CHAPTER 5

Monoclonal Antibodies – Addressing the Challenges on the Manufacturing Processing of an Advanced Class of Therapeutic Agents

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Abstract: Monoclonal antibodies (mAbs) were firstly described by Köhler and Milstein in 1975, and their potential as powerful therapeutic agents recognized in the following years. Currently, the US Food and Drug Administration (FDA) has already approved 56 monoclonal antibodies for the treatment of several diseases, including cancer (*e.g.* breast cancer, leukemia and prostate cancer), auto-immune disorders (rheumatoid arthritis and Crohn's disease), asthma, and cardiovascular and infectious diseases. Despite their advantages and therapeutic potential, the cost of manufacturing these biopharmaceuticals with high quality and purity level is still extremely high due to the absence of current cost-effective extraction/purification methods, and which has also impaired their widespread application as recurrent therapeutic agents. The upstream processing of mAbs has gone through several improvements in recent years, by using alternative expression systems or by optimizing the medium formulations and feeding strategies. On the contrary, the downstream processing is considered the bottleneck in the manufacturing of mAbs for therapeutic purposes at reliable costs, representing up to 80% of their total production costs – which is a frontier in clinical drug research. In the past years, several chromatographic and non-chromatographic alternatives have been explored for this purpose, resulting in the development of efficient platforms for the purification of mAbs, that are overviewed and discussed in this chapter. In summary, this chapter provides a vision on the current state of the art of the biopharmaceuticals market, on the production and use of mAbs as valuable therapeutic agents, including their use for the treatment of infectious diseases, while summarizing the mAbs-based products already approved by regulatory agencies. New insights concerning the development of new and alternative platforms or the extraction

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and purification of mAbs are also discussed, while envisaging the adoption of the most relevant techniques by the pharmaceutical industry to allow the widespread use of biopharmaceuticals in the near future.

Keywords: Affinity precipitation, Aqueous two-phase systems, Biopharmaceuticals, Biopharmaceutical market, Cancer, Chromatographic methods, Downstream processing, High performance tangential flow filtration, Infectious diseases, Ionic exchange chromatography, IVIG, Magnetic separation, Monoclonal antibodies, Multimodal chromatography, Non-chromatographic methods, Passive immunity, Pharmaceutical industry, Preparative electrophoresis, Purification, Therapeutic agents, Upstream processing.

INTRODUCTION

In recent years, human society has been facing several issues concerning the exponential emergence of drug-resistant microorganisms, diseases that are unresponsive to common drug therapies and the appearance of individuals with impaired immune systems who are unable to respond to conventional vaccines. These events fomented the search on effective alternative therapeutics, that is a crucial goal to be achieved within the next few years [1]. In this context, biopharmaceuticals have greatly improved the treatment of many diseases and sometimes are the only approved therapies available for a particular disease [2]. By definition, biopharmaceuticals are biological-based products, with high-molecular weight and complex molecular composition, obtained from heterogeneous mixtures from living beings, cells, animals or plants [3, 4]. Recombinant proteins, antibodies and nucleic-acid-based products are examples of biopharmaceuticals with application in several medicinal areas, such as in vaccination/immunization, oncology, cardiology, neurology and infectious diseases [2]. In what concerns antibodies, although being in many cases the only available therapy, the production cost of antibodies with high quality/purity for therapeutic applications still remains very high due to the absence of a cost-effective purification method, which has conditioned its application on a large scale and as a recurrent therapy [5].

Monoclonal antibodies (mAbs) are the most prevalent class of therapeutic recombinant proteins, used in the treatment of several diseases, including cancer (*e.g.* breast cancer, non-Hodgkin's lymphoma, leukemia, colorectal and prostate cancer), auto-immune disorders (rheumatoid arthritis and Crohn's disease), transplant rejection, asthma, and cardiovascular and infectious diseases [6]. Regarding the production of therapeutic mAbs, one of the most challenging aspects within the biopharmaceutical market is related with the need of quite high therapeutic doses, resulting in a crucial need of obtaining high amounts of pure mAbs [7]. These antibodies are biological products that can be produced by cell

lines and extracted from their extracellular supernatant, thus exhibiting a high content of impurities. The current production process of therapeutic mAbs typically comprises two main steps: (i) the upstream processing, that relies on the production of antibodies by cell lines derived from mammalian cells; and (ii) the downstream processing, which consists on the recovery, purification and isolation of mAbs from cells and cell debris, processing medium and other impurities [15]. In the past few years, the upstream processing of mAbs has greatly improved; however, the downstream processing has not evolved at the same pace and is considered the bottleneck in the production of therapeutic mAbs – representing, at least, up to 80% of the total production costs [8, 9]. At low product titers, upstream manufacturing is more expensive than the downstream, but higher titers shift the main manufacturing costs towards the downstream processing [10], and lead to a non-linear increase of overall costs for the manufacturing process [11]. Since these antibodies should be obtained from the complex matrix with an exceptional purification degree, the downstream process is the critical step in the overall process [12]. In this context, the downstream processing of mAbs represents a frontier in clinical drug research that should be overcome, so that their widespread use as valuable therapeutic agents could become a reality.

Objective

This chapter aims to review the current state of the art of the biopharmaceuticals market, the use of mAbs as powerful therapeutic agents for several therapeutic applications, and the description of the mAbs-based products already approved by regulatory agencies. New insights concerning the development of new and alternative chromatographic and non-chromatographic platforms for the extraction and purification of mAbs, including their advantages and limitations, are also presented and discussed.

BIOPHARMACEUTICALS MARKET

The biopharmaceuticals market has been growing since the human recombinant insulin Humulin®, produced by *Escherichia coli*, was approved by the Food and Drug Administration (FDA) in 1982, for the treatment of Diabetes Mellitus [13]. Later, in 1986, the human protein tissue plasminogen activator (tPA) became the first therapeutic protein obtained from mammalian cells approved in the market. During the same year, FDA also approved the first therapeutic monoclonal antibody (mAb), Orthoclone OKT3 (muromonab-CD3), produced *in vivo* by hybridoma cell technology [14]. Currently, there are more than 150 therapeutic proteins approved in the USA/EU, demonstrating their importance in the treatment of several diseases [15]. In addition, more than 500 recombinant proteins with potential as biopharmaceuticals are currently under clinical trials.

This increasing number of dugs candidates is being essentially driven by the biotechnological advances registered in relevant areas, such as genomics and proteomics, that allowed to discover new candidates [16].

The global market of biopharmaceutical products, estimated in US$199.7 billion in 2013, is projected to achieve US$497.9 billion in 2020 [17]. In the last 10 years, the commercial importance of antibodies has been steadily growing, being the most used type of biopharmaceuticals in different medical and scientific areas, due to their capacity to bind antigens with high affinity and specificity [18]. As medicine progresses into a new era of personalized therapy, the use of monoclonal antibodies to treat a wide range of diseases lies at the heart of this new forefront. For instance, mAbs represent the largest production segment in the global biopharmaceuticals market, accounting with US$75 billion in 2013, and expected to reach US$125 billion in 2020 [19]. In the period between 2010 and 2015, it was expected that mAbs were able to generate US$25 billion, as presented in Table **1**, thus enticing a growing number of companies to expand in this field [20]. During 2014, 36 of the top 50 pharmaceutical companies (excluding generics companies) had presence in the mAbs therapeutic sector.

Table 1. Combined global prescription for the top 50 pharmaceutical companies (excluding generic-drugs companies) by molecule type (2010 – 2015) [20].

Sales (US$ billion)							
Molecule type	**2010**	**2011**	**2012**	**2013**	**2014**	**2015**	**Differences in sales between 2010 and 2015**
Small molecules	413	414	402	398	399	401	-12
Therapeutic proteins	70	73	76	79	81	81	11
Monoclonal antibodies	46	52	57	62	67	71	25
Vaccines	24	25	28	30	31	32	8

Due to this robust market demand, the global mAbs market size will ascend by more than 12% in 2013-2017, reaching US$141 billion in 2017 [21]. By November 2016, a total of 56 therapeutic mAbs were already approved by FDA and 6 were still under revision. In Table **2**, the mAbs-based products already approved by regulatory agencies are summarized.

Propelled by the optimistic market prospect, by the advances of monoclonal antibody technologies and by the upcoming patents expiration of several key monoclonal antibody agents, for example infliximab (Remicade® from Johnson & Johnson, that will expire in the US in September 2018 and expired in Europe in February 2015), trastuzumab (Herceptin® from Roche, that will expire in the US in September 2019 and expired in Europe in February 2014) and adalimumab

(Humira® from Abbott, expired in the US in 2016), the research and industrialization of monoclonal antibody agents has become a global investment reality, and in which the future market competition is evident [21].

Table 2. Therapeutic mAbs approved or in review in the EU and US by regulatory agencies.

International non-proprietary name	Brand name	Target; Format	Indication (first approved or reviewed)	First approval year	
				EU	US
Avelumab	(Pending)	PD-L1; Human IgG1	Merkel cell carcinoma	In review	NA
Sirukumab	(Pending)	IL-6; Human IgG1	Rheumatoid arthritis	In review	In review
Dupilumab	Dupixent	IL-4R alpha; Human IgG4	Atopic dermatitis	NA	In review
Romosozumab	(Pending)	Sclerostin; Humanized IgG2	Osteoporosis in postmenopausal women at increased risk of fracture	NA	In review
Inotuzumab ozogamicin	(Pending)	CD22; Humanized IgG4; ADC	Hematological malignancy	In review	NA
Ocrelizumab	OCREVUS	CD20; Humanized IgG1	Multiple sclerosis	In review	In review
(Pending)	Xilonix	IL-1 alpha; Human IgG1	Advanced colorectal cancer	In review	NA
Bezlotoxumab	Zinplava	*Clostridium difficile* enterotoxin B; Human IgG1	Prevention of *Clostridium difficile* infection recurrence	In review	2016
Brodalumab	(Pending)	IL-17R; Human IgG2	Immune-mediated disorders	In review	In review
Sarilumab	(Pending)	IL-6R; Human IgG1	Rheumatoid arthritis	In review	In review
Olaratumab	(Pending)	PDGFRα; Human IgG1	Soft tissue sarcoma	In review	2016
Atezolizumab	Tencentriq	PD-L1; Humanized IgG1	Bladder cancer	In review	2016
Reslizumab	Cinqair	IL-5; Humanized IgG4	Asthma	2016	2016
Obiltoxaximab	Anthim	Protective antigen of B. anthracis exotoxin; Chimeric IgG1	Prevention of inhalational anthrax	NA	2016
Ixekizumab	Taltz	IL-17a; Humanized IgG4	Psoriasis	2016	2016
Daratumumab	Darzalex	CD38; Human IgG1	Multiple myeloma	2016	2015
Elotuzumab	Empliciti	SLAMF7; Humanized IgG1	Multiple myeloma	2016	2015
Necitumumab	Portrazza	EGFR; Human IgG1	Non-small cell lung cancer	2015	2015

(Table 2) contd.....

International non-proprietary name	Brand name	Target; Format	Indication (first approved or reviewed)	First approval year	
				EU	US
Idarucizumab	Praxbind	Dabigatran; Humanized Fab	Reversal of dabigatran-induced anticoagulation	2015	2015
Mepolizumab	Nucala	IL-5; Humanized IgG1	Severe eosinophilic asthma	2015	2015
Alirocumab	Praluent	PCSK9; Human IgG1	High cholesterol	2015	2015
Evolocumab	Repatha	PCSK9; Human IgG2	High cholesterol	2015	2015
Dinutuximab	Unituxin	GD2; Chimeric IgG1	Neuroblastoma	2015	2015
Secukinumab	Cosentyx	IL-17a; Human IgG1	Psoriasis	2015	2015
Nivolumab	Opdivo	PD1; Human IgG4	Melanoma, non-small cell lung cancer	2015	2014
Blinatumomab	Blincyto	CD19, CD3; Murine bispecific tandem scFv	Acute lymphoblastic leukemia	2015	2014
Pembrolizumab	Keytruda	PD1; Humanized IgG4	Melanoma	2015	2014
Ramucirumab	Cyramza	VEGFR2; Human IgG1	Gastric cancer	2014	2014
Vedolizumab	Entyvio	α4β7 integrin; humanized IgG1	Ulcerative colitis, Crohn disease	2014	2014
Siltuximab	Sylvant	IL-6; Chimeric IgG1	Castleman disease	2014	2014
Obinutuzumab	Gazyva	CD20; Humanized IgG1; Glycoengineered	Chronic lymphocytic leukemia	2014	2013
Adotrastuzumab emtansine	Kadcyla	HER2; humanized IgG1; ADC	Breast cancer	2013	2013
Raxibacumab	(Pending)	*B. anthrasis* PA; Human IgG1	Anthrax infection	NA	2012
Pertuzumab	Perjeta	HER2; humanized IgG1	Breast cancer	2013	2012
Brentuximab vedotin	Adcetris	CD30; Chimeric IgG1; ADC	Hodgkin lymphoma, systemic anaplastic large cell lymphoma	2012	2011
Belimumab	Benlysta	BLyS; Human IgG1	Systemic lupus erythematosus	2011	2011
Ipilimumab	Yervoy	CTLA-4; Human IgG1	Metastatic melanoma	2011	2011
Denosumab	Prolia	RANK-L; Human IgG2	Bone loss	2010	2010

(Table 2) contd.....

