medium [196]. The majority of the ligand–antibody interactions are either due to the complementarity of their shape, charge, hydrophobic, van der Waals and/or hydrogen bonding interactions [197]. It is necessary to identify the type of interaction in order to select the buffer conditions, to achieve highest association constant, e.g. in TAC, lyotropic salts are required to promote binding. The majority of ligand–antibody reactions are favored near neutral pH for which buffers such as PBS are commonly used [198]. Temperature may also

Table 2

Commonly used tags in recombinant antibody production vectors.

Tag name	Amino acid sequence	Vectors	References
C-myc-tag	EQKLISEEDL	pAK100, <u>pCANTAB3his</u> , <u>pCANTAB5his</u> , <u>pCANTAB 6</u> , <u>pCES1</u> , pCW93/H, pCW99/L, <u>pET23NN</u> , <u>pGEM-gIII</u> , pGP-F100, pGZ1, pHEN1, pHEN1-V λ 3, <u>pHEN2</u> , pHENIX, <u>pHG-1m/A27 Ik1</u> , pIG10, pIGT2, pIGT3, <u>pIT2</u> , pOPE40	[211–227]
FLAG-tag	DYKDDDDK	<u>pAPIIIκ scFv</u> , pCGMT, <u>pDN322</u> , <u>pDNEK</u>	[228–231]
Hemagglutinin (HA)-tag	YPYDVPDYA	<u>pComb3X</u>	[232]
His $_6$ -tag	HHHHHH	<u>pAPIIIκ scFv</u> , <u>pCANTAB3his</u> , <u>pCANTAB5his</u> , <u>pCANTAB 6</u> , <u>pCES1</u> , <u>pComb3X</u> , pDAN5, <u>pDN322</u> , <u>pDNEK</u> , <u>pET23NN</u> , pFAB5c-His, pFAB60, pFAB73H, <u>pGEM-gIII</u> , <u>pHEN2</u> , <u>pHG-1m/A27 Ik1</u> , <u>pIT2</u> , pOPE90	[211,213–217, 222, 224, 227,229,233–236]

Underlined vectors are those containing more than one affinity-tag.

