Journal of Chromatography A, 1221 (2012) 57–70

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Review

Technology trends in antibody purification

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ARTICLE INFO

Article history:
Available online 20 October 2011

Keywords:
Antibody purification
Chromatographic
Non-chromatographic
Remedial purification

ABSTRACT

This article reviews technology trends in antibody purification. Section 1 discusses non-chromatography methods, including precipitation, liquid–liquid extraction, and high performance tangential flow filtration. The second addresses chromatography methods. It begins with discussion of fluidized and fixed bed formats. It continues with stationary phase architecture: diffusive particles, perfusive particles, membranes and monoliths. The remainder of the section reviews recent innovations in size exclusion, anion exchange, cation exchange, hydrophobic interaction, immobilized metal affinity, mixed-mode, and bioaffinity chromatography. Section 3 addresses an emerging trend of formulating process buffers to prevent or correct anomalies in the antibodies being purified. Methods are discussed for preventing aggregate formation, dissociating antibody-contaminant complexes, restoring native antibody from aggregates, and conserving or restoring native disulfide pairing.

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DOI of original article: 10.1016/j.chroma.2011.11.003.
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doi:10.1016/j.chroma.2011.10.034

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1. Introduction

Antibodies have been technology drivers for over a century but never more than now [1–3]. Their commercial success and projected growth invite innovation from all sectors. Dramatic increases in cell culture productivity have meanwhile increased the stakes, and challenge purification technology to keep pace [1–13]. Even the most successful IgG purification method to date exhorts users to seek alternatives: bioaffinity chromatography with immobilized protein A consistently achieves high-capacity IgG capture without requiring modification of feed stream conditions, while delivering more than 95% purity, similar recovery, several logs DNA and virus clearance, and partial reduction of aggregate content, all without requiring significant process development. This is remarkable by any measure, but its price overshadows its performance and creates inspired motivation to replace it.

Other forces would seem to discourage innovation. A 2007 review entitled *Future of Antibody Purification* characterized purification technologies with the potential to change then-current industrial practices as *disruptive* [5]. The industry nevertheless needs technology improvements to survive increasing economic pressures, even if they are disruptive. Ironically, the evolution of the industry itself is the source of the strongest incentive for innovation in its history: the emergence of biosimilars.

Biosimilars are also known as follow-on biologics; clones of products for which patent protection has expired, permitting the entry of competing manufacturers. The influx of companies seeking to capitalize on this opportunity has created a globally competitive climate far more receptive to innovation. Importantly, the need for a competitive edge may drive innovation beyond cheaper faster ways to achieve current levels of purification, and extend to quality improvements in the antibody products themselves. Many recent advances offer opportunities to reduce contaminant levels by more than an order of magnitude with negligible increase of processing costs. It seems reasonable to expect that such improvements could reduce product antigenicity or mitigate patient risk in other significant ways. The impact of even one such success would likely accelerate new technology implementation and usher in a whole new cycle of innovation.

The first part of this review addresses trends in non-chromatography methods. Many regard protein A chromatography as a productivity bottleneck despite its effectiveness [5–8]. Some insist that *any* chromatography-based capture method would impose the same limitations, and that the industry can meet future demands only by embracing a philosophy of *ABC–Anything But Chromatography* [5,8]. Many alternatives are under active investigation, including novel precipitation methods, aqueous two-phase extraction (ATP), and ultrafiltration with charged membranes. These and related methods are covered in Section 2. Crystallization has been discussed in several reviews [5–7,9,10], but is not included because it has not demonstrated potential for antibodies.

Section 3 addresses trends in chromatography methods. Kelly et al. [11–13] have argued that chromatography is capable of meeting the industry's needs for the foreseeable future. Continuing advances support that view. These are discussed in subsections covering fluidized beds, simulated moving beds (SMB), and stationary phase architecture of fixed bed chromatography media. Recent advances in size exclusion (SEC), anion exchange (AEC), cation exchange (CEC), hydrophobic interaction (HIC), immobilized metal affinity (IMAC), mixed mode, and bioaffinity chromatography follow.

Section 4 addresses an emerging trend that will be referred to as *remedial purification*: enhancements that go beyond the basic task of fractionation, to prevent or correct anomalies in the antibody population. Four application areas already show promise: suppression of aggregate formation; enhanced contaminant reduction through dissociation of contaminants that form stable complexes with antibodies; restoration of native antibody from aggregates; and conservation/restoration of proper disulfide pairing. Beyond their immediate practical value, these advances offer a deeper understanding of the interactions among antibodies, contaminants, and purification materials that can be expected to promote development of even more effective technologies.

2. Trends in non-chromatographic methods

2.1. Precipitation

Precipitation remains a technique of interest because concentrating product to a solid offers the maximum degree of process volume reduction, with attendant benefits for subsequent purification steps [5]. One of the reasons it has not been implemented in current processes has been its reliance on bulky expensive hardware for centrifugation. Removing the supernatant by membrane filtration relieves that burden for both ammonium sulfate [14–16] and polyethylene glycol (PEG) precipitation [17,18]. Method development and lab-scale applications can be performed with disposable dead-end filtration units. Industrial operations can be scaled up with tangential flow [16–18]. Applications to date include purification of IgG from bovine serum [16], human serum [15], and mammalian cell culture supernatants [15,17,18]. Reported IgG recoveries range from 85 to 93%, with purity averaging about 85%.

Ito et al. [19,20] developed a dynamic variant of ammonium sulfate precipitation that integrates antibody precipitation and resuspension. A water channel is separated from an ammonium sulfate channel by a semi-permeable membrane. This creates an ammonium sulfate gradient in the water channel, causing proteins to be deposited as precipitates. Gradual reduction of salt concentration in the ammonium sulfate channel dissolves precipitated species in order of their solubility. The method has been applied successfully to fractionation of monoclonal antibodies but it remains to be seen if it can be adapted to commercial scale fractionation.

Antibodies may also be co-precipitated with negatively charged polymers [21–23]. Selectivity parallels CEC. In a direct comparison for intermediate purification, co-precipitation supported essentially the same recovery as CEC, and about 80% of the host cell protein removal [21]. As a capture step, recovery was also similar to CEC (82%) but inferior to protein A (95%). Host cell protein removal was only 15% as effective as CEC, and only 4% as effective as protein A. The authors noted that co-precipitation could be conducted in either a continuous centrifugation or filtration format.

Co-precipitation of non-IgG contaminants with positively charged polymers parallels the selectivity of AEC. These reagents selectively co-precipitate acidic host cell proteins, DNA, and various cell culture additives [22–25]. A polyallylamine precipitation-based multistep purification process achieved overall purity and recovery

roughly equivalent to a protein A-based process [24]. A polyarginine precipitation-based process achieved a similar result [25].

Co-precipitation methods raise several issues exemplified by the long-known method of co-precipitation with octanoic (caprylic) acid [1]. It has been evaluated as a potential protein A replacement but not implemented as a manufacturing method [26]. Reagent cost per batch of IgG exceeds the average cost per batch of protein A purification [26]. In addition, residual caprylate remains bound to the antibody. It has been shown to alter electrophoretic mobility of IgG in agarose gels [27], and block hemagglutination assays [28]. It has been measured at about 400 ppm of recovered IgG, and persists at about 40 ppm even after recovery of antibody from ammonium sulfate precipitates [29].