International non-proprietary name	Brand name	Target; Format	Indication (first approved or reviewed)	First approval year	
				EU	US
Tocilizumab	RoActemra, Actemra	IL-6R; Humanized IgG1	Rheumatoid arthritis	2009	2010
Ofatumumab	Arzerra	CD20; Human IgG1	Chronic lymphocytic leukemia	2010	2009
Canakinumab	Ilaris	IL-1β; Human IgG1	Muckle-Wells syndrome	2009	2009
Golimumab	Simponi	TNF; Human IgG1	Rheumatoid and psoriatic arthritis, ankylosing spondylitis	2009	2009
Ustekinumab	Stelara	IL-12/23; Human IgG1	Psoriasis	2009	2009
Certolizumab pegol	Cimzia	TNF; Humanized Fab, pegylated	Crohn disease	2009	2008
Catumaxomab	Removab	EPCAM/CD3; Rat/mouse bispecific mAb	Malignant ascites	2009	NA
Eculizumab	Soliris	C5; Humanized IgG2/4	Paroxysmal nocturnal hemoglobinuria	2007	2007
Ranibizumab	Lucentis	VEGF; Humanized IgG1 Fab	Macular degeneration	2007	2006
Panitumumab	Vectibix	EGFR; Human IgG2	Colorectal cancer	2007	2006
Natalizumab	Tysabri	a4 integrin; Humanized IgG4	Multiple sclerosis	2006	2004
Bevacizumab	Avastin	VEGF; Humanized IgG1	Colorectal cancer	2005	2004
Cetuximab	Erbitux	EGFR; Chimeric IgG1	Colorectal cancer	2004	2004
Efalizumab	Raptiva	CD11a; Humanized IgG1	Psoriasis	2004#	2003#
Omalizumab	Xolair	IgE; Humanized IgG1	Asthma	2005	2003
Tositumomab-I131	Bexxar	CD20; Murine IgG2a	Non-Hodgkin lymphoma	NA	2003#
Ibritumomab tiuxetan	Zevalin	CD20; Murine IgG1	Non-Hodgkin lymphoma	2004	2002
Adalimumab	Humira	TNF; Human IgG1	Rheumatoid arthritis	2003	2002
Alemtuzumab	MabCampat h, Campath1H; Lemtrada	CD52; Humanized IgG1	Chronic myeloid leukemia#; multiple sclerosis	2001#; 2013	2001#; 2014
Gemtuzumab ozogamicin	Mylotarg	CD33; Humanized IgG4; ADC	Acute myeloid leukemia	NA	2000#

(Table 2) contd.....

International non-proprietary name	Brand name	Target; Format	Indication (first approved or reviewed)	First approval year	
				EU	US
Trastuzumab	Herceptin	HER2; Humanized IgG1	Breast cancer	2000	1998
Infliximab	Remicade	TNF; Chimeric IgG1	Crohn disease	1999	1998
Palivizumab	Synagis	RSV; Humanized IgG1	Prevention of respiratory syncytial virus infection	1999	1998
Basiliximab	Simulect	IL-2R; Chimeric IgG1	Prevention of kidney transplant rejection	1998	1998
Daclizumab	Zenapax; Zinbryta	IL-2R; Humanized IgG1	Prevention of kidney transplant rejection; multiple sclerosis	1999#; 2016	1997#; 2016
Rituximab	MabThera, Rituxan	CD20; Chimeric IgG1	Non-Hodgkin lymphoma	1998	1997
Abciximab	Reopro	GPIIb/IIIa; Chimeric IgG1 Fab	Prevention of blood clots in angioplasty	1995*	1994
Muromonab-CD3	Orthoclone Okt3	CD3; Murine IgG2a	Reversal of kidney transplant rejection	1986*	1986#

* - Country-specific approval. # - Withdrawn or marketing discontinued for the first approved indication. NA - not approved or in review in the EU; information on review status in US not available.

Antibodies have proven an important role in the biopharmaceutical market, both economically and in terms of improving the treatment efficiency of various pathologies. Despite the effectiveness and safety of mAbs for human administration, particularly when they present high degree of purity and retain their specific activities, the access to this type of therapy has been hampered by high manufacturing costs, making it imperative to develop effective and economical methods to purify antibodies [22, 23].

ANTIBODIES

Structural and Functional Characteristics of Antibodies

Antibodies are glycoproteins that are found in plasma and extracellular fluids [18]. They are the major effectors of the adaptive immune system, and they are produced in response to molecules and organisms (such as bacteria, viruses, foreign molecules and other agents) which are neutralized and/or eliminated by these. This response is a key mechanism used by a host organism to protect itself against the action of foreign molecules or organisms. They are secreted by

specialized B-lymphocytes (plasma cells) and may also be called immunoglobulins (Ig) since they contain a structural domain found in various proteins. There are several Ig populations that can be found on the surface of lymphocytes in exocrine secretions and extravascular fluids [24]. Each animal can produce millions of different antibodies, and each antibody is able to bind specifically to a particular foreign substance known as an antigen [18]. B-lymphocytes carrying specific receptors, recognize and bind the antigenic determinants of the antigen and induce a process of division and differentiation, which transforms the B-lymphocytes into plasma cells that predominantly synthesize antibodies.

Antibodies comprise four polypeptide chains, namely two heavy chains (approximately 55 kDa each) and two identical light chains (about 25 kDa each), organized in "Y"-shape format. Each of these chains contains one variable region and multiple constant regions, associated by disulfide bonds and non-covalent bonds, resulting in a molecule with a molecular weight of approximately 150 kDa, as can be seen in Fig. (**1**). The antibody binding to the antigen occurs in the antigen-binding fragment (Fab) region through complementary-determining regions (CDR). CDRs are composed of different sequences of amino acids according to the type of antigen to which are associated, and are therefore referred as hypervariable regions [25]. The heavy chains are associated by disulfide bonds, located in a flexible hinge region, with approximately 12 amino acids (essentially proline, threonine, serine and cysteine), and are highly sensitive to enzymatic or chemical cleavage [24]. Each globular region, comprised by the folding of the polypeptide chains, is designated by domain.

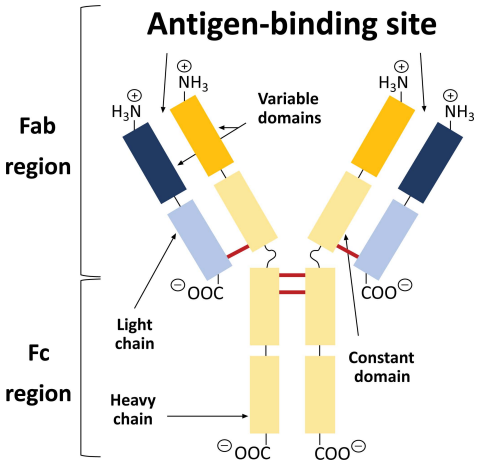
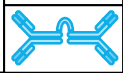
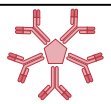


Fig. (1). Schematic representation of a conventional antibody. It comprises two identical heavy (yellow) and light (blue) chains, joined by disulphide bonds (red). Each chain contains a constant and variable region (coloured darker), where the antigen-binding site is located.

In mammals, there are five classes of Ig (IgG, IgM, IgA, IgD and IgE), depending on their primary sequence of amino acids, that differ on the functions performed in the immune system. Table **3** summarizes their main functions, structure and location.

Table 3. Classes of immunoglobulins: main functions, structure and location.

Class	Functions	Molecular structure		Location
IgA	Plays an important role in mucosal surfaces, such as lungs and gastrointestinal tract. Prevents colonization by pathogens. Also found in saliva, tears, sweat and breast milk.		Monomer (160 kDa)	Serum External secretions
			Dimer (390 kDa)	
IgD	Acts as an antigen receptor on B cells. Involved in the activation of basophils and mast cells to produce antimicrobial factors.		Monomer (175 kDa)	B cells surface
IgE	Helps in the protection against parasites. Binds to mast cells or basophils in response to allergic reactions.		Monomer (190 kDa)	Serum Surface of mast cells or basophils
IgG	Main class of antibodies in serum. Plays a crucial role in protecting against invasion of bacteria and viruses. The only antibodies able to cross the placenta to give immunity to the fetus.		Monomer (150 kDa)	Serum Intracellular Fluids
IgM	First antibody produced in an immune response. Protects against bacterial and fungal infections.		Pentamer (950 kDa)	Serum

Both IgA and IgG are subdivided in subclasses – isotypes – due to the polymorphisms verified in constant regions of the heavy chain [18]. Each Ig class determines a type and temporal nature of the immune response. Currently, two isotypes of IgA – IgA1 and IgA2 – and four isotypes of IgG – IgG1, IgG2, IgG3 and IgG4 - are known. In a biotechnology perspective, IgG is the most important class of antibodies, since they are the most abundant Ig in the blood (representing 75% of antibodies) [26].

Applications

Monoclonal antibodies (mAbs) have been the subject of intense investigation since their production was first achieved [27]. Their high specificity is an excellent advantage for therapeutic purposes since mAbs can only interact with a specific substance [18]. This specificity is also very attractive for numerous clinical trials and laboratory diagnostic tests, such as the detection and identification of analytes, cell markers, pathogens, among others [28]. However,

their monospecificity may also be considered a limitation, since the occurrence of minor changes in the structure of an epitope may affect the function of the antibody. Finally, an additional advantage of mAbs is related with the fact that, once the desired hybridoma has been generated, mAbs can be produced through a constant and renewable source, allowing a continuous and reproducible delivery of antibodies. Due to all these factors, mAbs, and in particular IgG, represent the most used type of antibodies for a large plethora of scientific and therapeutic applications. Below we describe some of the main applications of this biomolecule for such purposes.

At the diagnostic level, IgG antibodies are ideal biological recognition agents, and have thus been used in numerous analytical techniques, such as Western Blotting (immunoblotting), immunohistochemistry, immunocytochemistry, immuno-precipitation, enzyme-linked immunosorbent assay (ELISA), microarrays of antibodies, immunoscintigraphy, radioimmunological assays, flow cytometry analyzes, immunosensors, immuno-polymerase chain reactions (IPCRs) and real-time IPCRs [29]. These antibodies are also used as important tools in immunoaffinity chromatography [30], in the identification and localization of intracellular and extracellular proteins [18], and used for the detection of pathogens, adulterants, toxins, and/or other residues (drugs, chemicals or pesticides) in food products and in environmental analysis/monitoring [30].

Concerning their therapeutic applications, mAbs present a large potential for use in passive immunotherapy. Passive immunity is conferred by transferring specific antibodies against a particular pathogen to a host, and is distinguished from the active immunity because, in the last case, the immunity is conferred by the host's response to a given pathogen antigen, as depicted in Fig. (**2**). Antibodies can be administered as: (i) human or animal plasma or serum; (ii) pooled human immunoglobulin for intravenous (IVIG) or intramuscular (IG) use; (iii) high-titer human IVIG or IG from immunized or convalescing donors; and (iv) as monoclonal antibodies (mAbs) [31]. In particular, intravenous immunoglobulin (IVIG) is used as a replacement therapy in immunodeficient individuals, who are unable to create their own effective immune responses [32]. IVIG can also be used to suppress the pathological immune responses that occur in patients with autoimmunity [33]. IVIG formulations are prepared from the human serum IgG fraction, which is pooled from a minimum of 1000 up to 60,000 donors [34].

The therapeutic potential of antibodies in infectious diseases has also been investigated, and some of the biopharmaceuticals-based therapies presented on Table **2** refer to anti-infectives mAbs. Respiratory syncytial virus (RSV) infection is classified into the subfamily Pneumovirinae within the *Paramyxoviridae* family of enveloped, single-stranded, and negative-sense RNA viruses [35].

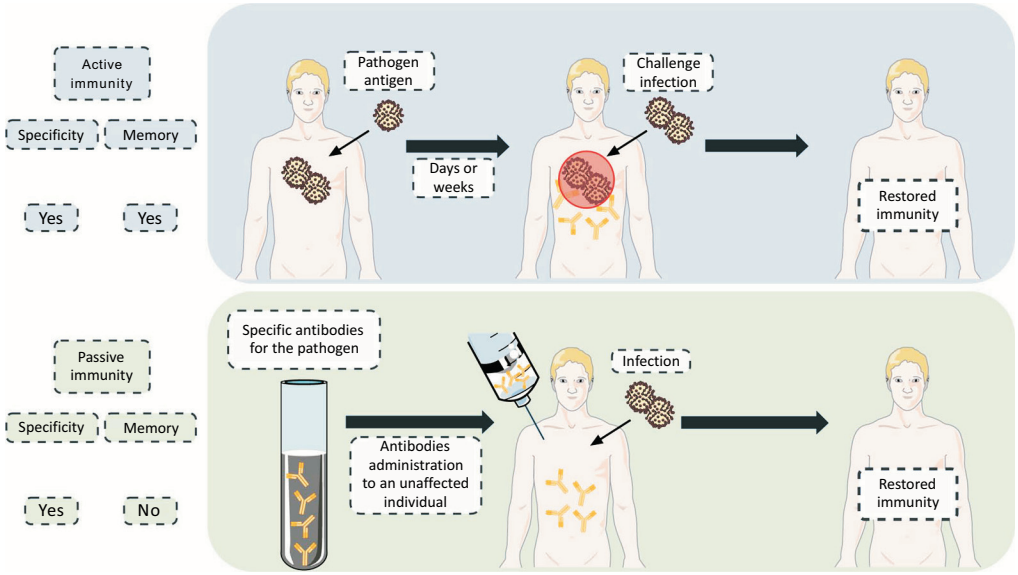


Fig. (2). Active immunity *vs*. Passive immunity.

This infection can result in a severe lower respiratory tract disease, and represents the leading cause of hospitalization of young children with respiratory tract diseases. RSV is considered an important target for antiviral development, because previous vaccine attempts have failed to elicit a long-lived protective immune response, and there is currently no approved vaccine against RSV [36]. Therefore, passive antibody therapies are used in high-risk infants to prevent and modify RSV infection [31]. The treatment for RSV infection has been limited to RSV-IVIG derived from plasma donors with high RSV neutralizing antibodies [31], and ribavirin, a nonspecific antiviral that interferes with virus transcription [37]. However, side effects associated to the use of ribavirin and the historical debate surrounding its efficacy illustrate the need for more potent and safe therapeutics to treat RSV infection. Palivizumab was the first mAb commercially available for immunoprophylactic use to prevent an infectious disease, and consists in a humanized mouse IgG1 against the RSV surface F glycoprotein [38]. Although the indications for RSV-IGIV and palivizumab are similar, palivizumab is preferred because of its lower cost and its easier administration (intramuscular *versus* intravenous administration for RSV-IVIG). Further, palivizumab does not interfere with measles and varicella vaccines and is less likely to transmit an infectious agent since it is not prepared from plasma. Motavizumab, an affinity-optimized monoclonal antibody developed from palivizumab, has also been under clinical trials [39]. Palivizumab was the first mAb commercially available (in 1999) in the EU for immunoprophylactic use to prevent an infectious disease, and

consists in a humanized mouse IgG1 against the respiratory syncytial virus (RSV) surface F glycoprotein [38]. *Bacillus anthracis* causes inhalational anthrax, and its endospores have been developed to be a highly lethal bioterrorism threat [40]. After the bioterrorist attacks of September 2001, which resulted in eleven confirmed cases of inhalational anthrax and five fatalities, the US government enacted new regulations to encourage the pharmaceutical industry towards the development of medical countermeasures against bioterrorist threats [41]. The anthrax toxin is a tripartite toxin that contains enzymatic and binding moieties. Lethal factor (LF) and edema factor (EF) have enzymatic activities. Protective antigen (PA) is the gatekeeper moiety that binds to cell receptors and then binds and translocates LF and EF into the cell [42]. In December 2012, the United States Food and Drug Administration (FDA) approved raxibacumab for the treatment and prophylaxis against inhalational anthrax [43]. Raxibacumab is a fully human IgG1 mAb that binds PA, thus blocking the binding of PA to its cell receptors, the binding of LF and EF, and the internalization of anthrax toxin [44]. So, the neutralization of PA appeared as an effective treatment and prevention strategy of the pathogenesis of inhalational anthrax. More recently, in 2016, another antitoxin therapy was licensed under the US FDA – obiltoxaximab – a chimeric IgG1(κ) mAb that prevents binding of PA to the cellular receptors [45, 46]. Also in 2016, bezlotoxumab was approved as anti-infective mAb for the prevention of *Clostridium difficile* infection recurrence. The debilitating symptoms of *C. difficile* infection (CDI) are caused by two exotoxins – *C. difficile* toxins A and B. Clinical trials have shown that the neutralisation of these toxins can prevent recurrence of infection, offering an antibacterial-sparing treatment option [47]. Bezlotoxumab consists in a human monoclonal antibody against *C. difficile* toxin B developed by Merck & Co, for reduction of CDI recurrence in adults who are receiving antibacterial drug treatment of CDI and are at a high risk for CDI recurrence [48, 49].