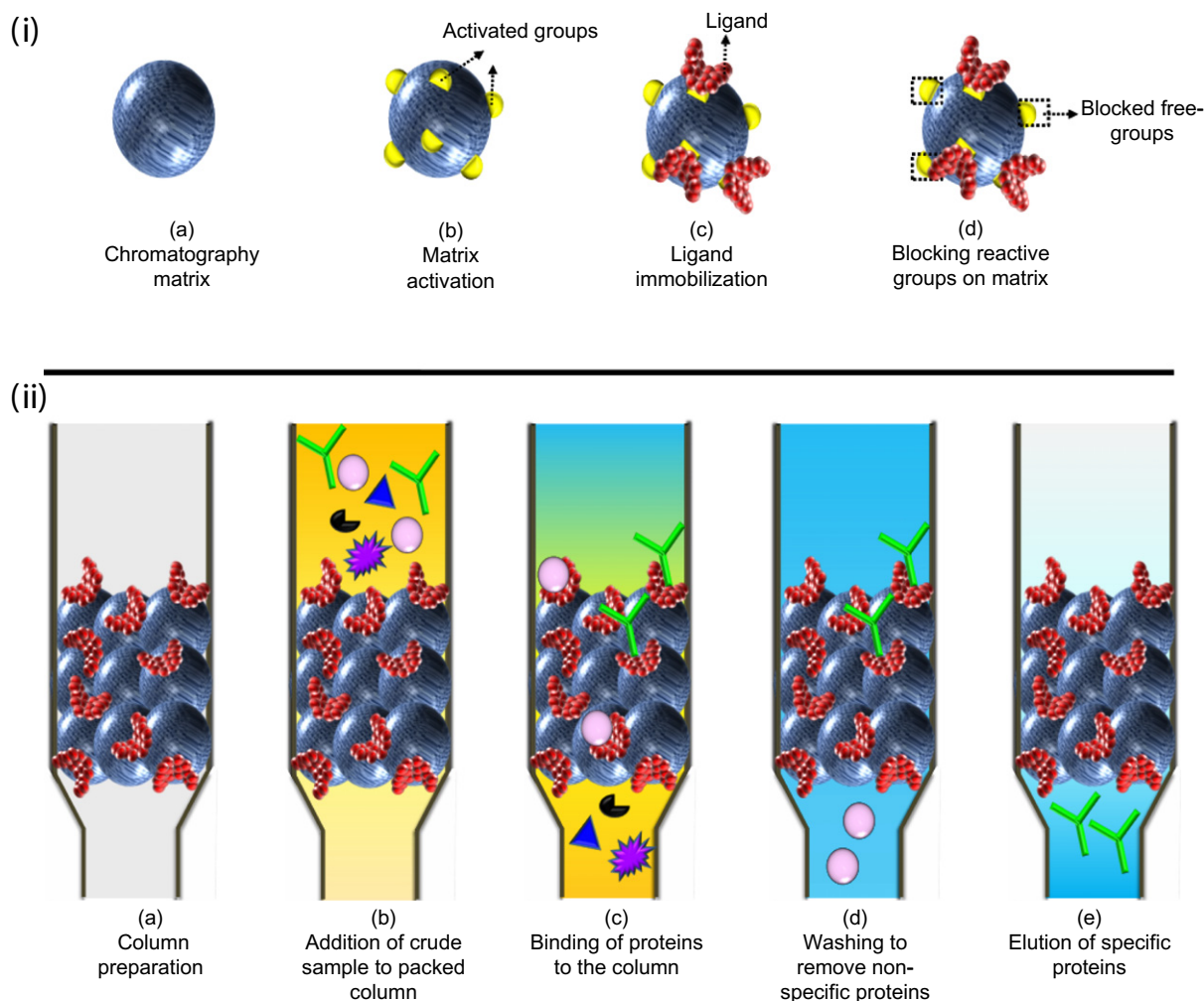


Fig. 4. Steps involved in antibody purification using affinity chromatography. Affinity chromatography is a multistep procedure for separating specific antibodies from a mixture of proteins. It involves the following steps: Preparation of absorbent medium: The matrix (a) is prepared by chemically activating it resulting in the generation of reactive functional groups on the surface (b). The chemistry can be selected based on the choice of ligand to be immobilized. Once the ligand is immobilized (c), unoccupied reactive groups are blocked (d), to prevent them from binding with the contaminations in the sample matrix. Purification procedure: A typical purification procedure consists of (a) column preparation, which involves the packing and equilibration of the ligand-immobilized matrix. Crude sample (containing a mixture of proteins and other biomolecules) is passed through the packed column (b). The specificity of the ligand causes the retention of specific proteins from the mixture (c). However, there is a possibility of some non-specific binding of proteins due to their charge. This can be removed by stringently washing the column prior to final elution (d). Specific protein can be eluted either competitively or by altering the pH that breaks the interaction between the protein and the ligand (e).

contribute to the binding depending on the type of interaction. Interactions based on hydrophobicity are favored at high temperatures, hence, promoting absorption, whereas, it is just the opposite for ionic interactions [199]. General considerations for sample preparation in order to achieve efficient specific antibody absorption on the ligand include:

- The removal of particulate material and control of the viscosity of the sample are necessary before starting the purification procedure as they could result in clogging of the column.
- Extraction of antibodies from cells should be carried out gently and the choice of buffer conditions and procedure will vary depending on the nature of the source (e.g. bacteria, mammalian cells or plant cells) and target protein (e.g. pI and stability) [196].
- Samples should not be very concentrated. When present in high concentrations antibodies tend to aggregate, causing them to be insoluble and blocking the binding site, thus, making it inaccessible for purification. Prior dilution of samples may be necessary in such cases [200].
- Occasionally, highly diluted samples can have slow binding rates and capture efficiency, especially for antibodies with low affinities. Samples can be concentrated, if required, using membranes or precipitation reagents such as ammonium sulfate, polyethylene glycol or caprylic acid [201].
- Identification and removal of contaminants that might interfere or compete with the antibody–ligand binding is necessary before applying the sample to the column. However, sometimes low concentrations of competing species are preferred to reduce non-specific interactions, e.g. 10–20 mM imidazole is used in binding buffer for IMAC purification of His-tagged proteins [202].
- Samples should be prepared in the binding buffer directly or buffer exchanged for increased binding efficacy. Similarly, columns should be pre-equilibrated with binding buffer before sample application.
- Flow rates employed during sample application are essential factors to consider. Antibodies possessing high affinity towards their respective ligand reach equilibrium quickly, whereas binders with weak affinity require slow flow rates to achieve

equilibrium. In such cases, incubation of the sample with ligand, prior to chromatographic separation, may be required to promote binding. Thus, for efficient binding, the sample application needs to be optimized based on the affinity exhibited by the required antibodies [198].

