Purification of Antibodies by Affinity Chromatography

Afinite Kromatografisi ile Antibadi Saflaştırılması

Review Article / Derleme

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ABSTRACT

Antibodies are becoming a dominant protein class for various human therapeutical applications and they represent the largest number of molecules in clinical trials today. Antibodies are purified using a combination of various purification techniques. The most popular technique is affinity chromatography based on specific interaction of immunoglobulin G (IgG) with affinity ligand. Affinity chromatography has several advantages since it is an easy, fast and selective procedure for capturing the target antibody. This review aims at describing different affinity techniques for purification of antibodies and reports the contributions of scientists working in this field and their important results. The main issues discussed in this review are: protein A affinity chromatography, histidine-ligand affinity chromatography, immobilized metal-chelate affinity chromatography, dye-ligand affinity chromatography, biomimetic affinity chromatography, negative chromatography, mixed-mode affinity chromatography, thiophilic chromatography, magnetically stabilized fluidized beds and aqueous two phase systems.

Key Words
Affinity chromatography, immunoglobulins, antibodies, antibody purification.

ÖZET

Antibadiler tedavi amaçlı uygulamalarda yoğun olarak kullanılan bir protein türüdür. Antibadiler değişik saflaştırma tekniklerinin bir arada kullanımı ile saflaştırılırlar. En yaygın kullanılan saflaştırma tekniği immunoglobulin G'nin (IgG) afinite ligand ile seçici etkileşimine dayanan afinite kromatografi tekniğidir. Afinite kromatografisinin kolay, hızlı ve hedef molekülü seçici olarak yakalama gibi birçok avantajları söz konusudur. Bu derleme makalede son yıllarda antibadi saflaştırma amacıyla kullanılan farklı afinite teknikleri ve elde edilen önemli sonuçları verilmiştir. Bu kapsamda temel ayırma teknikleri olarak; protein afinite kromatografisi, boya-ligand afinite kromatografisi, immobilize metal-şelat afinite kromatografisi, karışık-mod afinite kromatografisi, tiyofilik kromatografi, manyetik olarak kararlaştırılmış akışkan yataklar ve sulu iki fazlı sistemler ve önemli sonuçları tartışılmıştır.

Anahtar Kelimeler
Afinite kromatografisi, immunoglobulinler, antibadiler, antibadi saflaştırılması.

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INTRODUCTION

Antibodies or immunoglobulins represent glycoproteins having carbohydrate-recognition motifs and they play an important role in biochemistry, biotechnology and biotherapeutics [1]. Antibody based biotherapeutics and in-vivo diagnostics are gaining wider approval from many health authorities all over the world [2]. Monoclonal antibodies represent the fastest growing biopharmaceutical market segment with a potential to reach a total global sale of 50 billion USD per year [3]. Approximately, 30% of the biotherapeutic agents presently used in clinical trials comprises antibodies and Fc-fusion proteins [4]. Currently, 24 monoclonal antibodies, 2 monoclonal antibody fragments and 4 Fc fusion proteins have been approved for therapeutic use and over yet 150 are under clinical developement [5]. Immunoglobulin G (IgG) purified from human serum is frequently used to treat a variety of disorders such as primary and secondary immuno-deficiencies, infections, inflammatory and autoimmune diseases [6]. The administration of human IgG products has not only improved the quality of life, but also saved lives in various lethal instances. A large number of IgG products being under development necessitates certain protocols for standardization [7]. But the large quantities in which some of them will be required puts considerable economic pressure on both the current methods and the facilities required (Figure 1). The worldwide consumption of human IgG nearly tripled between 1992 and 2003 from 19.4 to 52.6 tons [8].

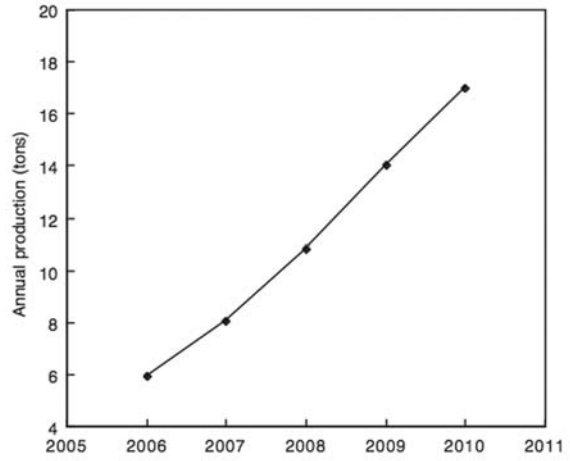

Figure 1. Annual production of monoclonal antibodies [5].

AFFINITY CHROMATOGRAPHY

Affinity chromatography is a method in which biospecific and reversible interactions are used for the identification, separation and purification of bioactive materials from crude samples (Figure 2) [9]. In affinity chromatography, a ligand molecule with a specific recognition ability is immobilized onto a suitable carrier, which is usually a polymer based material. The molecule to be purified is selectively captured by the ligand bound to the carrier by simply passing a solution of the target moleculess through the chromatographic column under favorable conditions. The target molecules are then eluted by using proper elutants, including specific solvents and / or free ligands, at optimal conditions such as pH, ionic strength, temperature, so that the interactions between the ligand and the target are broken and the target molecules are obtained in a purified form.

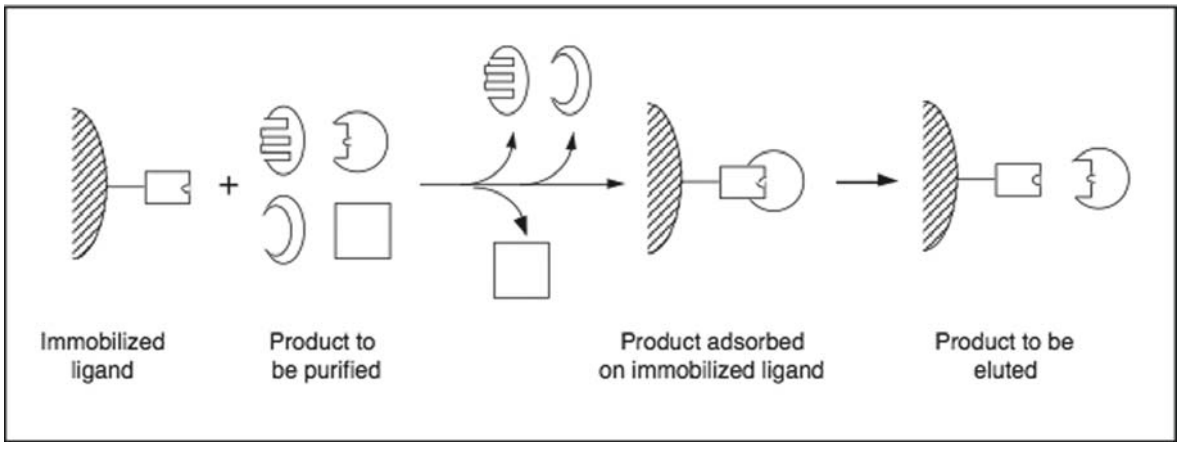

Figure 2. Principle of affinity chromatography.