Besides mAbs, two biopharmaceuticals given in Table **2**, based on bispecific antibodies (bsAbs) - blinatumomab and catumaxomab – should be also highlighted. A bispecific antibody is based on a conventional monoclonal antibody; yet, it can recognizes and binds to two different antigens or epitopes simultaneously. Thus, bsAbs show some advantages as therapeutic agents since they can potentially increase binding specificity by interacting with two different cell-surface antigens, further enabling the simultaneous blocking of two different pathways that exert unique or overlapping functions in pathogenesis, among others [50]. Their potential as new agents for therapeutic can be supported by the example of blinatumomab, that consists in a novel, bispecific T-cell engaging antibody that targets both tumor-associated antigens CD19 (expressed on B cells) and CD3 (a receptor on T cells) [51, 52], and was approved through an accelerated pathway for the treatment of acute lymphoblastic leukemia (ALL).

Blinatumomab consists of two single-chain recombinant antibodies with a small distance between the two that joins CD19 and CD3 by a flexible, non-glycosylated five-amino acid non-immunogenic linker [53]. Furthermore, catumaxomab was approved in the European Union (EU) in April 2009 for the intraperitoneal treatment of malignant ascites in patients with epithelial cell-adhesion molecule (EpCAM)-positive carcinomas [53]. Catumaxomab is a targeted immunotherapy characterized by its binding to three different cell types – tumor cells, T-cells and accessory cells – presenting two antigen-binding specificities – one for EpCAM on tumor cells and one for the CD3 antigen on T-cells [53, 54]. Despite these features, traditional combination therapies using mAbs can also modulate multiple therapeutic targets achieving similar effects, and in fact, the majority of the biopharmaceuticals going to clinical trials are still based on mAbs.

UPSTREAM PROCESSING OF MONOCLONAL ANTIBODIES

The clinical and commercial success of mAbs has led to the need of a large-scale production in mammalian cell cultures. This has resulted in a rapid expansion of the global manufacturing capacity, an increase in the size of reactors and to an increased effort to improve the process efficiency with a concomitant manufacturing cost reduction [55]. For this purpose, genetic engineering and cell engineering had allied themselves to develop new media and reactors that lead to the optimization of mammalian cell culture conditions at a larger scale. Hybridoma technology was the first technology that made possible the production of large quantities of mAbs from murine origin, but later new and very efficient expression systems were developed in order to allow the full exploitation of the antibodies potential.

Hybridoma Technology

Monoclonal antibodies were first recognized in the serum of patients with multiple myeloma, in whom clonal expansion of malignant plasma cells led to the production of high levels of identical antibodies, resulting in a monoclonal gammopathy [18]. The discovery of monoclonal antibodies produced by these tumors led to the idea that it may be possible to produce similar mAbs of any desired specificity by immortalizing individual antibody-secreting cells from an animal immunized with a known antigen [27].

In 1975, Georges Köhler and Cesar Milstein [27] developed a technique named hybridoma technology, that relies on fusing B-lymphocytes from an immunized animal (typically a mouse) with a myeloma cell line, and growing the cells under conditions in which the unfused normal and tumor cells cannot survive. In this procedure, spleen cells from a mouse that has been immunized with a known

antigen or mixture of antigens are fused with an enzyme-deficient partner myeloma cell line [25]. The myeloma partner used does not secrete its own Igs. The cells are then placed in a selection medium containing hypoxanthine, aminopterin and thymidine (HAT medium) that allows only the survival of the immortalized hybrid cells. These hybridomas are then grown as single cell clones and tested for the secretion of the antibody of interest. The clones with the desired specificity are selected and further expanded. Each hybridoma produces only one Ig and the antibodies secreted by each hybridoma clone are monoclonal antibodies which are specific for a single epitope on the antigen or antigen mixture used to identify antibody secreting clones.

In the late 80s, murine mAbs started their clinical development, however suffering from a large number of drawbacks. Murine mAbs exhibit a relatively short serum half-life when compared to human IgG and induced the development of human anti-mouse-antibodies (HAMA) in the patients, especially when repeated administrations were necessary. Also, murine mAbs are relatively poor recruiters of effector functions, which is critical for their efficacy, especially in oncology indications [56, 57]. In order to overcome these problems, Boulianne *et al.* (1984) and Morrison *et al.* (1984) developed mouse-human chimeric antibodies by genetic engineering techniques, through grafting the murine variable domain, specific for a given antigen, with the constant domains of the human antibodies [58, 59]. This new technique allowed to obtain molecules that were approximately 66% human, which consequently present a superior half-life in humans and a lower immunogenicity. Later, Jones *et al.* (1986) proposed changes and improvements to these antibodies by grafting only the murine hypervariable regions into the human framework, resulting in molecules that were approximately 95% human [60].

Whilst humanized mAbs appeared to overcome the inherent immunogenic problems of murine and chimeric mAbs, humanization has however some additional limitations, since it requires a laborious, complex and time-costing process. In order to obtain fully human mAbs, new techniques have emerged in the last years. One of these techniques is phage display, in which a library of bacteriophage expressing the antibody variable domains are screened against an immobilized target antigen in order to capture the phages that specifically bind to the antigen. Unbounded phage are washed away while bounded phage are eluted, propagated in *E. coli* cells and further used in another round of selection. At the end of the panning procedure, *E.coli* cells are infected with the phage from the last round and the individual clones correspond to individual monoclonal variable domains. An additional alternative technology consist on the creation of transgenic mice expressing repertoires of human antibody gene sequences, which allow the production of fully human monoclonal antibodies through the

hybridoma technology, with low immunogenic potential and properties similar to the human endogenous antibodies [61, 62]. Fig. (**3**) depicts several types of antibodies possible to be obtained through genetic engineering, as well as their classification according to the origin of their primary sequence.

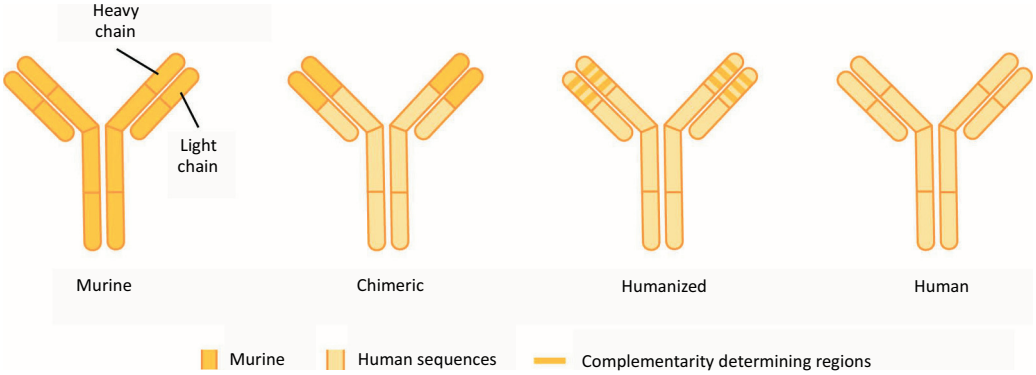


Fig. (3). Humanization of antibodies through genetic engineering. Therapeutic mAbs can be murine (100% murine protein) [suffix: -omab], chimeric (composed of approximately 35% murine sequences) [suffix: -ximab], humanized (only possess 5-10% of murine regions) [suffix: -zumab] or fully human (100% human protein) [suffix: -umab].

In clinical terms, there are no apparent differences between the monoclonal antibodies obtained using phage display techniques or transgenic mice. However, the detection process of an antibody by phage display allows a more direct isolation and also a higher control of the specificity and affinity of the antibody.

Recombinant DNA Technology

The development of very efficient expression systems is essential to the full exploitation of the antibodies therapeutic potential, both in terms of therapeutic efficiency and cost/widespread application [63]. The expression of functional, correctly folded antibodies or antibody fragments and its scale up to commercial levels is a major goal in the therapeutic antibodies development. Since antibody therapies may require large doses over a long period of time, the manufacturing capacity becomes an issue because the drug substance must be produced in large quantities at a cost and time efficiency which meet the clinical requirements. In response to the strong demand, companies have built large scale manufacturing plants containing multiple 10,000 L or larger cell culture bioreactors [64].

Therapeutic antibodies are mainly produced in mammalian cell expression systems due to their ability to produce large amounts of mAbs with a consistent quality and to adapt well to culture in large-scale suspension bioreactors [65]. Another reason, and probably the most important for the dominance of

mammalian cells, is their capability to perform the required protein folding, assembly and post-translational modifications, such as glycosylation, so that the produced mAbs would be chemically similar to human forms for increased product efficacy and safety. In particular, the cell line obtained from Chinese Hamster Ovary (CHO) is the most widely used cell line for the production of large-scale mAbs [66]. This cell line was first isolated in 1957 by Dr. Theodore T. Puck from a female Chinese hamster, and quickly gained recognition due to their ease and rapid growth/culture time and high expression [67]. Currently, this cell line appears as the production host of approximately 70% of total recombinant therapeutic proteins [68]. It is widely used as the main vehicle for the production of mAbs due the advantages summarized below:

1. CHO cells have proven a track record of producing safe, biocompatible and bioactive mAbs, enabling products from these cells to gain a more easily regulatory approval [69, 70];
2. Genetic modification techniques such as the use of dihydrofolate reductase (DHFR) or glutamine synthase (GS) can be used to attenuate the low specific productivity that hinders the production of recombinant proteins in mammalian cells [68, 71];
3. CHO cells present the appropriate molecular repertoire for the occurrence of native-like post-translational modifications with glycoforms comparable to those of humans, thus exhibiting similar molecular activity [72];
4. They grow in serum-free suspension cultures, often in stainless steel bioreactors or even disposable bioreactors [73];
5. Their scale-up is possible [72];
6. Human pathogenic viruses, such as HIV, influenza and polio, do not replicate in CHO cells, enhancing the safety of the produced mAbs [74];
7. They are easily genetic modified to optimize the production process [70].

Close to the CHO cells, the most commonly used cell lines for mAbs production are the murine lymphoid cells NS0 and SP2/0, since they are originated from differentiated B-lymphocytes that produce high amounts of immunoglobulins. However, these are not the preferred cell culture for mAbs production because the produced antibodies may contain immunogenic residues, resulting in a reduced half-life *in vivo* [75].

The use of cells from human sources is an option that allow to overcome the presence of antigenic groups in the antibodies produced. There are currently several possibilities being studied, namely human embryonic cells derived from the kidney HEK293, immortalized human amniocytes from CEVEC, and human embryonic cells derived from retinoblasts PER.C6 from Crucell [76]. Although HEK293 and human amniocytes are reported to be the most suitable for protein

production, PER.C6 are considered the most promising candidates since they are the most productive and can reach cell densities considerably higher than CHO cells, producing more than 27 g/L of protein in reactors [76, 77]. Despite of human cell lines being still subject to regulatory problems due to their low resistance against adventitious agents, several products derived from PER.C6 are currently in the clinical trials phase [76].

Highly productive cell lines result from a host cell line that has the desired characteristics, an appropriate expression system, and a good transfection and selection protocol. The selection of the appropriate expression system is determined by its ability to produce high titers, to consistently produce antibodies with the desired characteristics (*e.g.* the glycosylation pattern), to reach a high yielding cell line rapidly, and to grow in suspension. The process for the development of highly expressing cell lines begins with the transfection of mammalian cells with plasmid vectors carrying the heavy and light chain genes of the antibody of interest, and also selection marker genes, which allow the selection of the transfected cells conferring resistance to certain antibiotics or advantages in growth/development under certain deficient nutrition conditions [69]. In the case of CHO DG44 or DXB11 cells deficient in the enzyme dihydrofolate reductase (DHFR), a DHFR label is used, which confers the ability to reduce dihydrofolate to tetrahydrofolate, a metabolite required for nucleic acid metabolism, thereby allowing only cells that incorporated the vector with the DHFR gene to survive in a medium without hypoxanthine and thymidine [69, 78]. Amplification of the product can also be achieved by the addition of methotrexate to the medium, which inhibits the activity of DHFR, forcing cells to begin the amplification of the DHFR gene to ensure their survival, resulting in a simultaneous amplification of the mAb genes [79]. Another example is the glutamine synthetase (GS) marker, an enzyme that catalyzes the formation of glutamine from glutamate and ammonia, allowing the successfully transfected cells to survive in media lacking in glutamine. The use of this system with mammalian cells with endogenous levels of GS requires the use of methionine sulphoximine (MSX), a GS inhibitor. Similar to using MTX with DHFR, using MSX with GS forces cells to co-amplify the GS gene and the product gene [80]. The GS system has a time advantage over the DHFR system during development, and requires fewer copies of the recombinant gene per cell, allowing a faster selection of high-producing cell lines.