Aside from the toxicology of co-precipitating agents, it remains to be determined what clearance levels must be achieved to ensure that they do not contribute to long-term particle formation by acting as nucleation centers. McDonald et al. [21] reduced residual polyvinylsulfonic acid levels to less than 10 ppm by passing the antibody solution over an anion exchanger. Polymer bound to a porous particle exchanger could prove difficult to remove and might cumulatively alter media performance, but this would not be an issue with a single-use membrane exchanger.

See the 2009 reviews by Thömmes and Gottschalk [7] and Glynn [26] for more discussion of these and other agents.

2.2. Liquid:liquid extraction

Investigations into aqueous two-phase (ATP) extraction began to proliferate when the productivity limitations of protein A affinity chromatography started to reveal an opportunity for alternative methods [8,30,31]. The technique is based on the tendency of IgG to transfer spontaneously from various aqueous formulations into a more hydrophobic PEG-rich phase. A recent review by Azevedo et al. [8] provides a good introduction.

Several PEG–citrate–sodium chloride systems have achieved purity of 70–95% and recoveries greater than 95%, but final IgG concentrations averaged little more than 0.2 mg/mL [32–34]. Mao et al. [35] showed that host protein removal was more effective at pH 7.2. Removal of aggregates and fragments was more effective at pH 5.5. Oelmeier et al. [36], recognizing the time and labor intensiveness of screening experimental conditions, developed an automated cloud-point method that yields partition coefficients identical to values determined by manual methods.

Other investigations have explored application of two-polymer phases, like PEG and dextran. Antibodies partition in the PEG phase. One group coupled hydrophobic ligands to the terminal hydroxyls of PEG to increase the partition coefficient [37–40]. Purity and recovery were equivalent to PEG–citrate systems but final IgG concentration was still barely 1 mg/mL [40]. Other investigators have attached ligands that participate in more direct interactions with IgG, the most obvious being protein A [41]. Birkenmeier et al. [42] attached the metal chelator iminodiacetic acid. Zijlstra et al. [43] attached a dye ligand. Although these modifications increase partitioning efficiency, they impose the requirement for a dissociation step to remove the ligand-bearing polymer from the antibody. Residual *unmodified* PEG, on the other hand, could be beneficial. It increases antibody retention and binding capacity on cation exchangers and hydroxyapatite [44,45], highlighting their potential for product concentration and intermediate purification [18].

Zijlstra et al. [43,46–48] integrated ATP with cell culture in a system where cell production occurred in the lower phase, while IgG transferred spontaneously to the PEG phase. This avoided prolonged antibody contact with dead cell debris (Section 4.2) and radically streamlined the harvest process.

Azevedo et al. [49] argue that ATP already combines superior process economy with lower environmental impact than protein A, but issues remain. ATP involves different hardware and an entirely distinct process development and manufacturing paradigm. Combining aqueous two-phase capture with chromatography for subsequent purification would burden users with the need to support both. Variations in ATP performance resulting from variations among antibodies remain to be characterized. Virus and DNA reduction capabilities remain to be characterized. Intermediate and final purification steps to consistently ensure adequate product quality remain to be defined.

George and Stuckey [50] developed an IgG extraction system using reverse surfactant micelles in an isooctane solvent system. Extraction efficiency with an anionic surfactant was 80–90% but biological activity was reduced to 30%. Addition of the nonionic surfactant Brij-30 doubled activity recovery.

2.3. High performance tangential flow filtration (HPTF)

van Reis et al. [51–56] developed a technique based on electrostatic interactions between proteins and charged ultrafiltration membranes (100–300 kDa cut-off). Although potential exists for solutes to be retained by these interactions – which would constitute ion exchange chromatography – antibody selectivity is mediated by ion *exclusion*. A positively charged ultrafiltration membrane repels (rejects) positively charged proteins such as IgG monoclonal antibodies, despite them being small enough to pass through the pores. This permits their selective retention and concentration while weakly alkaline, neutral, and weakly acidic contaminants pass through the membrane.

Strongly acidic contaminants pose an apparent challenge. At conductivity values low enough to repel IgG, positively charged surfaces bind DNA. If DNA is present at low concentration, HPTF will compound its removal. If present at high concentration, DNA binding could reduce the charge potential on the membrane surface sufficiently to reduce the intensity of antibody exclusion. The same research group has demonstrated the principle, showing that IgG bound to the surface of a cation exchanger reduces charge potential with the practical result of reducing capacity [57,58]. This means that consistently effective use of HPTF as a capture method could require a sample-conditioning step to remove the bulk of the DNA.

Another limitation concerns variability among antibodies. The most alkaline IgGs will be excluded to the greatest degree, which also means that their rejection will tolerate lower pH and/or higher salt concentrations, with the benefit of allowing more contaminants to pass through the membrane. In short: higher purification potential for highly alkaline antibodies but lower potential for more acidic ones.

Chemical engineering aspects of HPTF have been advanced recently by Etzel [59].

3. Trends in chromatography methods

3.1. Application format

3.1.1. Fluidized bed chromatography

Fluidized bed chromatography involves the use of adsorbent particles dispersed in a liquid medium. The simplest example is batch chromatography but the format of industrial preference involves highly engineered up-flow columns that maintain the particles in an evenly dispersed state throughout equilibration, sample application, and washing. Elution, cleaning, and sanitization are usually performed after settling the bed. One version is referred to as expanded bed chromatography. The particles are engineered to embody a narrow range of densities. When they are dispersed upwards by the flowing buffer, denser particles tend to stratify towards the bottom of the bed, less dense particles towards the top.

Stable stratification permits formation of theoretical chromatographic plates that enable fair gradient fractionation of complex samples.

Operation of fluidized beds is more complicated than fixed beds, but they offer the valuable feature of selectively capturing antibodies while cells and debris pass between the dispersed chromatography particles. The economic and practical benefits of this shortcut have been discussed in numerous articles and reviews [5–7,9,60–68], but technical limitations in first generation materials sabotaged early hopes for broad implementation. One was that the density of the chromatography particles partially overlapped with the density of cells and debris that were intended to pass through, leaving the chromatography media virtually impossible to clean. Chronic frit fouling was also common [69]. These problems aside, general economic issues include higher equipment and media costs, plus higher buffer consumption than packed beds.

Second generation systems have overcome the physical limitations [70,71]. A rotating arm at the inlet port achieves effective flow distribution without requiring a frit. Cellulose-coated tungsten-carbide particles increase the density differential between cells and chromatography particles, and allow faster flow rates that achieve more efficient sweeping of debris through the system [70,72]. Capture efficiency is maintained by using taller beds to maintain sample residence time. Capacity of 10–20 g polyclonal IgG/L settled particles compares poorly with protein A in fixed beds, but compensates by suspending the need for clarification [70]. A recent study demonstrated the ability of the system to purify polyclonal human IgG from plasma with 80% purity and 93% recovery [73]. This was achieved with a mixed-mode ligand, avoiding the expense of protein A.