Table 1. Various techniques derived from affinity chromatography [9].

Affinity capillary electrophoresis
Immunoaffinity chromatography
Affinity electrophoresis
Lectin affinity chromatography
Affinity partioning
Library-derived affinity ligands
Affinity precipitation
Membrane affinity chromatography
Affinity repulsion chromatography
Metal-chelate affinity chromatography
Affinity tag chromatography
Molecular imprinting technique
Avitin-biotin immobilized syttem
Perfusion affinity chromatography
Covalent affinity chromatography
Protein A affinity chromatography
Dye affinity chromatography
Receptor affinity chromatography
High performance affinity chromatography
Tandem affinity purification
Histidine affinity chromatography
Thiophilic chromatography
Hydrophobic interaction chromatography
Weak affinity chromatography

The biorecognition "affinity" based approach was introduced in 1968 by Cuatrecasas, Wilchek and Anfinsen [10] to purify proteins, and today it still represents one of the most powerful techniques available for purification of bioactive materials [11-14]. The general biorecognition based approach was subsequently adopted for a variety of other affinity techniques (Table 1).

Purification of Antibodies

Several chromatographic techniques including protein A affinity chromatography, high performance liquid affinity chromatography, size exclusion chromatography, ion-exchange chromatography, hydrophobic interaction chromatography, hydroxyapatite chromatography and thiophilic adsorption have been used for the purification of IgGs [15-18]. Extensive effort has been spent on examining alternative methods and ligands with varying selectivity and complexity. In general, the more simple ligand, the more stable it is to harsh chemical procedures for cleaning, but with simplicity comes a lower degree of selectivity. Affinity ligands can be classified into biospecific and pseudospecific ligands. A general comparison of biospecific and pseudospecific ligands for antibody purification is presented in Table 2.

Staphylococcal protein A is one of the first molecules discovered IgG binding molecule and has been extensively used as biospecific ligand during the past decade. Nowadays, Protein A chromatography persists as the most common technique for antibody purification in large scales. However, as a result of their exceptional stability and selectivity allied to a low cost of production and low toxicity, it is to be expected that engineered pseudospecific ligands, tailored to specific needs, will gain importance in the near future, for both small and large-scale purification of native and recombinant antibodies.

Protein A Affinity Chromatography

There are a number of naturally IgG-binding proteins that have been described (Table 3). Of these, the most significant is protein A ligand which is a cell wall associated protein domain exposed on the surface of the gram-positive bacterium *Staphylococcus aureus* [20]. Protein A consists of a single polypeptide chain, molecular weigth 42000 and does not contain significant amounts of carbohydrate. It does however binds with different affinity to the Fc region of IgGs from a variety of sources, e.g. it binds to IgG from human, rabbit and pig with high affinity, binds horse and cow IgG with lower affinity and binds rat IgG only a very weakly region [21].

Table 2. Comparison of biospecific and pseudospecific ligands for antibody purification [19].

	Biospecific	Pseudospecific
Specificity	High	Low to medium
Selectivity	Very high	Low to medium
Capacity	Low	High
Elution	Difficult	Mild
Stability	Low	Very high
Toxicity	High	Low
Cost	High	Low

Table 3. IgG binding proteins from bacteria [7].

Protein	Source	Binding
Protein A	*Staphylococcus aureus*	Strong, Fc region
Protein G	Groups C and G streptococci	Strong, Fc region
Protein L	*Peptococcus magnus*	Binds to κ light chains
Protein P	*Closridium perfringens*	Binds to κ light chains
Protein D	*Branhamella catarrhalis*	IgG, Binds small amounts of IgG
Protein P	Groups A streptococci	IgA, Binds weakly to IgG

The interaction appears to be characterized by hydrophobic interaction together with some hydrogen bonds and salt bridges. Protein A consists of five homologous IgG binding domains. Each of five domains in protein A is arranged in an anti-parallel there α-helical bundle and the three dimensional structure is stabilized via a hydrophobic core [22]. Protein A binds two moles of IgG per mole protein A. It exhibits a very high specificity and can therefore be employed as a one-step procedure for the purification of IgGs [23]. Main disadvantage of protein A is that there is always a small degree of leakage of the protein ligand so that for therapeutic products additional purification steps are required. In addition, protein A carriers are expensive and difficult to handle, sterilize and preserve [24].

In addition to increased capacity and throughput, the other major concern for protein A carriers is their stability to cleaning agents. A new protein A resin is now available, where the asparagines residues in protein A have been engineered out, thus reducing deamidation under alkaline conditions, while maintaining the ability to bind IgG [25]. This and the development of more efficient protocols for regeneration of carriers, have increased the resins stability to base sanitizing solutions. Cleaning regimes consist of alternating cycles of low concentrations of NaOH and salt, resulting in the resin maintaining good yield out to 300 cycles [26], a significant improvement over the earlier protein A-Sepharose where the yield of IgG dropped to 50% after 300 cycles. It is interesting to compare the performance of various protein A affinity carriers for IgG purification (Table 4).

Table 4. IgG adsorption capacities for various protein A carriers.

Carrier	Protein A Loading	Q_{max} (mg/g)	[R]
Eupergit, Affigel	4.1 mg/mL	20.1	[23]
Cellulose beads	2.0 mg/mL	11.7	[27]
Cellulose nanofibers	30 mg/g	18.0	[28]
PHEMA beads	2.7 mg/g	24.0	[29]
Poly(caprolactam)	-	28.3	[30]
Polysulfone hollow fiber	3.1 mg/g	8.8	[31]
Sartobind-epoxy beads	5.9 mg/g	5.1	[32]
Poly(methyl methacrylate)	-	6.6	[33]
Naylon membrane	4.7 mg/mL	13.2	[34]
PHEMA cryogel	56 mg/g	98.7	[35]
Polyethersulfone-chitosan	6.4 mg/mL	41.1	[36]
Cellulose and acrylic composite	8.0 mg/g	15.0	[37]
PHEMA membrane	4.7 mg/g	9.8	[38]
Poly(GMA-EDMA)	5.9 mg/g	10.6	[39]
Cellulose fiber	6.0 mg/g	23.8	[40]
Agarose beads	-	36.1	[41]
Magnetic poly(MA-DVB)	24 mg/g	22.0	[42]
Chitosan-cellulose composites	6.3 mg/g	33.2	[43]
Poly(ether-urethane urea)	3.2 mg/g	2.7	[44]
Polyethersulfone	3.0 mg/g	11.4	[45]
Cellulose-GMA membrane	3.3 mg/g	15.3	[46]