After the selection of transfectants and amplification, single clones are chosen for the scale-up and characterization of product quality and long-term expression. It is important to remark that the integration and random amplification originate several heterogeneous products, making the process of production and selection of clones time-costly and very laborious [69]. In this context, recent developments

have emerged for the selection of clones with high production efficiency using automation, particularly to reduce the time dispended and to improve the consistency of the process. Flow cytometry applied to fluorescence-activated cell sorting allows a rapid monitoring of millions of cells to isolate specific subpopulations of several heterogeneous products, and can be applied to the separation of surface-labeled antibody-producing cells, since the levels of excreted proteins are proportional to the levels of proteins found on the surface of the cell [81].

DOWNSTREAM PROCESSING OF MONOCLONAL ANTIBODIES

As mentioned above, the upstream processing of mAbs has undergone numerous advances in the last years, namely in cell culture technologies that allow higher expression levels and higher cell densities [82]. However, the downstream processing has not evolved at the same pace, being currently considered as the bottleneck in the production of therapeutic mAbs [83]. This type of processing depends on chemical and physical interactions, which make it difficult to select a generic method, so they must be modeled, tested and developed from primary principles [8]. The main aspects that should be taken into consideration during the development of a purification process are the speed, and the total yield and purity of the target product. In addition, the process should fulfill several production criteria, such as robustness, reliability and capability to be scaled-up [12].

The explosion in the number of mAbs that went under clinical trial processes, created the need to apply a standard approach for their purification [84]. The purification process should produce mAbs suitable for human use, in which impurities such as host cell proteins, DNA, endogenous and adventitious viruses, endotoxins, aggregates and other species should be removed. Moreover, it is important to note that any treatment performed during the purification steps exerts stress on the protein, due to drastic changes in pH values, salt or protein concentrations, buffers, solvents, among others [85]. This stress can result in denaturation or aggregation of the antibody, with losses in recovery yields; therefore, the monitoring of the quality and functionality of the product is essential during all the processing steps using fast and appropriate analytical tools. Taken all together, the isolation and purification of the final mAbs products requires numerous and complex steps. All these steps represent a high contribution to the final costs of downstream processing, estimated to range between 50 and 80% of the total production cost of mAbs [4, 86]. With the increased antibody titer achievable through the upstream processing, higher amount of chromatographic resins, buffers and membranes will be consequently required, thus leading to higher costs associated to the downstream processing stage. Strube *et al*. [11] presented a comprehensive study on the cost distributions

in downstream processing according to product concentrations. Based on the assumption of a constant spectrum of impurities, the overall cost of goods (COG) decrease with increasing product titers. Therefore, downstream costs will have a higher impact on the overall production costs with increasing titer. In this context, all the efforts should be centered in the creation of a robust and generic process, with a limited number of steps, suitable for all mAbs candidates, by reducing the time and resources required, while fulfilling criteria of purity, quality, efficacy and safety of the therapeutic antibodies for therapeutic applications.

Standard Downstream Processing Platform

Fig. (**4**) depicts a general scheme of a typical downstream platform for the purification of mAbs, based in a common sequence of unit operations that have been developed and integrated aiming to accelerate the entry of these biopharmaceuticals into the clinical trials phase. This platform comprises 6 main steps: 1) clarification; 2) capture of mAbs; 3) viral inactivation; 4) polishing steps; 5) viral removal; and 6) final formulation of the bioproduct.

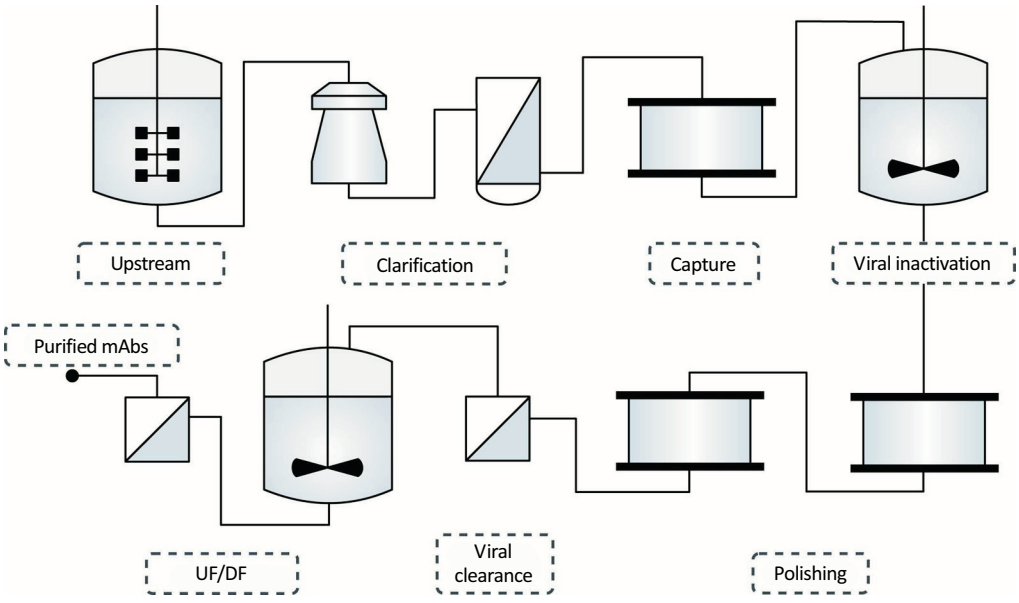


Fig. (4). Standard downstream platform for the purification of mAbs.

The first step in the recovery of an antibody from a mammalian cell culture is the **clarification step**. Since mAbs are typically produced using high density mammalian cell cultures, the removal of cells and cell debris from culture broth to

yield a clarified fluid suitable for chromatography is required [84]. In typical processes, the accepted range for solids concentration in a culture broth from mammalian cell cultures is usually 40-50%, and by the end of the clarification process, solids concentration is expected to be negligible, although turbidity may remain. This step is generally accomplished through the use of centrifugation, depth filtration and sterile filtration, although other approaches may be applied. Centrifugation is typically preferred over other clarification technologies, such as tangential flow microfiltration, due to its easy application at an industrial scale and ability to operate with large volumes (typically between 2-15,000L per batch) [12]. Clarification operations, in terms of capital cost and energy consumption, can account for up to 25% of the cost of the entire downstream process [87].

After a successful clarification, the medium containing the desired mAbs is subjected to a **capture step**, with protein A (proA) affinity chromatography being the gold standard in most industrial processes [88]. ProA is a naturally occurring polypeptide found anchored in the wall of the bacteria *Staphylococcus aureus* [89]. The molecular weight of the intact native molecule is 54 kDa. However, typically a recombinant proA is used for IgG purification, produced as a secreted extracellular protein in *E. coli*, devoided of its domain responsible for binding to cell wall, and with a molecular weight of 42 kDa [87]. The natural high affinity of proA for the Fc region of IgG-type antibodies is the basis of the purification of IgG, IgG fragments and subclasses [84]. In particular, IgG binds to proA at the junction between the heavy chain constant region 2 (C_{H2}) and 3 (C_{H3}) [90]. In Fig. (**5**), a schematic representation of this purification step using proA affinity chromatography is provided. This step typically involves the passage of the clarified cell culture supernatant over the column at pH 6-8, conditions under which the antibodies bind and unwanted components such as host cell proteins and cell culture media components and putative viruses flow through the column. An optional intermediated wash step may be carried out to remove non-specifically bounded impurities from the column, followed by the final elution of the product with a low pH elution buffer, ranging between 2.5 and 4. The IgG-proA binding mechanism primarily consists of hydrophobic interactions related to specific hydrogen bonds that are established as a function of the pH. At alkaline pH values, histidyl residues on the binding site of IgG–protein remain uncharged. At more acidic pH values, both histidyl residues in proA and on IgG become positively charged, and as they are close in the binding area, they mutually repel each other, thereby providing an easy way for the dissociation of the IgG from proA [87].

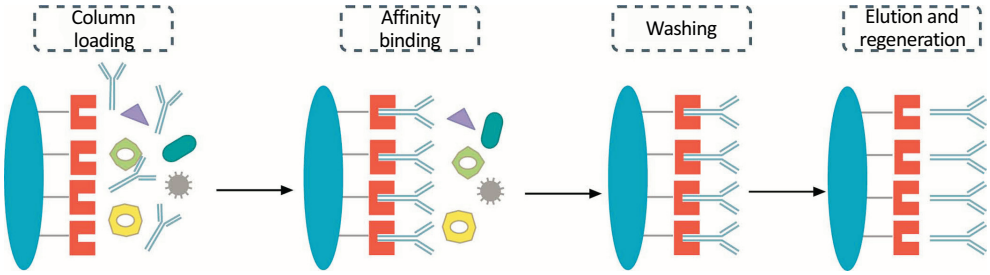


Fig. (5). Schematic representation of the mAbs capture step by protein A affinity chromatography.

The process ends with the regeneration of the proA column for future use in order to extend its life span, which generally does not exceed 200 chromatographic cycles [91, 92]. For the regeneration, solutions with acidic pH values (<3.0) or solutions containing chaotropic reagents (guanidine chloride or urea) to remove most of the antibodies or impurities that may remain in the column are used. The resins can also be sanitized with a sodium hydroxide solution (<100 mM) [12].

ProA affinity chromatography is reported to be highly selective for mAbs, resulting in purity levels higher than 99% obtained from complex cell culture supernatants [12]. Therefore, proA chromatography is widely used as the first stage of antibody purification due to its high selectivity and high removal capacity of impurities, namely host cell proteins (HCP), host DNA, virus, among others [84]. After this step, antibodies are found with high purity and to be more stable due to the elimination of proteases and other components of the culture medium which may cause the degradation of the product. This step, however, presents several limitations that have to be considered in the evaluation of the standard downstream process and other alternatives [12]. The ligand used is prone to proteolysis and the cleaved domains can adhere to product molecules creating an additional separation challenge. Conventional proA ligands cannot be exposed to alkaline conditions that are commonly employed to sanitize other column modes, thus requiring the use of high concentrations of chaotropes, such as urea, for column regeneration and sanitization. The use of high concentrations of chaotropes creates a cost issue as well as a disposal challenge. The need to elute the column at a low pH can induce product aggregation for some mAbs. Finally, and most significantly, the cost of proA resins is nearly an order of magnitude higher than conventional chromatographic resins. Table **4** summarizes the main advantages and disadvantages of the capture step of mAbs using proA affinity chromatography.

Table 4. Main advantages and disadvantages of the capture step of mAbs using proA affinity chromatography.

Advantages	Disadvantages
High selectivity for IgG, ensuring high yields and purities	Ligand leaching (toxic)
Use of acidic pH values for the elution step, also representing the first step of viral inactivation	Aggregation of antibody molecules under low pH values of the elution buffer
High dynamic binding capacity	Reduced flow in the loading step
Suitable to be included in a standard format	High resin costs

Since mammalian cells used in the manufacture of mAbs can produce endogenous retroviruses and are occasionally infected with adventitious viruses during the upstream processing, a **viral inactivation step** is always required [87]. All regulatory agencies impose a viral safety threshold for final formulations, although they generally do not state any preferred or recommended method for virus removal/inactivation. According to current regulatory standards, it is necessary to use two orthogonal steps, *i.e.* based on two different mechanisms, for viral reduction aiming at ensuring the safety of the products produced in mammalian cell cultures. Most industrial purification processes use inactivation at low pH values, taking advantage of the acidic pH used in the elution during proA chromatography [87]. Other viral inactivation options include the use of heat, solvents, detergents or ultraviolet radiation [93 - 95]; however, these approaches are not robust from a procedure and scale-up point of view.

Polishing steps are further addressed for a final removal of trace impurities of the solution containing the target mAbs [87]. The nature of the polishing steps is determined by the nature of the product and the impurities present, but usually one or two additional chromatography polishing steps are applied. Most mAb purification processes include at least one ion exchange chromatography step, for reducing high molecular weight aggregates, charge-variants, residual DNA and host cell proteins, leached ProA and viral particles. The use of anion exchange chromatography (AEX) is more common than the cation exchange chromatography (CEX), as this resin is often used in the flow-through mode (in which the product does not bind to the column whereas impurities are retained) [96]. Its operational pH is generally below the protein pI. This ensures that the mAb is positively charged and, therefore, easily washed away from the AEX matrix while negatively charged impurities remain attached. AEX can be used to remove impurities, such as the ones removed by CEX, but will also remove leached proA, HCP, endotoxins, and even viruses, which are bounded to the AEX column [97].

The effectiveness of **viral clearance** is determined by assays in which a model

virus (Murine Leukemia Virus - MuLV; Minute Virus of Mice - MVM) is injected into the culture medium to assess viral load before and after the viral removal operation [87]. Due to safety requirements, mammalian cell-derived products may contain less than one virus particle per million doses, which translates into approximately 12-18 $\log_{10}$ clearance for endogenous retrovirus and 6 $\log_{10}$ clearance for adventitious virus [84]. Filtration is a robust choice for viral clearance [87]. It is relatively insensitive to small variations in process parameters, which make it quite suitable for implementation in a standardized process. Virus filters are typically operated at constant pressure and, depending on the membrane, the volumetric charge may range from 200-400 L/m^2, before a significant flow decay is noticeable [98].

Finally, the purification process is completed when the product is placed in a predefined formulation buffer, which is usually accomplished with a final **ultrafiltration step in the diafiltration mode** (UF/DF) [87]. The type of membrane used, the imposed transmembrane pressure, the tangential flow velocity and the concentration at which diafiltration is performed can be standardized for practically all mAbs. Regarding the materials that can constitute the membrane, regenerated cellulose is the most commonly used material, due to its low fouling propensity and easiness of cleaning. Membranes can have different configurations, from the cassette format to hollow fiber modules, being this last one more suitable for the processing of viscous solutions or sensitive products to shear stresses. The optimization of this unit operation is of utmost importance since monoclonal antibody based drugs are generally administered in high doses. Due to this requirement, mAbs should be kept in containers of limited volume, where the product packaging and concentration step performs a fundamental role. Frequently, some additives like sugars or surfactants are also added to the final formulations in order to prevent physical degradation mAbs [85].

Alternative Downstream Processing Platforms

Downstream processing would never have developed as an individual sector of the bioprocessing industry without chromatography, whose inherent selectivity has made of it a key enabling technology in all bioseparation processes [8]. However, chromatography has been the major cost center, derived mainly from the high resin costs and relatively long processing times. Hence, lower cost alternatives have also been pursued [2]. Two viable options have been proposed for the downstream processing of mAbs: (i) the replacement of the proA affinity chromatography by other chromatographic processes; and (ii) the elimination at all of chromatography by non-chromatographic methods. In fact, a large number of alternative platforms for the IgG purification has been reported, which are summarized and compared concerning the recovery yields and purity levels in

Table **5**.