The use of magnetic particles represents another branch of the fluidized bed lineage. Initial applications have evaluated immobilized protein A [74,75], noting purity and recovery similar to conventional and fluidized bed applications, but requiring less time. Thiophilic magnetic microspheres (Section 3.3.6.3) were used to extract IgG from a variety of feed streams, including cell-containing culture media and whole blood [76]. The authors highlighted purity and recovery similar to protein A, with IgG binding capacity up to about 30 mg/mL of particles. One of the advantages of nanoparticles was illustrated in an application using phenylalanine as a ligand [77]. Average 158 nm nanospheres offered a surface area of 1874 m^2 per dry gram of particles. This enabled IgG binding capacities of 780 mg/g. 150 nm particles substituted with an imidazole derivative exhibited capacities up to 843 mg/g [78]. Refer to recent reviews by Peuker et al. [79], Franzeb et al. [80], and Hubbuch et al. [66,67] for comprehensive discussion.

3.1.2. Fixed bed chromatography

Fixed-bed applications continue to dominate the field, whether conducted with membranes, monoliths, or porous particles packed in columns. Most applications are run with a single bed. Bed dimensions can be increased if greater capacity is required, but chromatography media and buffer costs increase proportionally. Cycling can increase capacity without increasing media costs, but multiplies buffer costs and process time. Thömmes et al. [81] demonstrated feasibility for a continuous multicolumn system called simulated moving bed chromatography (SMB). SMB systems have proven to increase throughput while reducing chromatography bed volume and buffer consumption. Some authors [82] suggest that their efficiency justifies application of protein A as a single-use disposable.

Bisschops et al. [82] showed an SMB model that required 90% less protein A media and 37% less buffer than required by a traditional process. In experimental comparisons at the 5 mL column scale, SMB throughput was double the traditional format, buffer consumption was 27% less, and host cell protein reduction was improved by 15%. Other groups have evaluated 3-column SMB formats for IgG capture by cation exchange chromatography [83], for high-resolution cation exchange separation of antibody charge-variants [84,85], and for 2-step IgG purification procedures of cation exchange capture followed by polishing on a mixed mode column [86].

Shinkazh et al. [87] developed a novel physical format that merges the principles of SMB with fluidized bed chromatography. They pumped slurried porous particle media through a series of static mixers and tangential flow membrane modules. Purification, recovery, and reduction of buffer volumes were similar to fixed bed SMB systems.

See [82,83] for illustrated introductions that explain the physical organization of SMB, its performance and economic benefits; but note some unobvious features. For example, the larger the number of columns, the longer the system requires to reach steady state. SMB also involves regulatory issues that remain to be resolved, including in-process documentation, pooling and batch definition.

3.2. Stationary phase architecture

3.2.1. Diffusive microparticles

Chromatography on diffusive microparticles has been the norm in antibody purification for so long that they are commonly believed to define chromatography and its capabilities. In fact, the slow-flow-dependent capacity, recovery, and separation performance that cause some people to conclude that chromatography must be abandoned are not inherent to chromatography at all; they are all artifacts of diffusive particles. Dependence on diffusive mass transfer restricts flow rate (88–91). Eddy dispersion in the void volume degrades resolution independent of flow rate. Turbulent void flow creates shear forces in proportion with flow rate. These limitations notwithstanding, diffusive microparticles particles still offer the highest IgG-accessible surface areas and dynamic binding capacities of all fixed-bed stationary phases.

3.2.2. Perfusive microparticles

Perfusion chromatography media are defined as media that support intra-particle flow [88]. They are diffusive particles that employ large trans-particle channels to give solutes access to a larger surface area and add a component of convective mass transport. This conserves capacity and fractionation performance at higher operating flow rates than can be achieved with exclusively diffusive particles, but their absolute capacities are generally lower than the highest capacity diffusive particles, and they are still burdened with eddy dispersion and turbulent shear in the void.

A recent book by Carta and Jungbauer [91] provides a comprehensive chemical engineering treatment of porous particles and their impact on fractionation performance.

3.2.3. Adsorptive microfiltration membranes

The reason membrane adsorbers achieve high uptake efficiency at high flow rates is because solute mass transfer is convective [59,88,91,92]. Low operating pressure is a secondary benefit of thin membranes that facilitate the high flow rates at which convective transfer becomes advantageous. Convective mass transfer efficiency is unaffected by flow rate, solute size (diffusion constant), or viscosity, so capacity is relatively unaffected by these variables. Fractionation efficiency should be similarly unaffected but its benefits are rendered moot by poor flow distribution deriving from high dead-volume housing designs and variations in membrane thickness. Membrane adsorbers are consequently best suited to flow-through applications where substantial dilution at the sample boundaries is tolerable. Another important limitation is that membranes support much lower protein binding capacities than equivalent volumes of porous particles. This is a function of their

lower surface area. Anion exchange membranes for flow-through purification of IgG are consequently most effective after capture and intermediate purification have minimized the contaminating protein load. On the plus side, anion exchange membrane binding capacities for virus and DNA are 10–20 times higher than porous particles [93]. The technical basis for this paradox has been elucidated with monoliths (Section 3.2.4).

A novel cation exchanger represented as a membrane adsorber has been reported to support IgG binding capacities of 55 mg/mL [18], which the authors highlighted as up to 5 times the capacity of conventional membrane adsorbers. Elution, however, required 50 bed volumes, producing an IgG eluate with a concentration of only 1.1 mg/mL.

Antibody purification with membrane adsorbers was reviewed by Boi [94].

3.2.4. Monoliths

Monoliths are fixed chromatography beds cast as a single unit, characterized by a network of large highly interconnected channels [89,90]. They offer the uniform flow distribution of packed particle beds and the convective mass transport efficiency of membranes. This translates into consistent capacity and high-resolution fractionation regardless of solute size, buffer viscosity, or flow rate [91–93,95–98]. Monoliths easily accommodate flow rates of 10 bed volumes per minute without loss of performance. A valuable corollary of independence from flow rate is that dynamic binding capacity is largely liberated from the residence time requirements that burden porous particles. The lack of a void volume eliminates the turbulent void flow that contributes to molecular shear in particle columns [99]. It also eliminates eddy dispersion, which is a major impediment to high-resolution fractionation in particle columns. That, in combination with the lack of diffusive mass transfer limitations, allows monoliths to achieve high-resolution fractionation with short beds. Short beds, in combination with high porosity, enable low operating pressures at high flow rates. Short beds are maintained in large-scale monoliths by employing a radial-flow format.

Monoliths currently marketed for industrial applications have average 2 μm channels optimized for purification of large solutes such as DNA plasmids and virus particles. This is 20–50 times the average pore size in diffusive particle media. The large channel volume reduces the protein-accessible surface area that can be achieved in a 3-dimensional space, which limits IgG capacity to 10–25 mg/mL [89]. DNA binding capacity however is roughly 50 times higher, and virus binding capacity up to 100 times higher than porous particle media [89,93]. These data mark a well-characterized inverse relationship between solute size and capacity on monoliths and porous particles. Capacity on porous particles diminishes with increasing solute size because of slower solute diffusion constants and because larger solutes have access to a lesser proportion of the intra-pore surface area [100,101]. Capacity increases with solute size on monoliths because solute mass increases in proportion to 3-dimensional volume, not the 2-dimensional area a solute occupies on a chromatography surface [101]. The capacity crossover between monoliths and particles appears to be somewhere below 1 MDa for proteins [101]. This is consistent with reports of 25–69 mg/mL IgM dynamic binding capacity on strong anion and cation exchange monoliths, versus 10–25 mg/mL for IgG. [102,103]. A porous particle strong anion exchanger gave a capacity of only 13 mg/mL IgM under conditions where the corresponding strong anion exchange monolith achieved 29 mg/mL [103]. One study reported the reverse relationship [104], but based on a weak anion exchange monolith that has been shown in other studies to support much lower IgM capacities than strong anion exchange monoliths [103,105].