Histidine-ligand Affinity Chromatography

Pseudospecific ligands such as histidine, tryptophan, phenylalanine etc. can be used to purify a wide range of biomolecules [47]. They are small molecules with higher chemical and physical stability and lower cost. Hydrophobic amino acids tryptophan and phenylalanine with their predominant aromatic stacking properties, was shown to bind selectively proteins rich in aromatic residues [48]. Histidine was used as an affinity ligand in the purification of IgG [49]. The adsorption mechanism between histidine and IgG is based in several molecular interactions

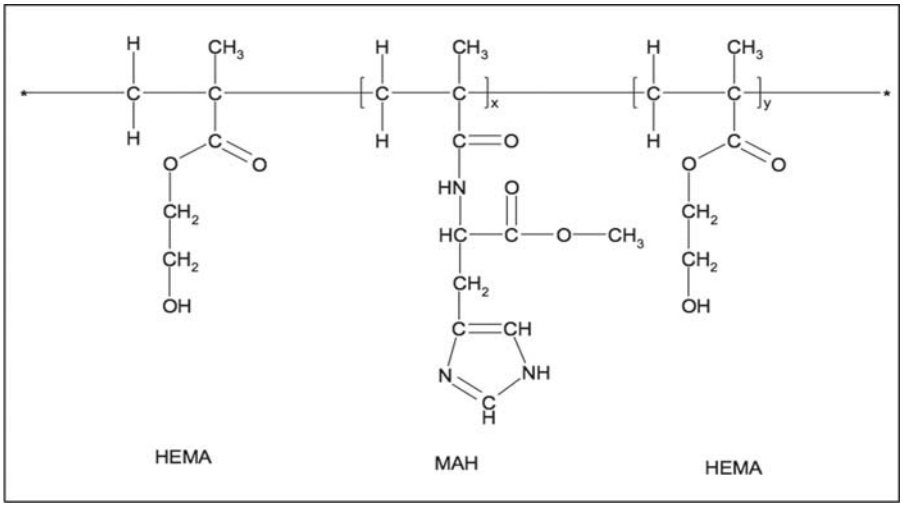

Figure 3. The molecular structure of PHEMAH.

such as hydrogen bonding, electrostatic and mild hydrophobicity, etc. The different physicochemical properties of histidine are because of the non-symmetric arrangement of its carbon atoms and its broad pKa range. Histidine is also characterized by its hydrophobicity and its capacity to transfer charge due to its imidazole ring. These properties, in addition to its role in the acid-base system, enable histidine to interact with its microenvironment by different mechanisms, for example as neighboring amino acid, pH, ionic strength and temperature [50].

Theoretical considerations and understanding of the interactions between protein molecules and the histidine coupled carrier have shown the mechanism to be water-mediated. This would involve changes in dielectric constant at the adsorption interface owing to the combined electrostatic, hydrophobic, and charge-transfer interactions between histidine and the specific amino acid residues available on the protein surface [51].

El-Kak et al used histidyl-bisoxirane-Sepharose gel, for separation of IgG_1, IgG_2 and IgG_3, three subclasses of human IgG, from human serum. They also studied the adsorption of IgG from a variety of animal species [52]. The high recovery and purification on histidyl-bisoxirane-Sepharose gel of IgG from all the sources tested compared well with results obtained by use of protein A-Sepharose gel.

The development of new carriers that capture at high capacities directly from plasma has not been abandoned so far. A series by Denizli and co-workers takes an extensive look at histidine carrying affinity supports [53-57]. Çanak et al. developed poly(2-hydroxyethyl methacrylate) beads with L-histidine as ligand [53]. IgG is adsorbed from human plasma in concentration of 196 mg/g carrier at a purity of 92%. IgG binds via Fab part and is eluted with 1 M NaCl, pH 4. Unfortunately, the binding behaviour was not flow independent and thus led to low binding capacities at reasonable flow-rates.

Denizli et al developed a new approach to obtain an efficient and cost effective purification of IgG from human plasma. Porous monolith was obtained by the bulk polymerization of 2-hydroxyethyl methacrylate (HEMA) and N-methacryloyl-(L)-histidine-methylester (MAH). Monoliths are an attractive new generation materials for purification of large molecules because they exhibit very low

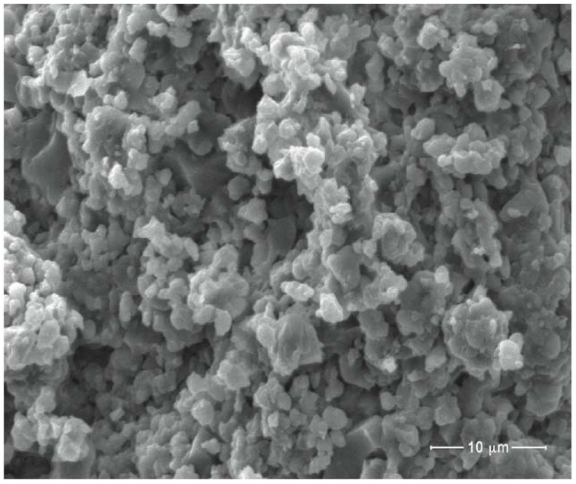

Figure 4. SEM photographs of PHEMAH monolith [57].

back pressure even at high flow rates and flow-unaffected binding properties [58]. Molecular structure and SEM photograph of PHEMAH monolith were given in Figures 3 and 4. It can be clearly seen that the PHEMAH monolith is composed of much smaller particles. The particles are 2 μm in size and irregular. The size of the large pores between clusters is 1 μm. There are also many pores whose diameter is 2 μm on the bulk structure of the monolith. These macropores reduce diffusional mass transfer resistance and facilitate convective transport. So the PHEMAH monolith had good flow properties. IgG adsorption amount from human plasma was obtained up to 96.5 mg/g with a purity of 95.3%. It was observed that IgG could be repeatedly adsorbed and eluted without significant loss in the adsorption capacity. The time consuming and high cost of ligand binding step has inspired a search for suitable low-cost carriers. This approach has many advantages over conventional techniques. An expensive and critical step in the preparation of affinity carrier is binding of an affinity ligand to the carrier. In this work, MAH group in polymer chain directly served as pseudo-specific ligand, and there is no need to activate the carrier for the ligand binding. Another problem is that of slow release of this covalently bonded ligands off the carrier. Ligand release is a general problem encountered in any affinity adsorption technique which caused a decrease in binding capacity. Ligand leakage also causes contaminations that will interfere with analysis of the purified biomolecule. Ligand binding step was eliminated in this approach. MAH was polymerized with HEMA and there is no ligand leakage.