Table 5. Most used chromatographic and non-chromatographic methods for the purification of IgG, compared in terms of recovery yield and purity level of IgG.

Purification method	Ligand	Recovery Yield (%)	Purity level (%)	Reference
Chromatographic platforms				
CEX, AEX, HIC (3 chromatographic steps)	-	85	> 99	[99]
CEX	Phosphonate	92-98	95	[100]
	Heparin	90	83	[101]
	Capto™ S	81-83	80-84	[102]
AEX	Advective Hydrogel Membrane	> 90	> 85	[103]
HIC	PVDF membranes	> 97	> 97	[104]
Multimodal chromatography	Capto™ MMC	92-93	95-96	[102]
	MEP-Hypercel™	76	69	[105]
	Phenylboronic acid	98	83	[106]
Affinity chromatography	Epitope imprinted macroporous membrane (5 cycles)	80-90	88	[107]
	Membranes based on Nylon 66 coated with low-molar-mass dextran or poly(vinylalcohol), pre-activated polysulfone (Ultrabind®) and regenerated cellulose (Sartobind®) membranes, carrying Protein-A ligands.	-	-	[108]
Immobilized metal affinity chromatography	Metal ions	-	-	[109]
Expanded bed adsorption chromatography	ProA	92	98	[110]
Continuous annular chromatography	ProA	77-82	-	[111]
	Hydroxyapatite	87-92	-	[111]
Non-chromatographic platforms				
Preparative electrophoresis	-	80	-	[112]
	-	80-90	-	[113]

(Table 5) contd.....

Purification method	Ligand	Recovery Yield (%)	Purity level (%)	Reference
Chromatographic platforms				
Affinity precipitation	Eudragit S-100	68	PF = 8	[114]
	GAPDH	98	PF = 1,8	[115]
	Bivalent peptidic hapten	> 85	> 97	[116]
Magnetic separation	Protein A coated magnetic particles	-	-	[117]
	Stimuli-responsive magnetic nanoparticles	64	> 98	[118]
	Starch-coated magnetic nanoparticles	69	> 99	[119]
High performance tangential flow filtration	Composite regenerated cellulose membranes, Biomax™ modified polyethersulfone membranes and conventional regenerated cellulose membranes	-	-	[120]
	Ultracell™ composite regenerated cellulose membranes chemically modified with bromo-propyl-trimethylammonium bromide	98	PF = 10	[121]

Legend: AEX – anion exchange chromatography; CEX – cation exchange chromatography; GAPDH – glyceraldehyde 3-phosphate dehydrogenase; HIC – hydrophobic interaction chromatography; PF – purification factor; ProA – protein A; PVDF – polyvinylidene fluoride.

Concerning the chromatographic approaches, **cation exchange chromatography (CEX)** was one of the first chromatographic techniques to be evaluated as the primary adsorption step in the purification of monoclonal antibodies, and as an alternative to the conventional Protein A affinity chromatography. This first adsorption step was already implemented in some industrial processes, namely in the production of the monoclonal antibody adalimumab, which is marketed under the trade name Humira®. and is mainly administered for the reduction of symptoms of chronic inflammatory diseases [122]. Recent advances in the production of cation exchange porous particles allowed the dynamic bonding capacities of these resins to increase to values greater than 100 mg IgG/mL resin, which are higher than those reported for Protein A resins (usually < 40 mg IgG/mL resin) that depends strongly on residence time [123]. Furthermore, this type of chromatography allows the removal of host cell proteins to comparable levels to those obtained with the traditional process, allied to the use of a chromatographic matrix an order of magnitude cheaper than proA resin [84, 87]. This alternative may be relevant also because proA chromatography requires the use of an acidic pH during the elution step, which may promote product aggregation and proA leaching from the column, as opposed to cation exchange chromatography which does not require an eluent with low pH values. In fact, the successful use of appropriate cation exchange columns has been already reported to resolve downstream bottlenecks, sustain cost-effective production, and manage

large quantities, with remarkable advances om the use of phosphonates bound to Zirconia particles [100] in the development of new ligands such as heparin [101] or sulfonate [102].

It was already demonstrated the potential of a platform constituted by 3 chromatographic steps for the purification of IgG, without the use of proA chromatography [99]. It has also been highlighted the use of other chromatographic variants, such as anion exchange chromatography (AEX) [103] and hydrophobic interaction chromatography (HIC) [104]. However, it is notorious that the use of a platform consisting of 3 chromatographic steps (combining CEX, AEX and HIC) allows the achievement of an exceptional purity level (> 99%) [99], which is not achieved by any of the techniques alone (CEX – 95% [124]; AEX – 85% [103]; HIC – 97% [104]). A major drawback in the use of CEX is that it requires samples with low ionic strength prior to loading, and due to the high amounts of salts used in the elution buffer, the corrosion of the metal instruments of the equipment may also be a problem. Furthermore, good binding capacities are not achieved when the cell culture supernatants are applied directly to the cation exchange resins, due to the moderate/high conductivities they present. For this reason, the use of cation exchange chromatography requires a previous diafiltration step of the supernatants or a dilution prior to the loading into the column. Moreover, the lack of pH control during elution may also affect protein stability causing precipitation. However, the increase on the dispended time and unit operations imposed by CEX is counterbalanced by the lower cost of the resins and by results of selectivity and purity comparable to those obtained with proA chromatography.

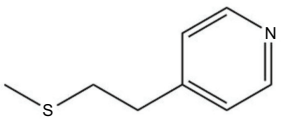
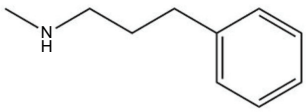
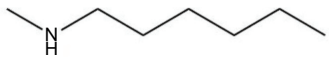
Hydrophobic interaction chromatography (HIC) is a useful tool for separating proteins based on their hydrophobicity, and is complementary to other techniques that separate proteins based on charge, size or affinity. HIC is a well-studied major polishing step in the purification of IgG-based products and is known for its capability to remove aggregated forms of the antibody [125 - 128]. HIC resins containing phenyl or butyl ligands [125, 129], and more recently these hydrophobic ligands have been used in combination with convective adsorbents, such as membranes and monoliths [104]. However, due to the high efficiency of proA affinity chromatography, HIC is mostly used as an intermediate purification step after the proA step or as a polishing step after ion exchange chromatography [84]. HIC in flow-through (FT) mode is efficient in removing a large percentage of aggregates with a relatively high yield. HIC in bind-and-elute mode normally provides effective separation of process-related and product-related impurities from the antibody product. The majority of host cell proteins, DNA and aggregates can be removed from the target antibody through the selection of a suitable salt concentration in the elution buffer or by the use of a gradient elution

method. For instance, Ghosh *et al.* [104] achieved the separation of humanized monoclonal antibodies from cell culture media using HIC with membranes composed of polyvinylidene, with a pore size of 0.1μm, obtaining a purity and recovery higher than 97%. Although HIC is a very powerful tool in mAbs purification processes, there are two main limitations when used in the bind-elution mode. In general, HIC resins have relatively lower binding capacity and lower step yield compared to the other chromatography steps used in mAbs purification. Furthermore, sufficient binding of mAb proteins to HIC resins is usually achieved with increasing salt concentrations in the binding buffers, and the elution product pool from the HIC step purification may still contain large amounts of salt, which often complicates sample manipulations and process flow transitions during large-scale manufacture. Efforts have been done in this context, for instance by Ghose *et al.* [130] who reported an unconventional way of operating HIC in the FT mode, just by using sodium citrate and modulating the pH of the mobile phase to alter the surface charge of the protein, and thereby influence selectivity [130]. Future trends will pass through the improvement of the properties of HIC resins on two main areas: resin pore size optimization to facilitate mass transport of mAb molecules towards the ligand binding sites to increase the binding capacity, and hydrophobic charge induction (HCIC) chromatography design to allow the mAb molecules binding to the resin at lower salt conditions.

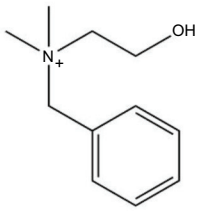
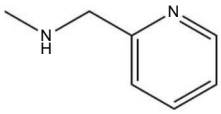
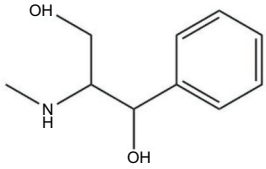
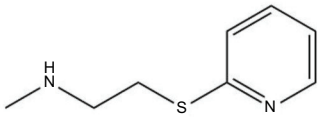
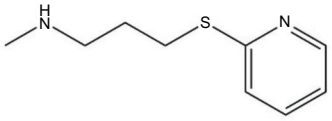
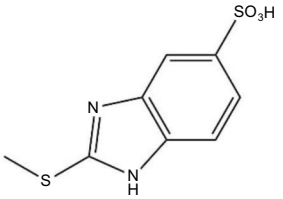
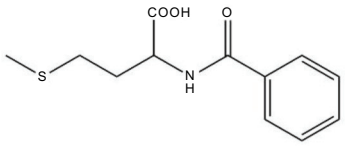
In the last decade, **multimodal chromatography** (MMC) has been receiving considerable attention [86]. MMC can be defined as a chromatographic method involving multiple types of interaction between the stationary phase and the mobile phase. The binding modes that are more frequently employed in multimodal ligands comprise ion exchange, hydrogen bonding and hydrophobic interactions [131], although others may be included for specific purposes, and the strength of each individual interaction can be manipulated accordingly. The selectivity and specificity 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 chromatographic purification problems [86]. However, considering the multitude of interactions that can be promoted with the ligand and all the factors that govern the different selectivities, the optimization of the conditions to be employed may be a complex process. The reported purification studies using these ligands usually start with a design of experiments (DoE) [132, 133], to determine the conditions that will allow to take full advantage of the multimodal potential. Monte Carlo simulations were also used as a tool to better understand and increase the process performance [134]. The screening of the appropriate ligand for a separation step is often based on the generation of a large library of ligands, which has been facilitated by increasing knowledge on both protein and ligand structures [86]. 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 [135]. The different moieties constituting the multimodal ligand should be wisely chosen, in order to ensure both high capacities and reasonable recoveries. 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 [136]. 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 [137]. 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 [138]. 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 [139]. Some multimodal ligands that have been routinely reported in the literature are summarized in Table **6**, as well the trade name under which some of them are commercialized.

Table 6. Examples of ligands that have been synthesized to be employed in multimodal chromatography [86].

	Name	pKa	Structure
Ligands positively charged	4-mercaptoethylpyridine (MEP HyperCel™)	4.85	
	Phenylpropylamine (PPA HyperCel™)	6.0 - 7.0	
	Hexylamine (HEA HyperCel™)	≈ 10	

(Table 6) contd.....

	Name	pKa	Structure
Ligands positively charged	*N*-benzyl-*N*-methyl ethanolamine (Capto™ adhere)	-	
	2-aminomethylpyridine	pKa1 = 2.2 pKa2 = 8.5	
	Aminophenylpropanediol	9.0	
	2-(pyridin-2'-ylsulfanyl) ethanamine	-	
	3-(pyridin-2'-ylsulfanyl) propanamine	-	
Ligands negatively charged	2-mercapto-5-benzimidazole sulfonic acid (MBI HyperCel™)	-	
	2-benzamido-4-mercaptobutanoic acid (Capto™ MMC)	3.3	

For example, Capto™ MMC is a weak cation exchanger with a phenyl, an amide, and a thioether group that allow hydrophobic, hydrogen-bonding and thiophilic interactions, respectively. These ligands are frequently referred as "salt-tolerant"

adsorbents, due to their ability to maintain high dynamic binding capacities in a range of different ionic strengths, from moderate to high values [140]. The negatively charged hydrophobic multimodal ligand has been recently patented for the capture of mAbs directly from cell culture supernatants [86]. The molecular structure of this multimodal ligand is shown in Fig. (**6**).

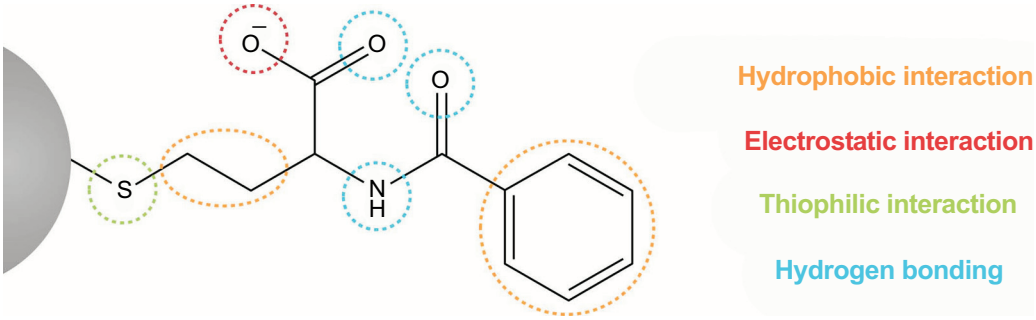


Fig. (6). Molecular structure of a multimodal ligand commercialized for monoclonal antibodies purification (Capto™ MMC), evidencing the several interactions that could be integrated.

Joucla and co-workers [102] have conducted a comparative study involving this promising multimodal ligand and a traditional cation exchanger to capture antibodies secreted by CHO cells. The use of Capto™ MMC led to an extraction yield ranging between 92-93% and a purity level of the antibodies of 95-96%. The antibody-selective MEP Hypercel™ sorbent has been extensively studied as well, as it provides similar binding capacities at approximately 25% of the cost, and without suffering from ligand contamination or instability [86]. In a study performed by Schwartz *et al.* [141], the isolation of a 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]), as well as the DNA content. 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 [142]. Equally favorable results were reported by Guerrier *et al.* [105], 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 69%, 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 aggregates 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. A more recent study relied on the use of phenylboronic acid (PBA) silica-based resins as a multimodal chromatography, and the authors proved a successful 100-fold scale-up with a recovery yield of 98% and a protein purity of 83% [106]. In summary, several characteristics that make these ligands attractive for application on an industrial scale can be highlighted, namely they are synthetic binders with a defined composition, they display enhanced chemical and physical stability, and can be modified or tailored to provide adequate selectivities allied to cost–effectiveness (resins can cost 5-10 times less than proA resin). However, it is difficult to predict the promoted interactions and to optimize the operating window, and it is also laborious and expensive to perform the screening studies and to use high-throughput screening platforms. Although the results are not as good as the obtained with proA chromatography, this technique is seen as a promising alternative for the established platform.