4.3. Washing

Washing is an essential step to ensure the removal of all unbound and non-specific proteins from the column before elution of specific antibodies. Non-specific interactions may be due to the interaction of the proteins in a crude sample with the matrix or ligand. These interactions are generally of low binding strength compared to specific interactions and can be dissociated by changing the buffer composition. General considerations for washing buffer are:

- Addition of salt (NaCl, Na₂SO₄, CaCl₂, MgCl₂, MgSO₄), or altering the pH of wash buffers can significantly affect the protonation/ionization state of the molecule, thus, affecting the hydrogen bonds and ionic interactions involved [200].
- Hydrophobic interactions can be removed by decreasing the salt concentration, addition of surfactants (Tween-20, Triton X-100) [203] or by adding organic solvents (methanol, ethylene glycol). Blocking agents (e.g. bovine serum albumin) can be used to reduce non-specific binding.
- Sometimes adding low amounts of the mimicking reagents to the washing buffer can outcompete the non-specific binding of contaminants to the ligand.
- Care must be taken in selecting the conditions of washing buffer, so as to avoid the elution of specific antibody.

4.4. Elution

Elution procedures are aimed at obtaining stable antibody with high yield and purity. High-affinity and specific interactions are preferred in order to achieve high resolution of protein, however, they pose trouble in selecting elution conditions required to obtain functional antibody. Elution is achieved by reducing the association constant of the ligand–antibody interaction. Biospecific or non-specific elution can be used for this purpose [204]. Biospecific elution is a gentle but slow method for elution relying on antibody displacement from the column by addition of a mimic that acts as a competing agent. Biospecific elution is again divided into two types, (i) normal role elution, in which the mimic competes with the ligand for binding the target antibody (normal role elution) and (ii) reversed role elution where the competition is between the target antibody and the mimic for binding the ligand, thus, eventually displacing the purified antibody in both processes [191].

Non-specific elution is a fast method of antibody elution which depends on weakening the ligand–antibody interaction by changing solvent conditions like pH, ionic strength and polarity. Addition of high concentrations of chaotropic salts (NaCl, MgCl₂ or LiCl), denaturing agents and detergents (guanidine hydrochloride, sodium dodecyl sulfate and urea) can be considered [205]. Organic solvents are useful in elution of low molecular weight compounds. Elution strategies are reviewed in detail by Firer [206]. However, certain general considerations for antibody elution are listed below:

- Despite the acceptance of some well established elution conditions for antibody purification, there is no universal method that can be used, due to the heterogeneity in the nature of physical forces involved in the antibody–ligand interaction. Elution conditions vary depending on the affinity of the ligand–antibody interaction and the intended use of the antibody.

- High affinity interactions ($>10^{-8}$) may require more than one elution procedure for the complete recovery of antibody.
- Biospecific ligand may preserve the binding activity of antibody but may require additional steps for purification to remove the competing agent from the purified antibody preparation.
- pH-based elution may be acidic or basic. Sometimes, depending on the pH of the solution used for elution, the binding characteristics of the antibody may be altered [207]. Therefore, it is necessary to analyze the binding properties of antibody following exposure to the eluant before opting for specific elution conditions. Acid elution is commonly employed for antibody purification but the pH must be neutralized to prevent denaturation of the purified antibody.
- Antibodies may aggregate at low pH; this can be prevented either by carrying out the elution at low temperature [208] or by the addition of elution-enhancing additives such as NaCl or Na₂SO₄ [85]. Urea is also reported to be effective in such cases [208].
- Low recovery issues with acid elution may be due to hydrophobic interactions between the ligand and the antibody, which can be solved by addition of organic solvents [209].
- When eluting with high concentrations of chaotropic salts, it is necessary to desalt or buffer exchange the purified product immediately to prevent denaturation [209].

5. Conclusions and future perspectives

With the growing competition in the development of antibody-based products, there is extensive pressure on the industry to develop high-affinity reagents and strategies to meet the demand–supply expectations, along with rapid and economical procedures to obtain high-quality products. Advances in antibody engineering has facilitated the generation of high affinity reagents. However, this has shifted the focus towards the upstream and downstream processes for large-scale production of the antibodies.

Upstream productivity was handled efficiently by using alternative production systems (e.g. bacteria, yeast, transgenic animals and plants), thus, reducing manufacturing costs. Downstream processing of antibody exhibited major challenges, since no single process, on its own, qualifies to meet the regulatory standards for antibody purification. This has led to increased economic pressure on purification procedures accounting for up to 50–80% of the total production costs [210].

Protein A affinity chromatography has, undoubtedly, been the predominant standard approach for antibody purification over the last few decades. However, it did not provide a solution to the cost and stability issues associated with purification procedures. The problem was further aggravated with the development of various recombinant antibody formats which necessitated more robust and novel approaches for purification.

The last two decades have witnessed significant advances in purification strategies using affinity chromatography, partially as a result of developments in novel antibody engineering and their attendant purification needs. The generation of *de novo* designed synthetic ligands and high-throughput purification techniques have provided a much needed alternative to the existing procedures. With the next generation ligand candidates and strategies in the pipeline, significant progress in affinity chromatographic purification of antibodies is expected in the near future.

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