Sun et al developed a novel amino acid affinity membrane by coating hydroxyethylcellulose on poly(vinylidene fluoride) hollow fibers to increase the membrane hydrophilicity and then bound with 1,6-hexanediamine as the spacer arm [59]. L-phenylalanine, L-tryprophan and L-histidine were used as the hydrophobic ligands. Hollow fibers in the membrane module (0.0188 m^2 surface area) adsorbed 21 mg human γ-globulin from 10 ml of human plasma with a purity of 83.9% in a single-pass mode [59]. The results also showed that among the three affinity membranes investigated, the L-phenylalanine-affinity membrane appeared to be the most favorable for the adsorption of human γ-globulin. The IgG adsorption capacities on amino acid functionalized several affinity carriers given in literature are listed in Table 5.

Immobilized Metal-chelate Affinity Chromatography

Immobilized metal chelate affinity chromatography (IMAC) of proteins, with metal chelate linked to Sepharose, was introduced by Porath et al in 1975 and since then it has been adopted for the purification of many therapeutic proteins, peptides, nucleic acids, hormones and enzymes

Table 5. IgG adsorption capacities for various pseudospecific affinity carriers.

Carrier	Ligand	Ligand Loading	Q_{max} (mg/g)	[R]
Poly(GMA-EDMA)	L-Histidine	81.8 μmol/g	12.5	[39]
PHEMA monolith	Histidine	47.8 μmol/g	96.5	[57]
PHEMA beads	L-Histidine	-	44.8	[60]
Bentonite particles	L-Histidine	541.3 μmol/g	89.6	[61]
Poly(HEMA-EGDMA) beads	IMEO	36.6 mg/g	78.9	[62]
PHEMA nanoparticles	IMEO	64.5 mg/g	843.0	[63]
PHEMA nanoparticles	Phenylalanine	420 μmol/g	780.0	[64]
m-Poly(EGDMA)	Histidine	70 μmol/g	320.0	[65]
Poly(ethylene vinyl alcohol)	L-Histidine	70 μmol/g	17.0	[66]
Poly(ethylene vinyl alcohol)	BDGE/L-Histidine	126 μmol/g	80.1	[67]
Polypropylene	L-Phenylalanine	20 mg/g	1.50	[68]
Polyethylene fiber	L-Phenylalanine	600 μmol/g	50.0	[69]
Poly(butadine-HEMA)	L-Histidine	4.2 μmol/g	23.6	[70]
Sepharose	Histidyl	65 μmol/g	10.5	[71]

Table 6. IgG adsorption capacities for various IMAC carriers.

Carrier	Ligand	Ligand Loading	Q_{max} (mg/g)	[R]
Poly(butadine-HEMA)	IDA/Cu^{2+}	196 μmol/g	39.3	[70]
Polymethylmethacrylate beads	Cu2+	39.5 μmol/g	54.3	[74]
CIM monolith	IDA/Cu^{2+}	-	15.4	[75]
	IDA/Cu^{2+}	-	8.5	
PHEMA beads	Cu^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+}	1.2 mmol/g	79.6	[76]
PHEMAH monolith	Cu^{2+}	16.3 μmol/g	104.2	[77]
Poly(GMA) beads	IDA/Cu^{2+}	628 μmol/g	171.2	[78]
Polyhydroxyethoxy) ethyl methacrylate	Ni^{2+}	785 μmol/g	2.0	[79]
Poly(GMA-EDMA) beads	Ni^{2+}	151 μmol/g	4.0	[80]
Poly(HEMA-GMA) beads	IDA/Ni^{2+}	490 μmol/g	8.4	[81]
	NTA/Ni^{2+}	600 μmol/g	6.4	
Poly(ethylene vinyl alcohol)	TREN/Ni^{2+}	114 μmol/g	204.6	[82]
Magnetic PEGDMA	MAH/Cu^{2+}	4.1 μmol/g	97.5	[83]
Poly(GMA-DEGMA-EDMA) terpolymer beads	IDA/Ni^{2+}	1580 μmol/g	1.5	[84]
Sepharose	TREN/Cu^{2+}	-	17.0	[85]
Alginate	Cu^{2+}	188 μmol/g	1.0	[86]

[72]. IMAC introduces a new approach for selectively interacting materials on the basis of their affinities for chelated metal ions. The separation is based on the interaction of a Lewis acid (electron pair acceptor), i.e., a chelated metal ion, with an electron donor atoms (N, O and S) on the surface of the protein. Proteins are assumed to interact mainly through the imidazole group of histidine and, to a lesser extent, the indoyl group of tryptophan and the thiol group of cysteine. Co-operation between neighboring amino acid side chains and local conformations play important roles in protein binding. Aromatic amino acids and the amino-terminal of the peptides also have some contributions [73].

Some IgGs show an innate affinity for metal ions due to a histidine-rich sequence. Recently, Denizli et al developed a novel IMAC carrier [74]. While IMAC conventionally uses a metal ion charged chelating group (which in turn is bound) it has been shown that Cu^{2+} ions directly charged poly(hydroxyethyl methacrylate-N-methacryloyl-(L)-histidine-methylester [PMMAH] (MAH consisting of imidazole groups) and this worked quite well as IMAC medium (Figure 5). This obviates the need for immobilizing any chelating groups like iminodiaceticacid (IDA). They reported that higher adsorption value was obtained from human plasma (up to 54.3 mg/g) with a purity of 90.7%. The IMAC beads allowed one-step separation of IgG from human plasma.

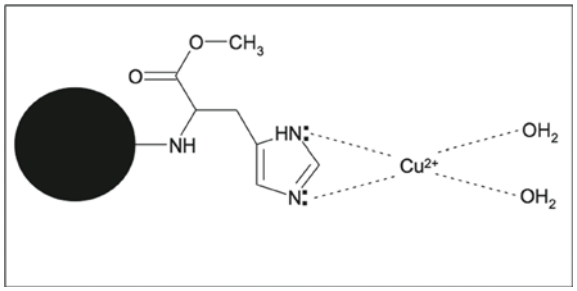

Figure 5. Chelation of Cu^{2+} ions through the PMMAH beads.

Prasanna et al successfully showed that Cu^{2+} and Ni^{2+} loaded CIM-IDA monolithic disk is a potential alternative for the purification of both polyclonal and monoclonal IgG [75]. Dynamic binding capacity at flow rate of 3 ml/min was estimated to be 15.4 and 8.5 mg of IgG/ml of support with Cu^{2+} and Ni^{2+} chelated CIM-IDA disk, respectively. Since monoliths are made from one piece of polymer resin, there is no need for column packing step. As a result of convective flow, dynamic binding capacities are higher even at higher flow-rates. The main advantage of the convective based system over the conventional gels is that they can be operated at high flow rates without compromising on the purity and the binding capacity of the product. Thus combining the advantages of monolithic disk with

pseudoaffinity ligand (metal ions), IgG from complex mixtures like human serum and mouse ascites fluid can be efficiently purified in a single step. IgG adsorption capacities of several IMAC carriers given in literature is listed in Table 6.