Affinity chromatography for purification was introduced in 1968, by Cuatrecasas and co-workers [143]. Affinity chromatography is based on biochemical separation relying on a reversible interaction occurring between the protein and the ligand, *e.g.* binding of an antigen to its specific antibody. The specificity of binding provided by the ligand to the target protein present in a complex mixture is behind the good results that can be obtained. The target protein is then eluted either by using competitive analogs, denaturing agents or by changing the pH, ionic strength or polarity of the eluent. Affinity chromatography is undoubtedly the most widely employed method for antibodies purification [29]. Over the past few decades considerable efforts were carried out 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. Recently, a type of affinity chromatography was proposed by Schwark *et al.* [107], where epitope-imprinted membranes targeting the C-terminal fragment of IgG heavy chain was developed and used for the purification of a commercial monoclonal antibody. Yields of extraction ranging between 80-90% were achieved from a cell culture broth after production of anti-IL8 antibody, and the depletion of host cell proteins using the best performing imprinted membrane under low-salt conditions reached 88% (0.7–1.2 log units), implying an effective removal of impurities from the cell culture supernatant. Castilho *et al.* [108] showed the potentiality of three affinity membranes as adsorbents for the purification of IgG, namely membranes based on Nylon 66 coated with low-molar-mass dextran or poly(vinylalcohol), as well as commercial pre-activated

polysulfone (Ultrabind®) and regenerated cellulose (Sartobind®) membranes, carrying Protein-A ligands, demonstrating high affinity for human IgG. Besides the high association constants, Protein-A adsorbers based on polysulfone and regenerated cellulose membranes showed also enhanced charge-to-charge consistency, simpler preparation procedure, membrane sterilisability, good selectivity for IgG purification from cell culture supernatants and good stability throughout repeated adsorption–elution cycles.

Immobilized metal affinity chromatography (IMAC) is also a widely used method for the purification of antibodies, since biomolecules with exposed His, Cys, Ser, Glu, Asp and Trp have affinity towards metal ions [144 - 146]. 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]. 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 Ni^{2+}, Cu^{2+}, Co^{2+}, Zn^{2+}, Fe^{3+} and Ga^{3+} [147]. 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. Fig. (**7**) exemplifies the interaction mechanism in IMAC, where a tridentate chelating agent IDA coordinates with the nickel atom, which also interacts with the histidine tail present in a random protein. It should be remarked that this histidine tail is not commonly present in antibodies; however, IMAC can be used to purify antibodies due to the presence of accessible histidine residues on the surface of the biomolecules that allow a similar interaction. Vançan and co-workers [109] compared four metal ions and buffer systems for the purification of IgG from human plasma and showed high purity absorption of IgG for all metals irrespective of the buffer system used. The ligands (metal chelates) in IMAC are of low cost and have high stability, capacity, simplicity, and selectivity. It is a versatile technique since the same ligand can be used for the purification of different proteins and the same chelating resin can be used to chelate different metal ions [148 - 150]. An important aspect in IMAC method is the occasional leakage of metal ions from the resin, leading to metal ions contamination of the final product. In this case, a column packed with metal-free matrix derivatized with a strong chelating ligand, such as TED (Tris(carboxymethyl)-ethylenediamine), could be used to trap any metal ions present in the eluate without altering the chromatographic time or the purification effectiveness [149]. Despite the IMAC potential as a less expensive alternative to biological affinity, a note of caution is in order: polynucleotides, endotoxin, and virus, have all been shown to bind to various immobilized metals. On the other hand, IMAC has also been used to selectively bind antibody fragments while endotoxins were removed by washing with a surfactant solution [151].

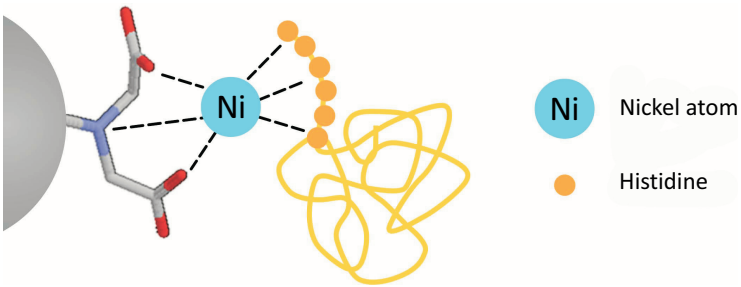


Fig. (7). Schematic representation of the interaction mechanism in immobilized metal affinity chromatography. The tridentate chelating agent IDA (iminodiacetic acid) coordinates with the nickel atom, which also interacts with the histidine tail present in the protein.

Regarding the chromatography-based methods, it is possible to highlight the work developed by González *et al.* [110] using **expanded-bed affinity chromatography**, allowing them to obtain extraction yields of 92% with a purity level of 98%. Finally, it is still possible to emphasize **continuous annular chromatography** as a viable alternative for IgG purification, as reported by Giovannini *et al.* [111] using proA as ligand, obtaining extraction yields ranging from 77% to 82%, and with the substitution of this ligand by hydroxyapatite, the extraction yields increased for the range between 87% and 92%.

Taking into account that most of the production costs of a biological product rely in the chromatographic steps, there is a great need for efficient, effective and economical (non-)chromatographic bioseparation techniques, capable of being scalable and that simultaneously lead to a high yield and purity degree, while maintaining the biological activity of the molecule. In this context, new non-chromatographic technologies have been developed as alternative strategies. The main techniques used in this field include preparative electrophoresis [112, 113], affinity precipitation [114 - 116], magnetic separation [117 - 119], membrane filtration [108, 152], and aqueous two-phase systems (ATPS) [153]. This last technique was not previously mentioned in Table **5**. However, due to their promising character, the main studied ATPS will be summarized and discussed below.

Preparative electrophoresis has proved to be a viable strategy for the purification of IgG, based on charge and size phenomena. Lim *et al.* [112] reported the use of gradiflow technology, by using a set of polyacrylamide separation membranes to separate molecules based on their size and charge. By tailoring the pH and pore size, the researchers [112] were able to separate IgG with 80% of recovery yield. Thomas *et al.* [113] developed a similar study, using the same gradiflow technology, through which they reported yields of IgG

extraction ranging from 80% to 90%. This technology proved to be a viable alternative since in addition to lead to acceptable recovery yields of antibodies, it is a method that does not depend on a variable binding interaction, it is applicable to a broad species spectrum, presents low cost and can be applied on an industrial scale.

Affinity precipitation has also been considered as an alternative for the conventional chromatography column. Precipitation has been described as a mature technology and is amongst the simplest and less expensive fractionation methods. Under mild conditions, proteins precipitation is reversible and subsequent redissolution can restore their functional characteristics. This technology can be used either to remove impurities or to isolate the target protein from a mixture [2]. In the first case, the differential solubility of proteins may be used to allow their fractionation. The resulting precipitate should be treated as the contaminant and removed during the recovery step. In the second case, the precipitation and subsequent redissolution of the target protein in a smaller volume of buffer not only reduces the processing volume, but also leads to a more concentrated target product pool, free from the soluble contaminants. Precipitation has, however, been recognized as a low resolution separation technique due to its lack of specificity. In addition, in large scale processes, the cost of the precipitating agents as well as the environmental impact of their disposal may also be an important bottleneck. Affinity ligands can, nevertheless, be used as ligand carriers to improve the selectivity of this technology. As demonstrated by Taipa *et al.* [114], IgG can be isolated from cell culture supernatants by precipitation of affinity with a heterobifunctional ligand derived from Eudragit S-100, yielding 68% of IgG with a 8-fold purification factor. Dainiak *et al.* [115] also studied IgG affinity precipitation, but using the glyceraldehyde-3-phosphate dehydrogenase ligand (GAPDH), with extraction yields higher than 98%, albeit with a purification factor of only 1.8 times.

More recently, Handlogten *et al.* [116] have shown to greatly improve the affinity precipitation process using a bivalent peptidic hapten, with a yield higher than 85% and a purity level higher than 97%. In summary, the target protein adsorbs to the polymer in a homogeneous phase, after which the entire affinity complex precipitates in a single step by manipulating the experimental conditions. After centrifugation, the affinity complex is separated from impurities and contaminants that remain in the supernatant. The target protein can, afterwards, be dissociated from the macroligand by manipulating the experimental conditions, in such a way that the polymer will become insoluble and precipitates. The target protein will be hence recovered from the supernatant, while the macroligand can be resolubilised for reuse [153].

Magnetic separation also consists in a versatile non-chromatographic method, capable to purify target biomolecules from crude extracts. The process is based on the use of magnetic adsorbents in combination with a magnetic concentrator, and an example of a magnetic separation process is depicted in Fig. (**8**). Holschuh *et al.* [117] demonstrated the feasibility of protein A coated magnetic particles in the preparative purification of a monoclonal antibody from a 100 L cell culture supernatant. The separation process was performed with a separation efficiency of more than 99% at a flow rate of 150 L/h. In comparison with conventional column and expanded bed chromatography, similar yields and purities were achieved but with much faster processing times. Later, Borlido *et al.* [154] used commercially available silica magnetic particles functionalized with phenylboronic acid (SiMAG-Boronic acid) to selectively capture mAbs directly from CHO supernatant with an overall yield of 86% while removing 88% of the CHO host cell proteins (HCP) and more than 97% of the CHO genomic DNA. In a different approach, thermo-responsive magnetic particles composed of a poly(methylmethacrylate) magnetic core with a N-isoproylacrylamide-co-acrylic acid polymeric shell were used as cationic exchangers for the purification of mAbs from a dialyzed CHO supernatant [118]. The antibody was able to be recovered with an overall yield of 64% and an HCP removal higher than 98%.

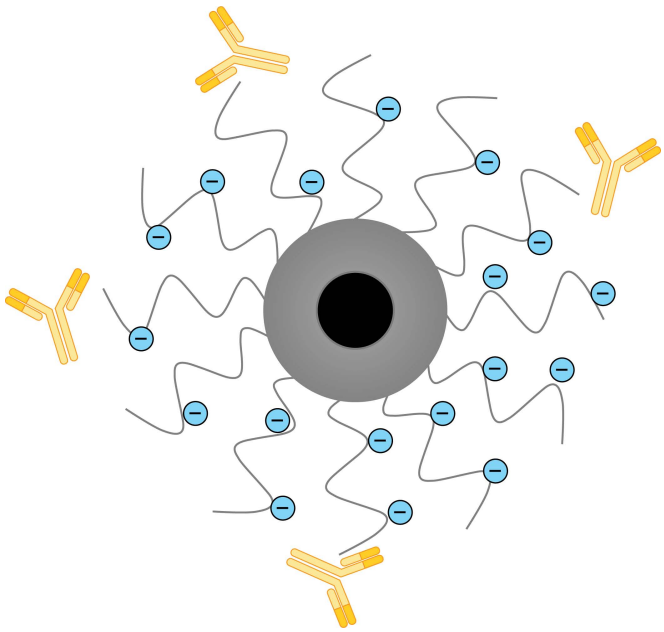


Fig. (8). Schematic representation of a magnetic separation process exemplifying the adsorption of antibodies on a modified magnetic nanoparticle with a negatively charged polymer.

More recently, Gagnon *et al.* [119] reported 69% of IgG recovery with more than 99% removal of HCP using magnetic starch-coated nanoparticles. Taking into account all the information mentioned before, it can be concluded that magnetic separation consists in a fast and smooth process, which combines the capability of scale-up with the possibility of automation, allows the separation and purification of mAbs in few minutes, and once the contaminants are tolerated, there is no need to perform any filtration or centrifugation prior to sample loading, which supports the potential of this technique for mAbs purification. However, the lack of large scale magnetic separators and the high cost of commercially available magnetic particles are the major challenges faced by this technology [155].

High performance tangential flow filtration (HPTFF) is a technique based on electrostatic interactions between proteins and charged ultrafiltration membranes [151]. Although potential exists for solutes to be retained by these interactions, antibody selectivity is mediated by ion exclusion. A positively charged ultrafiltration membrane repels (rejects) positively charged proteins such as IgG, despite them being small enough to pass through the pores. This permits their selective retention and concentration in the retentate, while weakly alkaline, neutral, and weakly acidic contaminants pass through the membrane, being collected in the permeate. Fig. (**9**) depicts a schematic representation of a HPTFF process using ultrafiltration (UF) membranes positively charged.

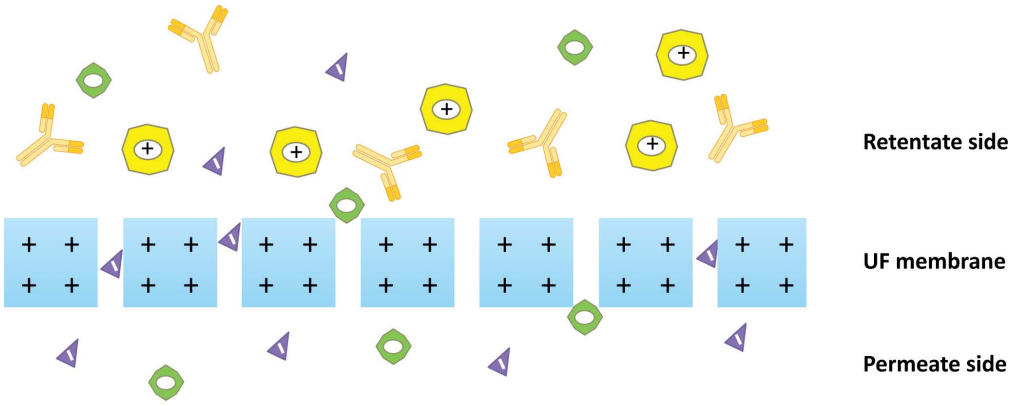


Fig. (9). Schematic representation of HPTFF process illustrated for the specific case of antibody purification using positively charged ultrafiltration (UF) membranes.