Ultramacroporous monoliths have been synthesized with channel sizes of 10–200 μm [106–109]. This permits unrestricted passage of cells and debris, offering yet another option for bypassing clarification of cell culture supernatants. Such large channels substantially reduce binding surface area for proteins, but by what increment is difficult to estimate since capacity in these studies was expressed in mg protein per dry gram of monolith. An ultramacroporous protein A monolith achieved 88 mg IgG/g [106]. One with a histidyl chelating ligand bound more than 100 mg polyclonal IgG/g from serum [107]. Albumin capacity on a chelating monolith co-synthesized with nanoparticles to increase surface area was over 675 mg/g [109], suggesting that similar modifications could support high capacity IgG capture as well.

3.3. Chromatography methods

3.3.1. Size exclusion chromatography (SEC)

SEC is not used for manufacture of purified antibodies because of its low productivity [4], but it remains an important part of the repertoire for lab scale purification of IgM, removal of aggregates from IgG and IgM, and analysis of both. It was disturbing therefore when a 2005 publication [110] revealed that phosphate buffers commonly used with the technique caused aggregate content to be underestimated. Aggregate flow down the column is retarded by nonspecific hydrophobic interactions with the solid phase. They elute later than they should, which causes them to co-elute, undetected, with the antibody. More accurate results were obtained by suspending nonspecific interactions through addition of arginine to the mobile phase. A 2010 review by Arakawa et al. [111] addresses the effects of diverse buffer options on SEC media of varying composition.

3.3.2. Anion exchange chromatography

The importance of AEC for removal of DNA, virus, endotoxin, leached protein A, and acidic host cell protein contaminants has made it a focal point for innovation, with the result that it has become one of the most flexible and powerful tools in the field. Murine IgGs are mostly more acidic than human, so fair binding is usually achieved at pH 8.0–8.5 [112]. Applications in bind-elute mode are advantageous because they eliminate contaminants that bind more weakly than the antibodies, in addition to those that bind more strongly. Human and chimeric IgGs with their higher isoelectric points typically bind too weakly to support high capacity or do not bind at all, but a relatively greater proportion of contaminant species bind more strongly than the antibody. This favors flow-through applications.

Applications in flow-through mode are commonly conducted on membrane exchangers because they offer higher throughput than porous particles with no compromise to DNA or virus removal, and because they are inexpensive enough to make disposal more economical than validating their re-use [113–116]. Lajmi et al. [117] observed that antibody aggregates fouled AEC membranes and reduced their processing capacity. They restored capacity with a 0.2 μm in-line guard filter. The majority of applications are still conducted on porous particles because they offer higher capacity for protein contaminants. As noted in Section 3.2.3, DNA and virus capacity is lower on particles than on membranes, but many rigorous studies have demonstrated effective virus clearance on particles [118–122]. Host cell protein removal is usually the limiting factor.

Kelly et al. [123] have endorsed an extension of flow-through mode that they call weak partitioning. This involves the application of IgG under conditions where its transport down the column is retarded, to the extent that a small subpopulation is retained. The goal is to disproportionately enhance retention of contaminants that interact more strongly with the exchanger than IgG.

The technique is conducted on porous particle exchangers to maximize removal of host cell proteins. The authors suggested that the improvement in performance was adequate to support purification of therapeutic grade IgG with just protein A and AEC.

Displacement mode applications represent an extension beyond weak partitioning. Antibody is loaded continuously under conditions where it binds weakly. Sample components that interact strongly with the exchanger accumulate and displace weakly retained antibody from the chromatography support. Brown et al. [124] overloaded both anion and cation exchange membranes under conditions where most of the IgG flowed through. Loading continued until contaminating proteins began to break through. Effective host protein removal was maintained with application of more than 3 kg IgG/L membrane. IgG recovery was greater than 99%. Host cell protein clearance was affected by pH and conductivity, as expected for ion exchangers. It was not affected by flow rate, consistent with convective mass transport. Breakthrough boundaries for contaminants were poorly defined, consistent with poor flow distribution in membrane adsorbers. This last point invites application of monoliths, which have been demonstrated to produce sharp solute boundaries in displacement applications [125–127]. Refer to [91] for introduction to the basic concepts of displacement chromatography.

3.3.3. Cation exchange chromatography

The high capacity of recently introduced porous particle ion exchangers has rejuvenated interest in CEC as a capture alternative to protein A [5,56,128–131]. Several have demonstrated dynamic binding capacity greater than 100 mg IgG/mL, with some approaching twice that [131]. Very recent introduction of essentially the same products on smaller particles can be expected to increase capacity yet further, and resolution with it, but at higher operating pressures that will likely require reduction of flow rate in large-diameter columns. Faude et al. [132] described a rapid method for estimating dynamic binding capacity. The old-fashioned way is described in [131].

Application of cell supernatants typically requires that conductivity and pH be reduced to achieve good binding capacity. Some workers recommend diafiltration to equilibrate filtered supernatants [129]. Others suggest PEG precipitation followed by resuspension in the target buffer [18]. Others suggest in-line dilution through the chromatography pumps [131,133]. Unexpectedly, conditions that would seem to favor the highest capacity, such as very low conductivity and/or pH, may have the opposite effect due to strong antibody binding reducing the surface charge potential on the exchanger [57,58]. Such conditions may be impractical in any case because of poor antibody solubility [131].

Staby et al. compared performance of numerous CEC media [134,135] but mostly prior to the introduction of new high capacity media. Comparison of several present generation exchangers is described in [131]. Strong cation exchangers are generally preferred for process applications because they offer better pH control (see below). Numerous studies have addressed media features including ligand density [136,137], porosity, and the use of grafting methods to increase charge density and extend exchange groups from the surface of the support [138–142].

Salt gradient elution dominates the literature, with several studies addressing their optimization [143–146], but pH gradients can offer worthy benefits [147]. IgG frequently elutes at neutral to weakly alkaline pH and low conductivity that facilitates application to a subsequent anion exchange step. IgGs that do not elute in pH gradients offer the opportunity to apply high pH washes that enhance removal of host cell proteins. The IgG can then be eluted with a very modest increase in salt concentration. IgG has also been eluted with hybrid pH/conductivity gradients [148]. Higher pH encourages more effective elimination of host cell proteins in all cases [143–149].

pH control has emerged as an unexpected challenge for CEC applications [131,150–153]. Positively charged hydrogen ions condense opposite negatively charged cation exchange ligands during equilibration. Sodium ions have a higher affinity for the exchange groups than hydrogen ions, so when salt is introduced, for example upon sample loading or elution, it displaces hydrogen ions into the mobile phase, causing pH to drop. Depending on the exchanger, the equilibration conditions, the buffer, and the salt concentration introduced, the drop may exceed 2 pH units and persist for several column volumes. If salt concentration is reduced, for example at the end of the sample load, pH spikes upward due to solid phase binding of hydrogen ions. Response is more intense with weak cation exchangers than strong ones, but many products marketed as strong cation exchangers have significant concentrations of carboxyl groups on their polymer backbones, so product names are not reliable indicators [131,150–153].