Recently, one interesting example for metal affinity devices is the particles embedded cryogels [87]. Cryogels are a very good alternative to bioseparation with many advantages including large pores, short diffusion path, low pressure drop and very short residence time [88-90]. But, due to the existing of large pores within the cryogel, the adsorption capacity for the biomolecules is low [91]. In actual bioseparation processes, it is of great importance to improve the binding capacity of supermacroporous cryogel. Therefore, particle embedding would be a useful improvement mode to use in the preparation of novel composite cryogels for increasing surface area [92-94]. This approach makes use of a combinatorial selection strategy to enhance adsorption capacity. Denizli and his coworkers reported the use of selective IgG adsorption with poly(hydroxyethyl methacrylate) cryogel with embedded poly(glycidyl methacrylate [PHEMA/PGMA-IDA-Cu^{2+} composite cryogel] [87]. The scanning electron photographs of PGMA beads, PHEMA and PGMA-IDA-Cu^{2+} embedded PHEMA cryogels were given in Figure 6. The presence of embedded beads can be seen clearly. IgG adsorption capacity of the PGMA-IDA-Cu embedded PHEMA cryogel from human serum was 257 mg/g.

Dye-ligand Affinity Chromatography

Textile dyes may also be used for antibody purification since they bind proteins in a selective and reversible manner [95]. Triazine reactive dyes are the most widely used ligands and they consist of polyaromatic sulphonated compounds containing a triazine reactive group, which facilitates their binding to insoluble matrices. Dye ligands can engage in ionic, hydrophobic, charge transfer and hydrogen bonding with proteins, establishing, most of the time, a mixed-mode interaction.

An older dye affinity application for IgG purification was achieved by Byfield et al [96]. A reactive dye, Remazol yellow GGL was attached to agarose beads by a sulphur-containing linker. This carrier was used for the purification of IgG from plasma. IgGs were adsorbed in the prsence of 20 mM phosphate buffer (pH 7.4) and were desorbed by increasing the ionic strength with 1 M NaCl. The adsorption capacity was 14 mg IgG/ml of resin with 40% recovery.

Wongchuphan et al investigated the applicability of dye ligands bound to an expanded bed chromatography quartz base carrier (Streamline)

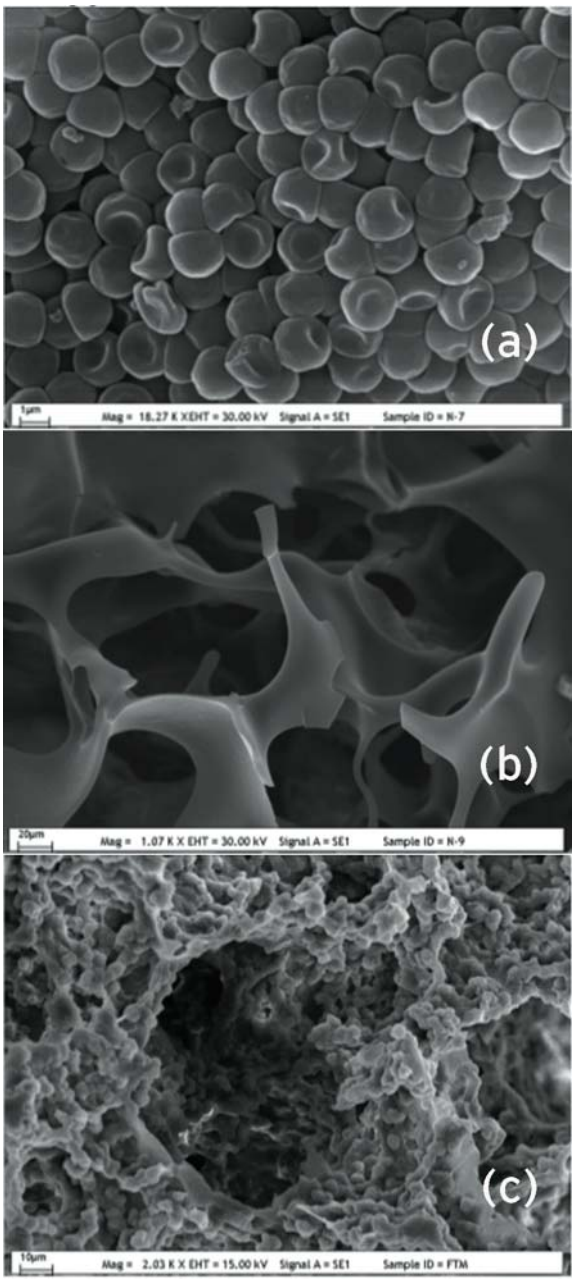

Figure 6. Scanning electron photographs of (A) PGMA beads; (B) PHEMA and (C) PGMA-IDA-Cu^{2+} embedded PHEMA cryogels.

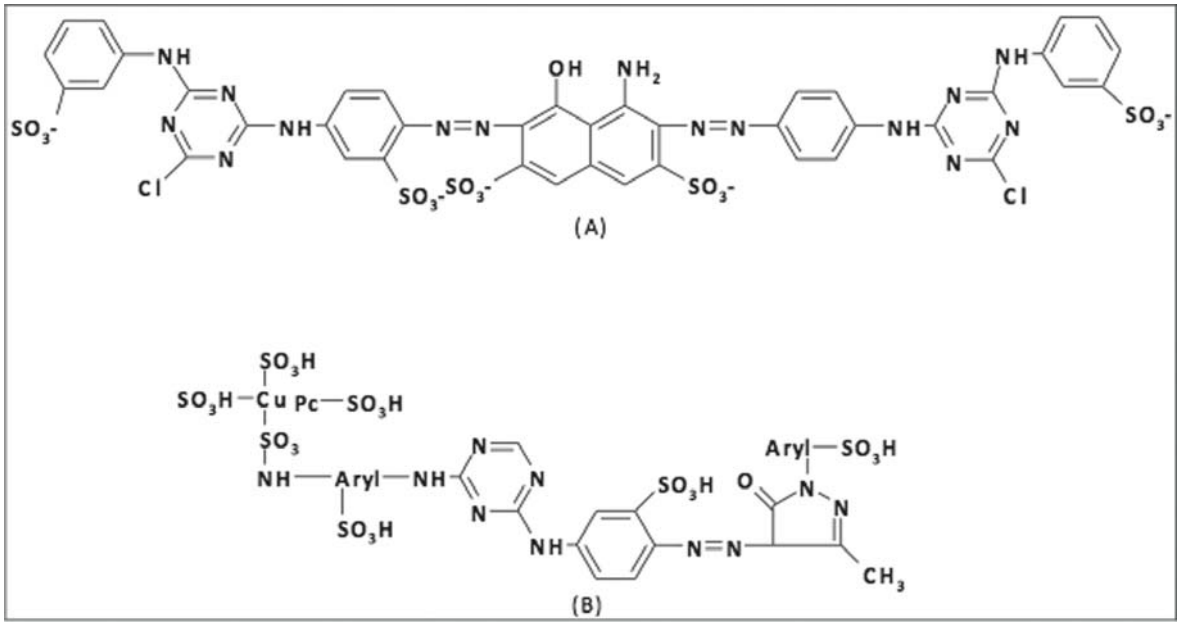

Figure 7. Molecular structure of Reactive Green 5 (A) and Reactive Green HE 4BD (B).

for the affinity separation of rabbit-IgG [97]. Reactive Green 5 bound onto carrier was selected for capturing rabbit-IgG. The molecular structure of Reactive Green 5 is indicated in Figure 7. The result indicated that Reactive Green 5-bound carrier has the highest adsorption efficiency for rabbit-IgG (49 mg adsorbed rabbit-IgG per ml carrier) at pH 7.0.