Concerning this technique, van Reis *et al.* [120, 121, 156] published several works concerning the use of membranes. In a first study [120], the authors were able to demonstrate that HPTFF using composite regenerated cellulose membranes, Biomax™ modified polyethersulfone membranes, and conventional regenerated cellulose membranes, leads to purification factors and yields for IgG-

BSA separations comparable to those of a range of commercial protein separation processes. In a further work, although the authors [156] did not focus on the purification of monoclonal antibodies, they proved that this technique is a powerful tool for the purification of proteins. The authors [156] reported an HPTFF-based process at pH 8.4 with a Biomax™ 100 negative membrane, which resulted in 94% BSA recovery yield with a 990-fold increase in the purification in linear scale-down systems representative of existing industrial scale systems. More recently, a scheme for the purification of a human pharmaceutical antibody fragment expressed in *E. coli*, using positively charged cellulosic membranes (Ultracell™ composite regenerated cellulose membranes chemically modified *in situ* using bromo-propyl-trimethylammonium bromide) was proposed [121]. HPTFF was shown to successfully enable the concentration, purification and formulation in a single unit operation, with a 10-fold removal of *E. coli* host cell proteins (HCP) and an overall process yield of 98%. The HPTFF performance was shown to be robust and reproducible, and the authors regenerated and re-used the membrane for seven times without loss of selectivity or throughput [121].

Finally, there is a classical technology, quite established in the pharmaceutical industry, named liquid-liquid extraction, that recurs to the use of organic solvents for purification purposes from aqueous media [2]. However, these organic solvents are in most cases very volatile, flammable and toxic, and proteins, in particular, present low solubility and high propensity for denaturation in the presence of these type of solvents [157]. Thus, their use in biotechnological processes is limited only to the recovery of low molecular weight products, such as antibiotics and organic acids from fermentation media [158]. Within liquid-liquid extraction techniques, **aqueous two-phase systems (ATPS)** can be considered. They have demonstrated an enormous potential and versatility for the downstream processing of biopharmaceuticals, such as monoclonal antibodies, high density lipoproteins, hormones, cytokines, growth factors, and plasmid DNA [2]. Due to their potential as a non-chromatographic alternative technique for the extraction and purification of mAbs, they are discussed below in more detail.

Aqueous Two-Phase Systems (ATPS)

ATPS, or aqueous biphasic systems (ABS), fall within the liquid-liquid extraction techniques since they allow the extraction/migration of (bio)molecules from one liquid phase to another, in which both phases are mainly composed of water. In 1896, Beijerinck reported for the first time the incompatibility and formation of two aqueous phases of solutions of agar with starch or soluble gelatin [159]. However, it was only in 1955 that ATPS were reported as a separation technique by Albertsson, who demonstrated that polyethylene glycol (PEG), potassium phosphate and water, as well as PEG, dextran and water formed two immiscible

aqueous phases aqueous above given concentration [160]. One of the phases is enriched in one of the solutes, while the other phase is enriched in the second phase-forming component. These solutes may be two polymers (for example PEG and dextran), a polymer and a salt (for example PEG and sodium phosphate) or other type of combinations [161]. The main advantages inherent to ATPS are related with the fact that it is a relatively simple technique, with a low cost associated, easy to operate and to apply on an industrial scale, with a high resolution capacity [2]. In addition, ATPS provide a highly biocompatible environment since both phases have a high water content and most of the polymers used have a stabilizing effect on the tertiary structure of proteins and on their biological activity [162].

The selective partition of a given product between the two coexisting phases represents the basis of the separation using a two-phase system [2]. This partition is controlled by several parameters, related with the properties of the system, the target solute, and the interactions between both. Concerning the system properties, several factors that influence the partition can be highlighted, namely the chemical nature of the phase-forming components, their molecular weight and concentration, system pH and ionic strength; regarding the properties of the target solute involved in the partition, it is possible to emphasize the charge, molecular weight, hydrophobicity and conformational characteristics. The complexity of the chemical and physical interactions involved in the partitioning process makes these systems quite powerful in contrast to other established separation techniques, since it is possible to achieve a high resolution only by manipulating the intrinsic properties of the system [163]. However, all this complexity of ATPS coupled with the fact that partition mechanisms are still poorly understood, undermine the prediction of the effectiveness of a particular ATPS [164].

Each ATPS has a single phase diagram under a particular set of conditions, such as temperature and pH [162]. As shown in Fig. (**10**), the binodal curve (ABCD) divides two regions: the monophasic region (below the curve), where the system presents only one phase, and the biphasic region (above the curve), that represents the solutes concentrations at which the system forms two aqueous immiscible phases [161].

In this figure it is also possible to identify the M point, which corresponds to a mixture point in the biphasic region, and whose composition of each phase acquires the designation B and D, since they are the end-points (nodes) of a specific tie-line (TL) where the mixture point is comprised. It is possible to select several mixing points along the same TL, differing only in the total system composition and volume ratio of the phases, while keeping the exact composition of the two coexistent phases (B and D) [162]. The tie-line length (TLL) is a

numerical indicator of the composition difference between the two phases and is generally used to correlate trends in the partitioning of solutes between both the phases [161].

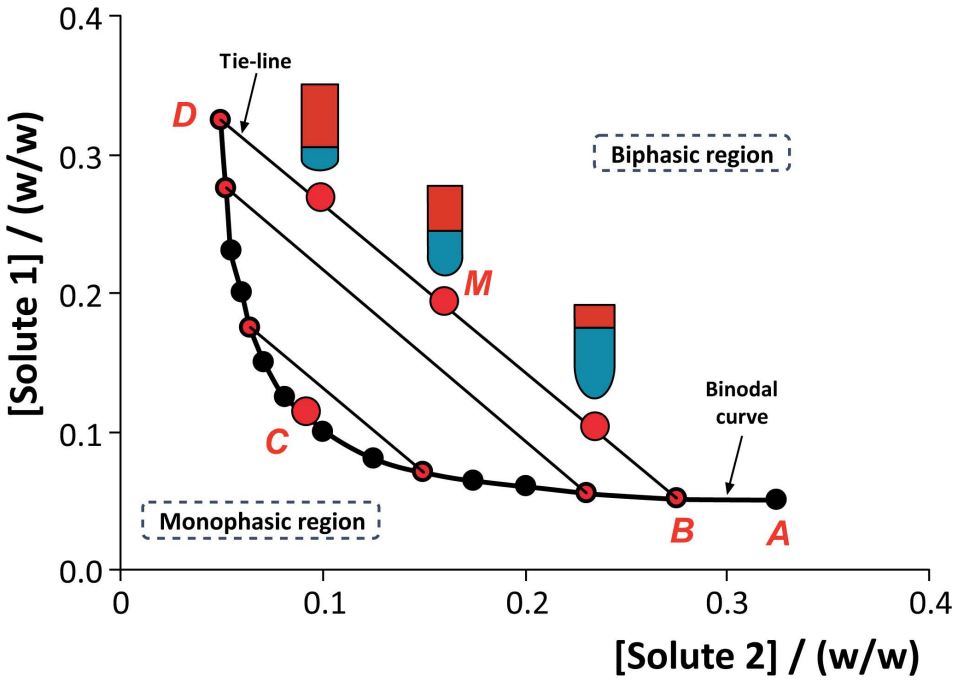


Fig. (10). Schematic representation of a phase diagram of an ATPS composed of two different solutes, in weight fraction, and where the concentration of water is omitted.

One of the most common polymers used in the formation of ATPS is PEG [165]. PEG is a polyether diol, commercially available in a wide variety of molecular weights [166]. This polymer is widely used mainly due to its interesting characteristics, namely its high biodegradability, low toxicity, low volatility, low melting temperature, high miscibility in water and low cost [167]. Moreover, PEG significantly accelerates the renaturation of proteins, allowing the recovery of their biological activity, and consequently presenting a stabilizing role in their structure [168]. However, there are other polymers that can be used in the formation of ATPS, such as dextran. This is a more expensive hydrophilic polymer when compared to PEG, although also commercially available in a wide variety of molecular weights [2, 169]. Regarding the separation of the phases in a solution containing a mixture of polymers, the phenomenon has been treated from a more fundamental point of view through the application of several theories that involve the thermodynamic properties of the polymers in solution [162].

In Fig. (**11**) is represented an example of a process based on the use of ATPS for the extraction and purification of antibodies from a complex matrix containing several impurities, where the antibodies are extracted to the upper phase, while the other impurities are retained in the opposite layer of the system.

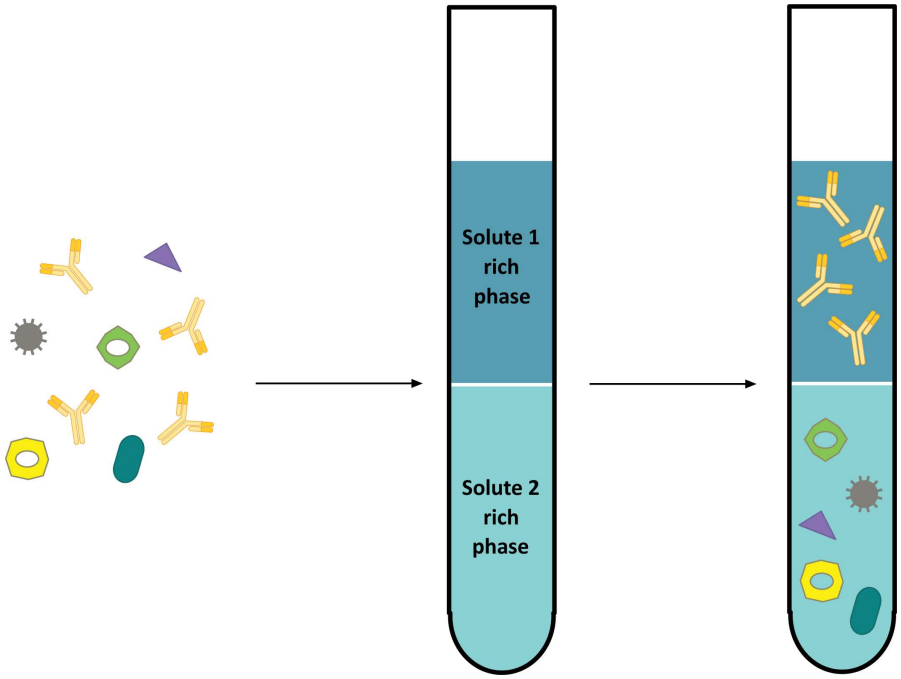


Fig. (11). Schematic representation of the extraction/purification of antibodies using ATPS.

The first study that suggested the use of ATPS for antibody purification was conducted by Andrews *et al.* [170], in 1990, using a proA-modified PEG molecule. However, the costs of this ligand impeded the approach proposed to be used on a wider scale [170]. Later, in 1992, Sulk *et al.* [171] proposed an extraction procedure using an ATPS consisting of 5% of PEG 1540 and 22% of phosphate buffer coupled to a final purification step using thiophilic absorption chromatography. The IgG1 monoclonal antibody against horseradish peroxidase from hybridoma cell culture supernatant was recovered in the PEG-rich top phase, with a process overall yield of 71%, with 90% recovery in the ATPS step and a purification factor of 6.2. Latter, Andrews *et al.* [172] described a polymer/salt ATPS that allowed the recovery of a murine IgG1 from a hybridoma cell supernatant in two steps. The extraction of IgG to the PEG-rich phase was performed with an ATPS composed of 15% of PEG 1500, with a low concentration of phosphate and NaCl (14 and 12%, respectively) and at pH 5.5, with a recovery yield of 90% and a purification factor of 2.7. IgG was

subsequently reextracted to a fresh phosphate solution, and the most hydrophobic compounds, which partitioned alongside with IgG, were further removed by hydrophobic interaction chromatography, using an ammonium sulphate solution as the mobile phase. In the same line, Ziljstra *et al.* [173, 174] coupled triazine dye mimetic green to PEG in order to selectively recover IgG from hybridoma cells grown in the dextran-rich bottom phase. More recently, in 2015, Muendges *et al.* [175, 176] published two studies on the extraction and purification of IgG from CHO cell lines through a single-stage and by using a multi-stage aqueous two-phase extraction. In the single-stage approach, the authors [176] were able to extract IgG1 from a CHO cell culture supernatant with a system composed of PEG 2000 and sodium phosphate at pH 6 with an yield higher than 90% and purification factor up to 3.1. However, the authors found that it is necessary to carry out a multi-stage approach to achieve a higher purity of IgG1 [177].

More recently, Aires-Barros and co-workers [17, 155, 163, 178 - 191] reported the successful use of ATPS for the extraction and purification of antibodies from different supernatants of cell lines, namely hybridoma and CHO cells. Table **7** presents a comparison between the performance of different ATPS in terms of recovery yields and purity levels studied by Aires-Barros and co-workers [17, 155, 163, 178 - 191]. ATPS based on PEG/phosphate [163, 178 - 181, 190], PEG/sodium citrate [182, 183], PEG/dextran [17, 155, 184 - 188, 191], and UCON/dextran [189], have been used for the extraction and purification of antibodies from real matrices.

Table 7. ATPS investigated by Aires-Barros and co-workers for the extraction and purification of antibodies, compared in terms of system performance, namely IgG recovery yield and purity level.

Composition of ATPS	IgG origin	Recovery yield (%)	Purity level (%)	Ref.
8% PEG 3350 + 10% phosphate + 15% NaCl, pH 6	AM	76	100	[163]
12% PEG 6000 + 10% phosphate + 15% NaCl, pH 6	CHO	88	PF = 4.3	[178]
12% PEG 6000 + 10% phosphate + 15% NaCl, pH 6	HYB	90	PF = 4.1	[178]
8% PEG 3350 + 10% phosphate + 10% NaCl, pH 6 5 steps: ATPS + 4 countercurrent steps	CHO	89	75	[179]
PEG 3350 + phosphate Pilot scale with continuous extraction using ATPS in countercurrent	CHO	85	50	[180]
PEG 3350 + phosphate + NaCl, pH 6 3 steps: extraction, reextraction and washing	CHO	80	97	[190]
PEG 3350 + phosphate + NaCl, pH 6 3 steps: extraction, reextraction and washing	PER.C6	100	97	[190]

(Table 7) contd.....