It stands to reason that if conductivity changes affect pH control, pH changes must affect conductivity control. This has been demonstrated recently by Fogle and Hsiung [154]. Discontinuities in mobile phase pH and conductivity can also breach process control with anion exchangers. Their effects can be nearly eliminated in flow-through applications by matching the pH and conductivity of the equilibration/wash buffer and sample.

Aggregate removal by CEC and AEC was reviewed in 2009 by Yigsaw et al. [155]. A more recent article by Suda et al. [156] describes development of conditions for aggregate removal by anion exchange conducted in flow-through mode.

3.3.4. Hydrophobic interaction chromatography

The most frequent application of HIC has been for aggregate removal. Some examples have been reported on porous particle media [157–159], others on membrane adsorbers [18,160–162]. Both tend to be used in flow-through mode.

HIC is also effective for removal of DNA and host protein contaminants, but applications tend to be restricted because of the high salt concentrations at which antibodies elute. This is unnecessary since low conductivity elution can be achieved with glycine [163]. Glycine lacks the ability to promote high capacity binding, but a 2 M wash is able to conserve retention of IgG that was initially loaded in conventional binding salts such as ammonium sulfate. Since glycine is zwitterionic from about pH 4 to 8, it contributes nothing to conductivity. A reducing gradient elutes antibody at conductivity values suitable for direct application to ion exchangers. Glycine may also be used to modulate selectivity in flow-through applications.

3.3.5. Immobilized metal affinity chromatography (IMAC)

IMAC is operationally more complex than other methods because it requires the extra step of immobilizing a metal ion. It is chemically more complex because of the diversity of adsorbents that may be used to immobilize the metal, the diversity of metals ions that may be employed to mediate selectivity, and the diversity of elution methods that may be applied. IgG binding is dominated by a highly conserved histidyl cluster at the junction of the second and third constant domains of the heavy chain [164]. Many combinations of adsorbent-metal-elution method permit IgG to be purified to greater than 90% in a single step, but capacities have yet to rival protein A and are frequently less than 10 mg/mL.

Most applications are conducted on supports substituted with iminodiacetic acid (IDA) and loaded with nickel or copper [165–170]. Use of cobalt, zinc, and iron are less frequent. Prasana and Vijayalakshmi reported IgG binding capacities of 14–16 mg/mL for either polyclonal or monoclonal IgG on copper-loaded IDA monoliths at flow rates up to 9 bed volumes per minute [168].

Capacity with nickel was barely half that. Alternative metal adsorbents are occasionally used, such as nitriloacetic acid [171], aspartic acid [170], or Tris(2-aminoethyl)amine [172].

A research group headed by Denizli et al. [106,107,173–175] synthesized a solid phase that was novel in two respects. Instead of immobilizing a chelating ligand to an inert support, they used a used a histidyl methacrylate derivative to synthesize chelating groups directly into the polymer backbone. Loaded with copper, it improved IgG capacity over IDA-based supports. Practical access to the technology is presently limited to researchers equipped to synthesize their own chromatography media, but the ligand can be evaluated by immobilizing histidine on a pre-activated affinity support. Bayramoglu et al. [176] demonstrated that a 6-carbon spacer increased binding capacity.

Martin et al. [177] investigated copper, zinc, cobalt, and calcium on IDA for purification of IgM monoclonal antibodies. IgM was believed to adsorb to histidine residues in the third constant domain of the heavy chain. Elution required only 5 mM imidazole, suggesting a weak interaction, but purity was reported as homogeneous by native PAGE and equivalent to IgM purified by SEC.

Despite IMAC's potential as an inexpensive alternative to biological affinity a note of caution is in order: polynucleotides [178], endotoxin [178,179], and virus [180–182], have all been shown to bind various immobilized metals. On the other hand, IMAC has also been used to selectively bind antibody fragments while endotoxin was removed by washing with a surfactant [183].

IMAC was reviewed by Block et al. [184].

3.3.6. Mixed mode chromatography

Mixed modes represent a broad and increasing diversity of ligands that exploit the combined functions of two or more chemical mechanisms. The influence of their primary mechanisms can be demonstrated fairly easily, for example electrostatic and hydrophobic interactions, but the practical contributions and control of secondary functionalities are poorly understood, including metal coordination, π–π bonding, hydrogen bonding, and van der Waals forces. These introduce a strong element of unpredictability that is compounded by variations in ligand density and physical configurations among ligands of similar chemical character. Despite these variations, mixed modes can be grouped by their dominant functionalities into three subsets that produce characteristic results with IgG: those that augment anion exchange with hydrogen bonding, those that augment metal coordination with electrostatic interactions, and those that augment hydrophobic interactions with other functions.

3.3.6.1. Hydrogen bond-enhanced anion exchangers. Mixed modes of this class are commonly called salt-tolerant anion exchangers. Their ability to bind virus and DNA at moderate salt concentrations makes them strong candidates to replace traditional anion exchangers as the preferred final polishing media in IgG purification. They derive from recent pioneering work by Etzel et al. [59,185], who compared a traditional quaternary amine membrane adsorber (Q) against membranes substituted with experimental anion exchange ligands. All reduced virus concentration in the flow-through by 5 logs when sample was applied in the absence of salt. Addition of 50 mM NaCl diminished virus removal by about 100-fold on the Q. Removal efficiency was undiminished with the others up to 150 mM NaCl. Salt tolerance was attributed to hydrogen bonding. Faber et al. [186] reported results similar to Etzel et al. [59,185]. Woo et al. [187] reported the ability of a polyallylamine-substituted membrane to achieve DNA, endotoxin, and virus removal of more than 3, 4, and 4 logs at up to 250 mM NaCl.

Comparing experimental salt-tolerant monoliths with a commercial quaternary amine monolith (QA), Etzel and Riordan [188] reported the same trends they observed with membranes, but more than twice the protein binding capacity. Gagnon et al. [103] compared DNA elution characteristics of a commercial salt tolerant monolith (ethylene diamine, EDA) with a QA monolith. DNA eluted from QA at about 0.6 M NaCl but from EDA at more than twice that concentration.

These ligands also exhibit unique selectivities among proteins. Brne et al. [105] showed that IgM retention on an EDA monolith was essentially unaffected by a decrease of operating pH from 8.0 to 6.5. Albumin eluted earlier than IgM at all pH values, but its retention diminished with decreasing pH, yielding a progressive increase in separation. Another series of experiments showed that IgM retention at pH 7.2 was stronger on EDA than on either DEAE or QA monoliths. EDA achieved baseline separation, versus DEAE and QA where albumin eluted as a leading shoulder on the IgM peak.

Bresolin et al. [189] immobilized Tris(2-aminoethyl)amine on agarose beads, producing a twin ethylamino ligand. They used it at low conductivity for flow-through purification of IgG from human serum, achieving 90–95% purity in a single step. Selectivity for DNA and virus at elevated salt concentrations remains to be characterized.

3.3.6.2. Metal coordination mixed modes. Hydroxyapatite (HA) is a mineral of calcium and phosphate that presents surface calcium residues capable of participating in metal coordination bonds with protein polycarboxyl domains or phosphate residues on nucleotides, endotoxins, and lipid envelope viruses [190–192]. Calcium coordination is largely unaffected by conductivity, endowing apatites with a high degree of NaCl tolerance at low phosphate concentrations [192]. HA surface phosphates participate in cation exchange interactions with protein amino residues [190–192]. HA surface hydroxyls are theoretically capable of hydrogen bonding but have not been shown to make a significant contribution to biomolecule retention. In fluorapatite, the hydroxyls are replaced by fluoride groups [193].