Human IgG is composed of four isotypic subclasses termed IgG_1, IgG_2, IgG_3 and IgG_4 [98]. One of the indications of IgG administration is to enhance resistance of the patient against infections [99]. Most often, IgGs directed to bacterial pathogens belong to the IgG_2 subclass. Hence, in situations of bacterial infections, it would be advantageous to use enriched IgG_2 preparations. At present, there is no established method for the preparation of IgG_2-enriched solutions suitable for human therapy. Yavuz et al prepared Reactive Green HE 4BD bound poly(hydroxypropyl methacrylate) [poly(HPMA)] gel beads for separation of IgG subclasses [100]. Molecular structure of Reactive Green HE-4BD was given in Figure 7. They reported that the IgG adsorption capacity of the dye-bound poly(HPMA) gel beads was determined for a commercially available IgG solution to be 4.2 mg/g for IgG_1, 64.5 mg/g for IgG_2, 7.1 mg/g for IgG_3, and 10.8 mg/g for IgG_4. The Reactive Green HE 4BD-bound poly(HPMA) beads have a significant adsorption capacity for IgG_2. The quantity of adsorbed IgG_2 is three times higher than the quantity of the other subclasses, IgG_1, IgG_3, and IgG_4. The same adsorption behaviour was observed when the albumin free human plasma used. The quantity of adsorbed IgG_2 is higher than the quantity of the other subclasses, IgG_1, IgG_3, and IgG_4. Adsorption capacities for albumin free human plasma were obtained as 6.4 mg/g for IgG_1, 67.8 mg/g for IgG_2, 5.2 mg/g for IgG_3, and 8.6 mg/g for IgG_4.

IgG binding chloroazine dyes offer good selectivity and high binding capacities as well as excellent chemical stability. These have been used to purify polyclonal IgG from ethanol precipitates from plasma [101]. Unfortunately, they bind additives such as phenol red and Pluronic® from cell culture fluids and as a result are not recommended for primary recovery. This does not render them ineffective, but does diminish their usefulness in the eyes of many users.

Biomimetic Affinity Chromatography

In order to further develop affinity chromatography for IgG, different peptide based ligands derived from combinatorial libraries have been designed and different approaches have been used for the construction. Compared to protein A, peptide ligands should display not only reduced production costs, but also increased resistance to chemical and biological attacks, reduction in the

amount of contaminants of biological nature, high capacity for large-scale purification. Fassina et al prepared a peptide library with the aim to mimic an IgG-binding domain from protein A [102]. By coupling of the peptides to a solid support, they showed that one of the new peptides (TG19318) was functional as affinity ligand for IgG purification. Under optimized conditions, antibody purity after affinity purification was close to 95% and maximal column capacity reached 25 mg IgG/ml support. Teng et al described a fully characterized IgG binding ligand comprizing a triazine scaffold substituted with 3-aminophenol and 4-amino-1-naphtol [103]. They showed that this synthetic protein A mimic ligand interacted with human IgG and could purify IgG selectively from diluted human plasma. They achieved adsorption capacities in the range of 26.8 mg per g moist gel. IgG was eluted with glycine-HCl buffer with a recovery of 67-69% and a high purity of 99.2. The bound ligand was able to withstand incubation in 1 M NaOH for 7 days without loss of binding capacity for IgG. Chhatre et al evaluated a prototype agarose based affinity carrier utilizing a bound synthetic ligand designed to replace protein A as an IgG affinity capture resin and compares its purification characteristics with four commercially available matrices for the recovery of polyclonal antibodies from crude hyperimmune ovine serum [104]. The novel carrier was found to show the highest dynamic capacity (29.2 mg/ml) of all matrices under evaluation-30% higher than the other commmercial carriers evaluated. IgG yield of over 85% and purities of over 90% were achieved consistently over multiple loading cycles. The high binding capacities and eluted IgG purities observed suggest that this synthetic ligand alternative to protein A may be suitable for the commercial scale purification of therapeutic monoclonal antibodies from mammalian cell culture.

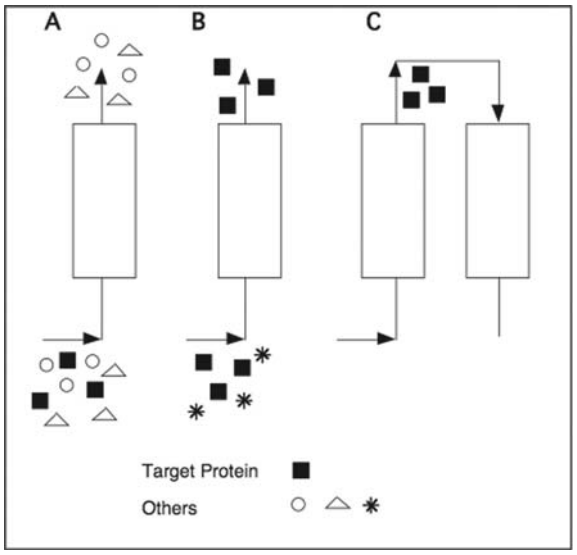

Figure 8. Alternative strategies for adsorption: (A) "positive binding"; (B) "negative binding"; and (C) two-step (negative and positive).

Negative Chromatography

The negative chromatography aims at allowing the product to pass through the column, retaining only the contaminants or impurities [105]. There are two alternative protocols that can be followed in the adsorption step (Figure 8). In the first protocol, which is the so-called positive binding, adsorbent carries a suitable ligand which specifically binds the target protein. Ideally only few contaminating proteins should bind to the column under optimized adsorption conditions. These non-specifically adsorbed contaminating proteins should be removed from the column by using flushing equilibrating buffer before the elution step. Alternatively, a negative binding protocol can be applied, in which contaminating proteins (other than the target protein molecules) or others are adsorbed in the column preferentially. Higher recoveries and purities can be achieved by a two-step process, in which the protein mixture is first passed through a negative binding column, and the effluent of this column is then directed to the second "positive" binding column. Negative chromatography aims to obtain the product in the flowthrough and washing steps, while the contaminants or impurities remain adsorbed and can be recovered in elution steps.