Composition of ATPS	IgG origin	Recovery yield (%)	Purity level (%)	Ref.
10% PEG 3350 + 12% sodium citrate, pH 6 3 steps: ATPS + HIC + SEC	CHO	90	100	[182]
8% PEG 3350 + 8% sodium citrate + 15% NaCl, pH 6 2 steps: extraction, back-extraction	HYB	99	76	[183]
PEG 150-GA + dextran 500000	CHO	93	PF = 1.9	[185]
10% PEG 3350-GA + 5% dextran 500000 2 steps: ATPS + CEX	CHO	73	91	[187]
8% PEG 3350-GA + 5% dextran 500000 + 10mM potassium phosphate, pH 7	CHO	97	94	[186]
8% UCON 2000 + 6% dextran 500000 + 20% TEG-GA 2 steps: extraction and reextraction	CHO	85	88	[184]
7% PEG 3350 + 5% dextran 500000 + 1.3% TEG-GA, pH 4	CHO	96	43	[188]
7% PEG 3350 + 5% dextran 500000 + 1.3% TEG-GA, pH 4 5 steps: ATPS + 4 countercurrent steps	CHO	95	85	[188]
8% UCON 50HB-3520 + 5% dextran 500000, pH 5 2 steps: extraction and reextraction	-	82	-	[189]
7% PEG 3350 + 5% dextran 500000 + 300 mM NaCl, pH 3	HYB	72	-	[17]
7% PEG 6000 + 5% dextran 500000 + 150 mM NaCl, pH 3	HYB	84	-	[17]
8% PEG 3350 + 5% dextran 500000 + 200 mM NaCl + GA-APBA-MP	CHO	92	98	[155]
7% PEG 3350 + 5% dextran 500000 + LYTAG-Z	HYB	89	42	[191]

Legend: ATPS – aqueous two-phase system; AEX – anion exchange chromatography; AM – artificial mixture; CEX – cation exchange chromatography; CHO – chinese hamster ovary cells; GA – diglutaric acid (COOH); GA-APBA-MP – gum arabic coated particles modified with aminophenyl boronic acid; HIC – hydrophobic interaction chromatography; HYB – hybridoma cells; LYTAG-Z – choline binding polypeptide tag fused to the synthetic antibody binding Z-domain; PEG – polyethylene glycol; PER.C6 – human embryonic cells derived from retinoblasts; PF – purification factor; SEC – size-exclusion chromatography; TEG-GA – triethylene glycol diglutaric acid; UCON – ethylene oxide/propylene oxide.

The addition of NaCl to a PEG/phosphate-based ATPS enabled the separation of IgG from an artificial mixture of albumin and myoglobin [163], and the purification of IgG from both hybridoma and CHO cell supernatants [178]. Using this type of systems the authors simulated a countercurrent chromatography process to separate IgG from the remaining impurities [179]. In addition, this type of ATPS has already been tested on a pilot scale and showed to be equally efficient [180]. On the other hand, this ATPS was also incorporated into a microfluidic platform proving its reduction to the microscale [181]. The use of a

battery of mixer-settlers separators to carry out a continuous multi-stage extraction of IgG from supernatants of two different types of cell lines was also investigated, and where promising results were obtained [190]. Essentially due to the concerns associated with the use of phosphate salts, other alternatives have been studied, namely ATPS consisting of PEG and sodium citrate, in the presence and absence of NaCl, for the recovery of human antibodies from both a hybridoma and a CHO cell supernatants [182, 183]. The authors [182] demonstrated that ATPS can be integrated in a process involving HIC and size-exclusion chromatography (SEC), and that it is also possible to apply a back-extraction/purification step that allows not only an improvement on the IgG purification but also allows the separation of the antibody from the polymer [183].

In order to improve the IgG specificity to the PEG-rich phase in PEG/dextran ATPS, PEG functionalized with various ligands [185 - 187] or including some additives, such as triethylene glycol-diglutaric acid (TEG-AG) or a choline binding polypeptide tag fused to the synthetic antibody binding Z-domain (LYTAG-Z), have been studied [184, 188, 191]. With this approach, an increase the systems selectivity for IgG and higher yields of extraction have been obtained. Included in the versatility of the techniques applied for the purification of IgG, a hybrid process combining ATPS with magnetic separation was also developed [155].

In order to be applied and administered to humans, the extracted and purified antibodies must have an exceptional purity degree. According to the CFR-Code of Federal Regulations, purity means the relative freedom of foreign matter in the final product, not being harmful to human use or harmful to the product [192]. Therefore, impurities must be kept to minimum levels in order to minimize the associated risks, in particular immunogenic problems that may arise from the presence of those contaminants. The systems composed of 8% of PEG 3350, 10% of phosphate buffer with 15% of NaCl [163], and 10% of PEG 3350 with 12% of sodium citrate [182], are quite relevant in this line since they allow to obtain 100% purity of IgG. However, it is important to note that the first system [163] aimed at purifying IgG from an artificial mixture containing IgG, albumin and myoglobin (the major protein impurities present in serum-containing cell culture supernatants), so it is difficult to extrapolate this result to the reality, since a real matrix is much more complex. In the second system [182], 100% purity was reached as the result of 3 extraction steps: 1 step with ATPS and 2 subsequent chromatographic steps (HIC and SEC). This result, when compared to the 3-step chromatographic platform [99] presented in Table **5**, which allowed the recovery of 85% of IgG with a purity higher than 99%, is more promising since it reduces one chromatographic step, allowing even higher yields of IgG (90%) with a comparable purity level. Comparing these results with the isolated use of HIC

[104], it is found that it is not possible to reach the same level of purity, since the authors have shown only 97%, and, despite being a high purity level, it is not able to compete with the current established platform. In this context, ATPS appears to be an interesting alternative that deserves to be explored in more detail, since excellent results were already attained.

Another advantage of ATPS is that clarification, purification and concentration can be integrated in a single step. In fact, the productivity, yield and economy of bioprocesses can be considerably improved by process integration, facilitating the development of scalable and efficient bioprocesses [193]. Thus, there is nowadays a strong demand for intensification and integration of process steps to increase yield, reduce the process time and cut down in running costs and capital expenditure. Besides these advantages, integrated processes still wait for broad industrial application, since it has a complex development, and there is a need for detailed process knowledge of the applicant [194]. Efforts have been made in this line, and there are already several works described in the literature that report the integration of clarification, capture, purification and concentration in one ATPS step [17, 191, 195, 196]. Platis *et al.* [196] successfully developed and optimized an ATPS composed of 12% of PEG 1500 and 13% of phosphate buffer at pH 5 for the purification of mAb 2F5. Through the incorporation of this system in a downstream processing protocol, the authors [196] achieved a clarification of the plant extract, removal of the plant derived compounds, such as phenolics and alkaloids, and partial purification of the antibody (3–4-fold purification, with 95% recovery at the bottom phase). Later, the same authors [195] used a system composed of 13.1% of PEG 1500 and 12.5% of phosphate buffer at pH 5 for the purification of anti-HIV mAbs 2G12 and 4E10 from unclarified transgenic tobacco crude extracts. Both mAbs partitioned to the bottom salt-rich phase with 85 and 84% yield and 2.4- and 2.1-fold purification factor, respectively. Furthermore, the ATPS was integrated in an affinity-based purification protocol, using proA, yielding antibodies of high purity and yield [195]. Therefore, a simple and effective way for the bioprocessing of therapeutic antibodies in two steps (ATPS + affinity chromatography) was proposed by the authors, suitable for analytical or clinical purposes. Also Silva *et al.* [17] proposed an ATPS composed of 7% of PEG 6000, 5% of dextran 500,000 and 150 mM of NaCl at pH 3, able to recover approximately 84% of IgG with only 0.1% of cells in the top phase, phase in which the antibody was retained, and with a clearance of cells higher than 99.8% . More recently, Campos-Pinto *et al.* [191] proposed a strategy to integrate the clarification and the primary recovery of mAbs from a complex medium containing Hybridoma cells, based on the use of a single-step ATPS composed of 7% of PEG 3350 and 6% of dextran 500,000 and the dual tag ligand LYTAG-Z. Based on the capacity of this system to tolerate solid components/impurities, allowing the simultaneous clarification of the media, purification and

concentration, ATPS are thus promising downstream processed when compared, for example, with chromatography, that requires a limpid and clear solution to be loaded.

Other researchers have also recently suggested new methods for the separation and purification of IgG from various non-conventional matrices. Rito-Palomares *et al.* [197] studied ATPS consisting of PEG 1000 and phosphate buffer at pH 9 for bovine blood processing in an approach comprising two steps: extraction and reextraction. Through this approach, the authors [197] verified that soluble proteins such as bovine serum albumin (BSA), haemoglobin and IgG partition into the PEG-rich top phase, while the cell debris (such as clotting factors and blood cells) are partitioned mostly into the salt-rich bottom phase. A back extraction of the soluble protein into a second phosphate-rich bottom phase resulted in a maximum overall protein recovery of 62%, and the authors [197] proved the recycling of the PEG-rich phase up to 5 cycles. It is also possible to highlight the investigations of Vargas *et al.* [198] that reported a new method of plasma fractionation using an ATPS formed by PEG 3350, potassium phosphate and NaCl at pH 6. Wu *et al.* [199] found that the system consisting of 12% of PEG 4000, 18% of hydroxypropyl starch (HPS) and 10% of NaCl at pH 8 is promising for the primary recovery of IgG from a HSA containing feedstock, attaining an yield of 99.2% with a purification factor of 5.28 in a single step. With the addition of a back extraction step the authors [199] reported a 84.0% yield with a purification factor of 5.73. More recently, Freire and co-workers [200] investigated the use of ATPS formed by bio-based ionic liquids (ILs), composed of ions derived from natural sources, and biocompatible polymers for the extraction and purification of IgG from rabbit serum. The authors [200] reported the recovery of *ca.* 85% of antibodies with a 58% enhancement in the IgG purity when compared with its purity in serum samples. The same research group [201] also reported the use of ILs as adjuvants in the formation of ATPS composed of polyethylene glycol and a buffered salt. The best results were attained with a system composed of 25% of PEG 400, 25% of $C_6H_5K_3O_7/C_6H_8O_7$ and 5% of 1-butyl-3-methylimidazolium acetate ($[C_4mim][CH_3CO_2]$) where the complete extraction of IgG in a single-step was achieved with the purity in the polymer-rich phase enhanced by *ca.* 37% as compared to the IL-free ATPS.

One of the major research groups that works directly within the monoclonal antibody purification area using ATPS is the Platis and Laubrou research group in Athens, which study transgenic plants, more specifically the tobacco plant [195, 196, 202]. Plant biotechnology has demonstrated that transgenic plants are suitable hosts for expressing recombinant biomolecules. Several reagent-grade recombinant proteins from transgenic corn (trypsin and avidin) [203, 204] and rice (lysozyme and lactoferin) [205, 206] have been commercialized, while

clinical trials for plant-derived therapeutic proteins (*e.g.* interferon alpha-2b) are underway [207]. Future progress in utilizing transgenic plants for biopharmaceuticals production will depend on the efficiency of the purification methods, since in those cases, purification steps also play the most significant fraction of the final products [208]. The tobacco plant has the same glycosylation pattern as humans, whereby the production of humanized monoclonal antibodies through this plant is possible and viable. The main benefits are the low production costs and the low risk of human contamination, since they are of plant origin. ATPS proved to be a beneficial system for the purification of plant proteins, which would otherwise have to resort to several chromatographic steps, namely ion exchange chromatography (IEX), molecular exclusion and affinity chromatography [202]. The authors [196] studied systems composed of PEG 1500 and phosphate buffer, which allowed the removal of potentially harmful secondary metabolites of the tobacco plant. Moreover, it was possible to couple this purification method, essentially for the removal of polyphenols, with CEX and metal affinity chromatography, increasing even more the purification levels [202], and there is already evidence of the selective partition of mAbs and plant cells fragments to opposite phases [195]. The purified mAbs were always analyzed by protein electrophoresis, ELISA and western blot, demonstrating the activity of mAbs and proving their purity concerning degraded variants, polyphenols and alkaloids [195, 196, 202].

In summary, some promising results have been achieved in the extraction/purification of IgG from various complex matrices, in particular from biological matrices, such as bovine blood, hybridoma and CHO cell culture supernatants, rabbit serum or even transgenic plants. The differences obtained with the various studies are related with the modification of the ATPS phase-forming components, their concentration, pH, presence or absence of additional ligands, addition of electrolytes and use of additional or combined/hybrid purification steps. It is however important to note that the need to resort to additional extraction/purification steps makes the process more complex and less cost-effective. In general, it is noticeable that one of the most used phase-forming components of ATPS is PEG, possibly due to its previously discussed biocompatibility characteristics. Nevertheless, the search on new affinity ligands, electrolytes and phase-forming components is very important since it can lead to significant improvements on the selectivity of ATPS for target biomolecules.

CONCLUDING REMARKS

This chapter provides a global vision on the current state-of-the-art regarding the manufacturing and possible applications of monoclonal antibodies, while highlighting new trends to improve their purification process. The most used

technologies for the production of mAbs were presented and discussed, with the mammalian cell technology established as the golden standard for the upstream processing of mAbs. In the downstream side, new and alternative process strategies are still under development, in order to reduce the number of steps in the isolation and purification process of mAbs aiming at turning the biotechnological process more competitive for the biopharmaceutical industries. Attempts to replace the golden-standard and expensive proA affinity chromatography from the process have been made, with the major goal of reducing the costs associated to the downstream processing of mAbs. Several chromatographic and non-chromatographic methods have been proposed to embrace the challenge of obtaining mAbs at a lower cost. Non-chromatographic techniques appear to be the most promising group of alternatives, since they generally allow to achieve high recovery yields and high purity levels, allied with simplicity, robustness, and lower energy consumption. In particular, ATPS can be highlighted as a promising non-chromatographic approach, since they are a simple technique, with a low cost associated, easy to operate and to apply on an industrial scale, with a high resolution capacity and able to provide a biocompatible environment. This technology allows the intensification of the downstream process, as clarification, purification and concentration can be integrated in a single step. Several promising works have already proved the efficiency of this technique concerning the extraction and/or purification of mAbs directly from biological matrices; however, it is evident that the study of new systems aiming the selective extraction and consequent purification of IgG should be performed by manipulating the ATPS components, the extraction conditions or the exploration of different electrolytes/ligands. It has been reported that using ATPS, the purification costs can be reduced by at least 39%, which emphasizes the potential of this technique [209]. All the works discussed in this chapter correspond to strategies proposed in recent years to overcome the major bottleneck in the processing of cost-effective mAbs. This information should encourage both the search on new and more efficient alternatives and the improvement of the existent technologies in order to boost the manufacturing process and the widespread use of mAbs as conventional therapies.

CONFLICT OF INTEREST

The authors (editor) declare no conflict of interest, financial or otherwise.

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