The cation exchange component of HA confers the same pH control challenges as dedicated cation exchangers (Section 3.3.3), but with the added consequence that HA becomes unstable at pH values below 6.5. If a sodium chloride step is introduced in the absence of adequate buffering capacity, pH can drop below 6.5 for sufficient duration to cause calcium loss. Dattolo et al. [194] have analyzed and modeled the phenomenon in detail, while Cummings et al. [195] have developed methods to maintain pH control. The primary tactic consists of re-equilibrating the column before elution with a buffering cation such as Tris or histidine. This replaces most of the hydrogen ions associated with HA phosphates and moderates pH reduction upon introduction of sodium ions.

HA's primary contribution to the field has been aggregate removal, which it combines with highly effective removal of acidic contaminants. Elution with simple phosphate gradients offers adequate fragment and aggregate removal for some IgGs, with better than 10-fold reduction of non-antibody contaminants [190,196]. Elution with NaCl gradients at low phosphate concentrations reduces aggregate levels of most IgG preparations to less than 0.1%, even from initial levels as high as 40–60% [190–193,197–199]. NaCl gradients also reduce host cell protein contamination by 2 logs, DNA by more than 3, virus by more than 4, endotoxin by more than 4, and leached protein A to levels beneath the detection limit of current commercial assays.

Fragment and aggregate removal capability by either phosphate or NaCl gradients are improved in the presence of PEG [45]. PEG increases retention in proportion to solute size. In addition to improving resolution among fragments, intact native antibody and aggregates, PEG enhances virus removal by a factor of about 5 [200]. Morrison et al. [201,202] have explored the use of displacers that selectively weaken phosphoryl cation exchange or

calcium coordination to increase resolution and improve recovery of non-aggregated antibodies. They were able to achieve more than baseline resolution from preparations containing up to 40% aggregates, resulting in 100% recovery of non-aggregated antibody.

Murakami et al. [203] explored the use of HA modified with polyethyleneimine (PEI) and found that it increased retention of nucleotides and acidic proteins. PEI is believed to bind to HA phosphate and replace its native cation exchange ability with a PEI-mediated anion exchange functionality that works cooperatively with calcium coordination. Since calcium coordination is little affected by conductivity, this creates another class of salt-tolerant anion exchangers. IgG binding via calcium coordination can be weakened or suspended by the presence of modest phosphate concentrations.

HA is sometimes used for capture of IgMs and occasionally for IgGs [103,104,191]. Sodium chloride resistance by calcium coordination confers salt-tolerance that helps to achieve good antibody capacity without dilution, but stronger binding of DNA preferentially consumes capacity, requiring that the column be under-loaded to maintain purification performance and avoid product losses. Antibody purification with apatites was reviewed in [191], and aggregate removal in [190]. Refer to recent articles by Nakagawa et al. [204,205] and Wensel et al. [199] for HA retention relationships among subclasses and light chain types.

Charef et al. [206] reported a metal coordination mixed mode that is a throwback to the original discovery of IMAC. Metal immobilization on a solid phase was first observed on carboxy cation exchangers. Carboxyl groups are randomly distributed, leaving some spaced appropriately to approximate the configuration of carboxyl groups in chelating agents such as citrate, EDTA, and others. The rest remain simple cation exchange groups. Charef et al. discovered that IgG and other proteins exhibited different fractionation properties on a carboxy cation exchanger if it was saturated with different metal ions. Adsorption of copper or nickel caused polyclonal IgG to elute in two separate fractions. Adsorption of zinc retained only albumin. Adsorption of iron retained albumin and IgG.

3.3.6.3. Hydrophobic mixed modes. Hydrophobic mixed modes have been the primary focus in the search for alternatives to protein A. They have evolved along three lines. The first involves immobilization of already-existing ligands such as amino acids and textile dyes. Both are referred to as psuedobiospecific and psuedoaffinity ligands, as well as by their commercial or proper names [207,208]. The second approach can be described as employing variants or modifications of basic hydrophobic ligands with the intent of refining IgG specificity and/or reducing the requirement for salt in the binding buffer. Some examples are named for their proposed mechanism of IgG binding, like thiophilic interaction and hydrophobic charge induction chromatography (HCIC). Others are named for their structural features, or receive commercial names devoid of useful information. The third approach involves synthesis of complex ligands designed with the intent of achieving the pinpoint selectivity and high capacity of protein A with a more economical construct that can tolerate washing with concentrated NaOH. Examples are often referred to as bioaffinity ligands; some as biomimetics or just mimetics.

Fassina et al. [209–214] described a peptide mimetic designated TG19318, consisting of a tetradentate lysine core with identical cationic/hydrophobic tripeptides attached to each arm. It bound IgA, IgE, IgM, and IgG from all human subclasses. Binding capacity for polyclonal human IgG was reported up to 25 mg/mL; recovery 85–95%; purity 90–95%.

A group led by Lowe developed a biomimetic based on a tridentate triazine scaffold with anilino and tyramino substitutions [215–217]. They emphasized their goal to mimic a doublet of protein A hydrophobic residues in the binding interface with IgG [217]. Binding capacity based on purified polyclonal IgG was more than 50 mg/mL, but capacity for monoclonal antibodies produced by mammalian cell culture has not proven competitive [70,73,76,218]. This has been attributed to interference by nonspecific hydrophobic interactions with cell culture additives such as antifoams and steroids [5]. Zamolo et al. [219] and Horak et al. [220] reported that a triazole spacer was effective for reducing interference by the antifoam Pluronic F68. Dynamic binding capacity at 10% breakthrough was 11.4 mg/mL; recovery 86%. Boi et al. [221] immobilized the same ligand/spacer on a membrane, with the result of reducing capacity to barely 3 mg/mL (see Section 3.2). The best purity data also come from polyclonal antibodies produced in serum [222]. Rigorous studies characterizing purification of monoclonal IgG from cell culture supernatants reveal mediocre performance [218].

Yang et al. [223–225] described a cationic/hydrophobic hexapeptide ligand capable of site-specific recognition. It interacted with amino acid residues in a specific region of the third constant domain of IgG heavy chain. They reported 94% purity and 85% recovery. Host DNA was reduced by about 4 logs; host proteins by 2–4 logs. Dynamic binding capacities of this and other hexapeptides in the series were up to 20 mg IgG/mL. Whereas mimetics strive to offer the simple bind-wash-elute approach typical of protein A, obtaining the best capacity and purity with these peptide ligands required optimization of sodium chloride and caprylate concentrations. Other research with peptide ligands highlights the importance of hydrophobicity. An immobilized tetrapeptide lacking strongly hydrophobic residues produced electrophoretically pure monoclonal IgG, but dynamic binding capacities of only 4–9 mg/mL [226].

Immobilized tryptophan, phenylalanine, and histidine make no claims to site-specific recognition, but their capture results show similarities to both so-called bioaffinity and biomimetic ligands [227–230]. Denizli [227] remarked on the mixed mode nature of all three amino acids, noting their hydrophobicity, ability to participate in base stacking, hydrogen bonding, and electrostatic interactions. Naik et al. [230] reported the ability of immobilized tryptophan to achieve purity and recovery of 90% and 85% with polyclonal IgG from serum, and monoclonal IgG from cell culture supernatant. Dynamic binding capacity was not reported.