Recently, tris(2-aminoethyl)amine (TREN) bound agarose gel was evaluated for the purification of IgG from serum by negative chromatography [105]. The non-biological ligand TREN is a quadridentate chelating ligand with four nitogen atoms, three of which are primary in nature and the fourth one is tertiary. Due to its high amine content, TREN serve as an anion exchanger. A one-step IgG purification process allowed the recovery of 73.3% with purifty of 90-95%. The IgG binding capacity was relatively high (38.2 mg/ml).

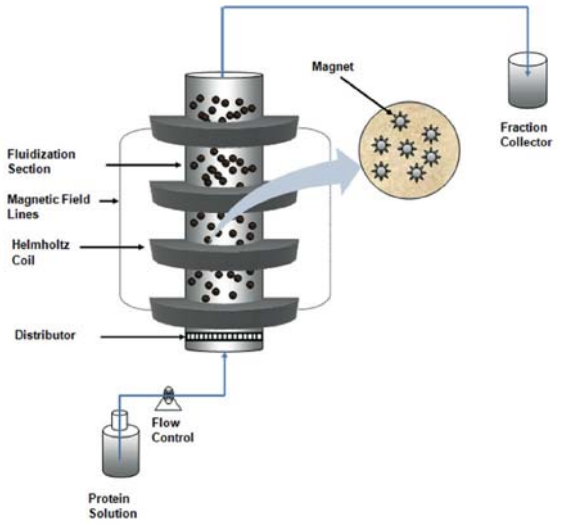

Figure 9. Schematic representation of MSFB system.

Bresolin et al demonstrated that it was possible to purify IgG using both human plasma and w-aminodecyl-agarose by applying the principle of negative chromatography in a single step [106]. The nature and the pH of the buffer influenced the adsorption of IgG and serum (or plasma) proteins due to the occurrence of mixed-mode (ion-exchange and hydrophobic) interactions. High purity IgG was obtained with a 75% recovery in Hepes 25 mmol/L pH 6.8 feeding human serum. IgG adsorption capacity of w-aminodecyl-agarose gel is 19.6 mg/ml. The results indicated that the use of w-aminodeycl-agarose is a potential technique for purification of IgG from human serum.

de Souza et al evaluated the feasibility of using the ligand aminohexyl bound on agarose gel for the purification of IgG from human plasma by negative chromatography [107]. They observed that the simple aliphatic amine aminohexyl binds IgG as the complex amine TREN. High purity IgG is obtained (100.9% IgG purity) when plasma was diluted 10 times. The binding capacity capacity of albumin was high and a positive cooperativity was observed for IgG binding.

Magnetically Stabilized Fluidized Beds

Magnetic materials are nowadays well-known and have been investigated intensively due to their potential applications in many areas, such as biology, medicine, colloid sciences [108-111]. The magnetic character implies that they respond to a magnet, making sampling and collection easier and faster, but their magnetization disappears once the magnetic field is removed. In addition, magnetic beads promises to solve many of the problems associated with chromatographic separations in packed bed and in conventional fluidized bed systems [112]. Advantages of magnetic columns according to conventional affinity chromatography include the efficient fluid-solid mass transfer properties, low pressure drop, good fluid-solid contact, elimination of clogging and continuous countercurrent operation [113]. Different chromatography based methodologies have been addressed as an attempt to circumvent such problems [114]. For example, both monolithic chromatography and magnetically stabilized fluidized beds (MSFB) are more efficient for processing viscous fluids, being suitable for antibody purification from cell extracts [115]. The main benefits of MSFB would reduce the number of steps required for product recovery by allowing direct capture of product from the cell suspension (Figure 9).

In a novel process integration, magnetic poly(EGDMA) carriers having MAH have been used for IgG purfication from human plasma in a MSFB [116]. This type of fluidized bed combines the best characteristics of both packed and fluidized beds, including efficient fluid-solid mass transfer properties, elimination of particle mixing, low pressure drop and elimination of clogging. They reported that higher amounts of IgG were adsorbed from human plasma (up to 320 mg/g) with a purity of 87%. IgG molecules could be repeatedly adsorbed and desorbed with these sorbents without noticeable loss in their IgG adsorption capacity. The magnetic carrier could be repeatedly used for ten cycles without significant loss in its binding capacity. In addition, MAH reduced immunogenicity, limited toxicity and no leakage from affinity carrier.

Öztürk et al used the magnetic poly(HEMA-EGDMA) beads (150–250 μm diameter in spherical form) carrying the pseudo-specific ligand, 3-(2-imidazoline-1-yl)propyl (triethoxy silane) (IMEO) (Figure 10) [62]. IgG purification was performed on a magnetically stabilized fluidized bed system using BioRad economic column (diameter: 1 cm, length: 10 cm). During the experiment, the magnetic beads in

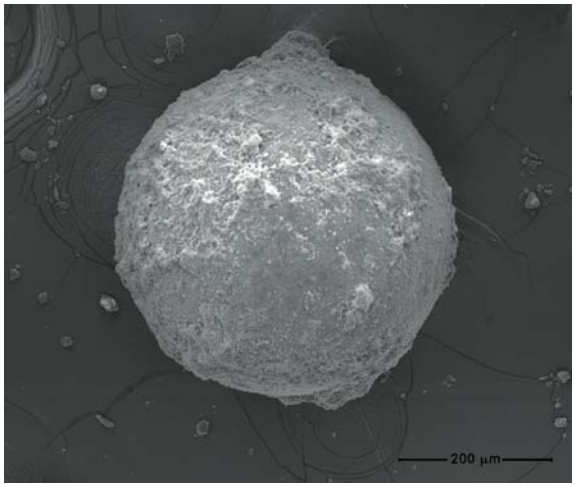

Figure 10. SEM of magnetic poly(HEMA-EGDMA) beads prepared by modified suspension polymerization method [62].

the column were exposed to magnetic field which surrounded the column ($B_{rms} \approx 24$ Gauss, $B_{p-p} \approx 33$ Gauss, $\phi = 50$ Hz). IMEO immobilized m-poly(HEMA-EGDMA) beads were used for the affinity adsorption of IgG from human plasma. The non-specific IgG adsorption onto the plain m-poly(HEMA-EGDMA) beads was very low (about 0.4 mg/g). Higher adsorption values (up to 55 mg/g) were obtained when the m-poly(HEMA-EGDMA)/IMEO beads were used from human plasma. The adsorption capacity from human plasma in magnetically stabilized fluidized bed decreased drastically from 78.9 mg/g to 19.6 mg/g with the increase of the flow rate from 0.1 ml/min to 3.5 ml/min.