Thiophilic interaction chromatography and HCIC have been promoted for capture [231–233], but have not offered adequate performance to enter the mainstream [218,234]. HCIC has subsequently shown modest ability as a polishing tool, including aggregate removal [235–239], but its performance has been eclipsed by a support employing a N-benzyl-N-methyl ethanolamine ligand: dominantly a hydrophobic anion exchanger [235–240]. This combination provides a third pathway to create salt-tolerant anion exchangers. As in combinations with hydrogen bonding or metal coordination, the positive charge promotes binding of acidic host proteins, leached protein A, DNA, endotoxin, and virus. IgG interacts less strongly than these species, but development of conditions that achieve good contaminant reduction without compromising IgG recovery can be challenging. The ability of this ligand to reduce modest aggregate levels to less than 1% has prompted some to suggest the feasibility of 2-step purifications consisting of protein A followed by mixed mode [240], or CEC followed by mixed mode [86].

Others polishing candidates show promise but remain to be broadly evaluated. Riske et al. [241] followed protein A capture with immobilized Cibachron blue. Loaded with 180 mg IgG/mL, it reduced host protein contamination by 2–3 logs, reduced aggregates by up to 85%, and reduced antibody fragments to less than 0.1%. Bresolin et al. [242] and de Souza et al. [243] evaluated hexyl- and decylamino ligands that also exhibit broad-spectrum host protein removal. They reported remarkable 1-step flow-through purification of polyclonal IgG from serum, which highlights their

polishing potential for monoclonal IgG following capture by other means.

Efforts continue to develop a hydrophobic mixed mode that challenges or exceeds protein A. Mountford et al. [244] synthesized a heterocyclic that achieved aggregate removal coincident with high-purity capture. They emphasized that this was only one of an extensive new family of options. Characterization of its dynamic binding capacity with a significant diversity of monoclonal antibodies awaits, along with definition of recovery, purification performance, and aggregate removal capabilities at high IgG loads.

Recent reviews by Denizli [227], Roque et al. [245], and Ngo et al. [246] are pertinent to this and the following section. Denizli's review is especially valuable for its insightful discussion of retention mechanisms.

3.3.7. Bioaffinity chromatography

In this review, the term bioaffinity applies strictly to protein ligands of biological origin, the classic example being protein A. Protein A has three features that appear likely to maintain its dominance for the foreseeable future: induced fit, a flexible multivalent tentacle arrangement, plus three-decades-and-counting of vendor competition to maximize capacity. Claims of bioaffinity will likely continue to be made for aspiring synthetic ligands, but protein A embodies the ultimate hallmark of biological specificity: its binding at the cleft between the second and third constant domains of the heavy chain induces a conformational change in the second domain that causes the antibody to lock down on the protein A [247,248]. This is what permits a ligand with low non-specific binding properties to achieve strong specific binding. Each protein A molecule has five binding domains, each domain with roughly equivalent potency, separated by sections of flexible random coil that allow the respective domains to conform to and simultaneously bind multiple IgGs [249]. Vendors have achieved a 250% increase in dynamic binding capacity over first-generation diffusive particle-based products, with some now claiming to offer greater than 50 mg/mL.

Chemical engineering studies suggest that few opportunities remain to substantively increase the productivity of porous particle media [250–252]. Their key limitations are diffusive mass transfer and the fact that the protein A itself takes up much of the intrapore space. The dramatically higher throughput supported by monoliths and membranes suggests them as alternatives, but neither has yet offered the binding capacity to challenge particles [253–255]. Simple models nevertheless show that a single 8 L monolith with a capacity of 10 g/L can produce 20 kg of IgG in 27 h, versus a 35 L particle column with a capacity of 35 g/L producing 20 kg in 85 h [256]. The monolith, now having been used for 250 cycles, may be economically discarded, thereby avoiding sanitization, storage, and associated validation costs. Comprehensive cost models emphasize that the major expense of bioprocessing derives from the facility itself [257]. Short processing time supports better facility utilization and higher overall facility capacity, making monoliths even more attractive. The downside is that the combination of low capacity and high cycle count requires 3 times the buffer volume. Data-driven comparisons are needed to properly evaluate practical feasibility, but it is obvious that higher capacity would shift the advantage dramatically towards monoliths.

The random coil sequences in second-generation protein A ligands have been re-engineered to eliminate the residues most vulnerable to proteolysis and alkaline hydrolysis [258]. This reduces ligand leaching and permits sanitization with modest concentrations of sodium hydroxide [259]. Additives have been identified that, used in combination with NaOH, conserve 90% of initial IgG binding capacity for at least 50 cleaning cycles [260]. Recombinant modifications to the binding domains have eliminated Fab binding [261], with two favorable results: elution conditions are uniform among different IgGs, and they can be eluted at more moderate pH than from natural protein A [261]. Avoidance of extremely low pH reduces the potential for inducing aggregate formation.

Protein A elution with arginine further favors recovery of non-aggregated IgG (Section 4.1) [262,263]. Arginine also enhances virus inactivation [264–266]. The use of secondary washes has become a routine tactic for maximizing contaminant reduction (Section 4.2) [112,267–269]. pH gradient elution supports limited removal of aggregates [112,270–272]. See reviews by Vunnum et al. [273] and Hober et al. [258] for broader discussion.

Camellid mammals produce IgG analogs comprising only heavy chains. The variable region terminus (VHH) embodies antigen-binding characteristics analogous to the heavy/light chain terminus of most other mammalian IgGs. Recombinant bioaffinity ligands based on these domains offer purification performance similar to protein A [274–278], but even at best, they embody the same cost and productivity issues as protein A. Their main value is enablement: they allow a proven-effective purification paradigm to be extended to antibodies that do not consistently bind to protein A, such as IgG_3, IgA [279], and IgM.

An immobilized VHH ligand recognizing IgG from multiple species has made it possible to consolidate purification of mouse, rat, and other-species IgG on a single platform. A VHH ligand against bovine IgG eliminates it from other-species-IgG grown in bovine serum-supplemented culture media. Commercial availability of these ligands has quieted speculation that alternative bacterial ligands (protein L, protein P) might fulfill a prominent role in the field [5]. Protein G will likely persist, but IgG damage from its extremely low elution pH places it at a disadvantage to VHH ligands. They are also more tolerant of sanitization with NaOH [275].

4. Remedial purification

4.1. Prevention of aggregate formation

It was typical until the last decade to observe light-to-moderate turbidity during pH neutralization of protein A eluates. This was generally assumed to reflect aggregate formation due to conformational stress arising from exposure to very low elution pH. Precipitation was often not apparent while pH remained low because IgG adopts a stable alternative conformation under those conditions [280,281]. Formation of excess aggregates during pH neutralization has now been largely eliminated by elution with arginine. A succession of publications provides a comprehensive rationale for how it works [217,262,263,282–292]: a hydrophobic phenylalanine–tyrosine doublet in protein A comprises the largest portion of the binding interface with IgG [217]. Arginine interacts strongly with these residues, and accumulates at high concentration in their vicinity, thereby relaxing hydrophobic interactions between protein A and IgG, and moderating the pH required for elution [292]. As eluate pH is neutralized, condensation of arginine at hydrophobic sites forms a barrier against hydrophobic interactions among IgGs, leaving them to regain their native conformation at reduced risk of aggregate formation [292]. Aggregate levels in arginine eluates average 1–2% lower than those obtained with other eluents [262,263,282].