Mixed-mode Ligands

Glycolipids such as gangliosides and glycosphingolipids carry out vital functions (i.e., signal transduction, cell recognition and cell proliferation) in biomembranes through carbohydrate interactions [117]. Some of these glycolipids exhibit a high affinity towards glycoproteins as a result of "multivalent of cluster effect" and thus focused on as a new affinity ligand for IgGs [118]. Im et al described for the first time that mannosylerythritol lipid (MEL-A), a yeast extracellular glycolipid, carrying poly(2-hydroxyethyl methacrylate) beads (50-150 µm in diameter) showed a significant binding affinity towards a natural polyclonal human-IgG [119]. The structure of MEL-A produced by *Candida antartica* is given in Figure 11. The binding affinity was nearly the same as that observed for ganglioside GM1. PHEMA itself showed no selective binding for hIgG. However, the bound amount of hIgG to the PHEMA-MEL-A increased drastically depending on the bound amount of MEL-A. The bound amount of hIgG (12.6 mg per g of polymer) was 2.7-fold higher than that of human serum albumin with the PHEMA-MEL-A.

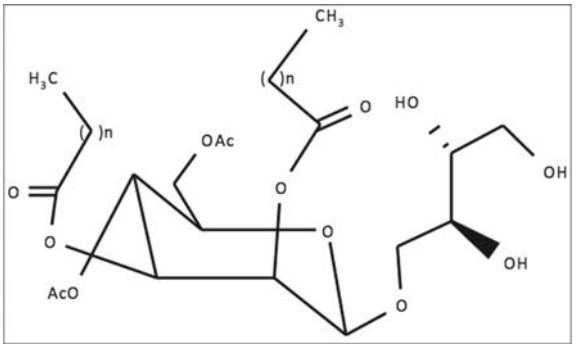

Figure 11. Structure of mannosylerythritol lipids produced by *Candida antartica* [118].

Thiophilic Affinity Adsorption

Thiophilic adsorption chromatography was developed by Porath et a. in the 1980s [120]. Hutchens and Porath have shown during thiophilic adsorption of proteins for both functional groups present in the ligand structure, thioether sulphur and the adjacent sulphone group, in a cooperative manner [121]. Recently, Qian et al explored a potential non-chromatographic

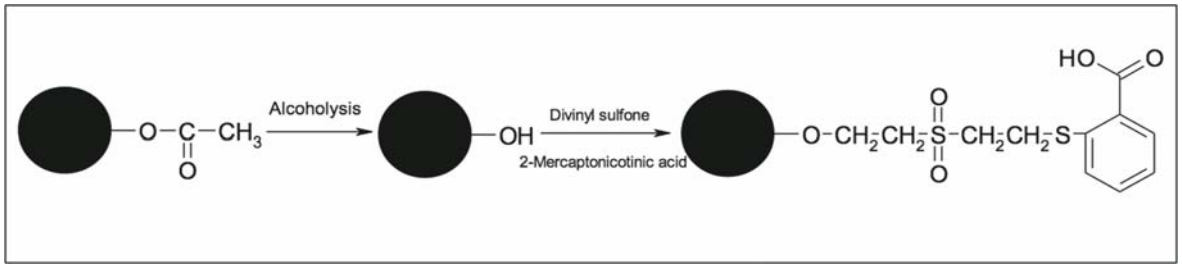

Figure 12. Preparation of thiophilic magnetic carriers.

Table 7. IgG adsorption capacities for various thiophilic carriers.

Carrier	Ligand	Q_{max}	[R]
Agarose	Divinylsulphone	6.3 mg/ml	[125]
	2-Mercapto ethanol	6.9 mg/ml	
Sepharose CL-6B	4-(1H-imidazol-1-yl)aniline	59.0 mg/ml	[126]
Cellulose beads	2-Mercapto-5-benzimidazolesulfonic acid	30.0 mg/ml	[127]
Poly(ethylene vinyl alcohol)	Mercapto methyl imidazole	16.0 mg/ml	[128]
	Mercapto purine	14.9 mg/ml	
	Mercapto nicotinic acid	10.3 mg/ml	
	Mercapto methyl pyrimidine	4.9 mg/ml	
Poly(ethylene vinyl alcohol)	DVA/2-Mercaptoethanol	3.2 mg/ml	[129]
Cellulose beads	Mercapto ethyl pyridine	30.0 mg/ml	[130]
Sepharose	2-Mercapto ethanol	4.0 mg/ml	[131]
Poly(styrene-vinyl acetate)	2-Mercapto nicotinic acid	14.0 mg/ml	[132]
Agarose	Divinylsulphone	28.1 mg/ml	[133]
Agarose	Divinylsulphone	150.0 mg/g	[134]
Agarose	DVS/2-Amino pyridine	30-60 mg/ml	[135]
Silica	Mercapto thiazoline	25.0 mg/ml	[136]
Agarose	Mercapto thiazoline	30.0 mg/ml	
Sepharose	Sulfanyl ethylamine	55.6 mg/ml	[137]

technique to isolate IgG from human serum based on the utilization of thiophilic magnetic poly(vinyl acetate-divinyl benzene) beads [122]. After the thiophilic ligand of 2-mercaptonicotinic acid was modified on the surface, these magnetic beads exhibited a strong specificity towards IgG in a salt-independent manner. Then, antibodies could be directly isolated from human serum in batch-wise mode with the assistance of magnetic decantation. The driving force for this selective recognition was attributed to electron-donor acceptor interaction [123]. The purity of the isolated antibody exceeded 94%. The bioactivity of antibodies was sufficiently preserved that the bioactivity purity of IgG exceeded 99% [124]. Prominent advantages of this method, such as strong specificity, rapid processing, mild conditions, conventional equipment and excellent reusability, make this non-chromatographic technology embody great potentialities to isolate the antibodies on a large scale (Table 7).

Aqueous Two Phase Systems

Aqueous two-phase separations using polymer-salt (poly(ethylene glycol) (PEG) phosphate and PEG citrate) systems have been applied successfully for purification of monoclonal antibodies from cell culture harvest systems [138]. Liquid-liquid extraction in aqueous two phase system is a powerful, non-chromatographic, unit operation for the separation of biomolecules, which has been successfully applied in the purification of different biological materials, such as cells, viruses, organelles, nucleic acids, proteins and enzymes [139]. Azevedo et al described the feasibility of using aqueous two phase systems composed of poly(ethylene glycol) and sodium citrate for the primary recovery of antibodies from an hybridoma supernatant for the first time [140]. The partial purification of IgG from a hybridoma cell culture supernatant in a serum-containing media was achieved using 15% NaCl and recovering the antibody in the top phase.

The partition of human antibodies in aqueous two-phase separations of PEG and phosphate was systematically studied using first pure proteins systems [141]. It is possible to selectively recover IgG in the top phase with high yield and purity using high concentration of NaCl and low concentrations of phase forming components. A back extraction step was also performed, and IgG was recovered with a total yield of 76% and a purity of 100%.

CONCLUDING REMARKS

Purification techniques for antibodies have a long history of highly qualified attempts to obtain them in an active and high purity [142]. In this review, we have outlined the developments in the purification of antibodies. It has been demonstrated that a variety of affinity techniques including both specific and pseudospecific can be used for purification of antibodies.

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