4.2. Dissociation of antibody-contaminant complexes

Many practitioners assume that the majority of non-antibody contaminants persisting after protein A chromatography derive from nonspecific interactions of those contaminants with protein A columns during sample loading, and their subsequent co-elution with the antibody. The assumption is largely accurate with silica

and controlled-pore glass supports [273], but in 2008 Shukla and Hinckley [268] published a study of a polymer-based protein A, documenting that more than 90% of the contaminating host cell proteins were carried through protein A as complexes with IgG that had formed in the cell culture supernatant. They dissociated and removed the majority of them with secondary washes that combined various proportions of urea, isopropanol, and Tween-80. This led them to suggest that the association was dominantly hydrophobic with a potential contribution by hydrogen binding. The greater importance of the study however, was that it revealed a previously unrecognized phenomenon with serious potential to affect purification performance and product quality.

Luhrs et al. [293] and Mechetner et al. [294] observed that antibodies against human histones formed stable complexes with host histones released from dead cells during cell culture. The bound host histones prevented the antibodies from binding their target human histones. The magnitude of the problem was proportional to the level of cell mortality at harvest. Conventional purification by protein A or protein G affinity chromatography failed to dissociate the foreign antigens. The phenomenon was further complicated by the fact that histones bind DNA, which is released from dead cells with histones in the form of chromatin. Host DNA and host histones imposed different kinds of aberrations on potency assays, resulting in erroneous estimates ranging from 15% to 558%. By applying a 2-stage method of passing clarified supernatant through an anion exchanger, then applying a 2 M NaCl wash before eluting the affinity columns, they were able to restore potency to the range of 90–101%.

Gagnon et al. [103] characterized chromatographic behavior of IgM–DNA complexes. DNA comprised up to 24% of the complex mass, but low levels of at least a dozen host cell proteins were apparent on reduced SDS-PAGE [103]. The complexes co-eluted with purified IgM on analytical SEC, indicating that the associated DNA fragments were small. Experimental data showed that complexes were associated through a combination of electrostatic interactions, metal coordination, and hydrogen bonding. Related studies suggest that van der Waals forces were also involved. Luscombe et al. [295] determined that van der Waals comprised 65% of the binding contacts between DNA and DNA-binding domains on proteins. The remainder was divided evenly between direct and water-mediated hydrogen bonds. DNA itself is very weakly hydrophobic, so hydrophobicity was considered unlikely to make a significant contribution to complex stability [103]. DNA and contaminating proteins were observed to form similar complexes with IgG in a follow-up study [296].

Rao and Pohl discovered that ferric ions create IgG populations with atypical early- and late-eluting peaks on a weak cation exchanger [297].

These studies provide broad documentation that antibodies and contaminants do not coexist in biological solutions solely as separate independent entities, and they warn of important consequences. IgM–DNA complexes with the highest DNA content failed to bind to CEC under conditions that supported 100% retention of purified IgM [103]. This was attributed to DNA-mediated neutralization of positively charged binding sites on the antibody. Complexes with intermediate DNA content eluted before purified IgM on CEC. HIC results paralleled CEC but were attributed to DNA fragments occluding hydrophobic sites on the antibody and imposing a hydrophilic influence in their place. Complexes eluted after IgM and before DNA on AEC. Complexes with low DNA content eluted in the same position as purified IgM on AEC, CEC, HIC, SEC, and bioaffinity.

No single method was able to achieve complete dissociation at high column loads, but all methods achieved some [103]. Bioaffinity with a strong secondary wash, AEC, and HA were the most effective [103,296]. The effectiveness of AEC and HA was attributed to the ability of their strong DNA interactions to competitively dissociate DNA from the antibody. A urea wash prior to elution of AEC improved dissociation. This was attributed to urea's ability to weaken hydrogen bonding between IgM and DNA without diminishing electrostatic interactions between DNA and the exchanger [103,298]. Only monolithic anion exchangers were able to dissociate complexes [103]. A porous particle exchanger merely fractionated them. The difference was attributed to greater than 10 times higher charge density on monoliths. NaCl, urea, and combined washes enhanced dissociation on HA [296]. The combination was suggested to simultaneously weaken hydrogen bonding and electrostatic interactions between the antibody and DNA, without weakening metal coordination bonds between DNA phosphates and HA calcium. An EDTA wash prior to elution of CEC also enhanced complex dissociation [103,297].

These studies suggest that complex composition, abundance, and stability vary most strongly with the characteristics of the antibody and the level of host cell mortality at harvest. More investigation is required to reveal how widely such complexes occur, how they affect purification performance, and specifically how they affect product quality. Shukla and Hinckley [268,299] suggested that complexed contaminants are the most relevant for development of anti-host protein assays because they are the most likely to persist through a purification process. The other side of that coin is that complexation could have potentially serious consequences for product quality and patient safety. Contaminants associated with antibodies in stable complexes could create novel antigenic determinants that favor development of therapy-neutralizing antibodies [299].

These findings collectively suggest simple but powerful revisions to the philosophy and practice of purification process development: assume that some portion of the product exists in stable associations with contaminants. Assume that such complexes have the potential to evade traditional purification methods. Favor chromatography methods and materials with elevated ability to dissociate complexes where practical. Employ dissociating washes where possible.

4.3. Restoration of native antibody from aggregates

Aggregates are a burden from many perspectives. Developing methods to remove them increases the expense of process development. Aggregate removal represents a direct loss of product [9]. That loss is compounded if poor fractionation requires sacrificing non-aggregated antibody to ensure adequate reduction of aggregate levels. If not removed completely, residual low-level aggregates may act as nucleation centers for creation of particles during product storage [300]. Restoration of native antibody could address all these concerns.

Pressure-induced disaggregation and refolding has been applied effectively with many proteins [301–305]. High pressure is believed to dissociate aggregates by insertion of water between hydrophobic protein–protein interfaces [306,307]. It also enhances electrostriction of water, to the extent of disrupting salt bridges [308,309], but hydrogen bonding and secondary structures remain unaffected [309–311]. Hydrostatic pressures of about 2000 bar cause disaggregation [301–308]. Roughly double that pressure is required to unfold native structures [311–314]. An Fc-fusion protein was exposed to pH 3 to create 14.5% aggregates [301]. Subsequent treatment at 2000 bar in the absence of NaCl reduced aggregate content to less than 1%. Treatment in the presence of 250 mM NaCl reduced aggregates to only 6%, highlighting a necessary method development component to the technique.

Seefeldt et al. [301] compiled a comparison of disaggregation efficiency by pressure and chaotrope-based methods. Pressure was disproportionately more effective for the majority of proteins, but Xu et al. [315] showed that chaotrope-mediated disaggregation

may have practical value nevertheless. They described 50% dissociation of IgG aggregates when protein A was eluted with guanidine. The recovered antibody was comparable to the reference with respect to physicochemical and pharmacokinetic properties.

4.4. Conservation or restoration of native disulfide pairing

A surge of recent publications has exposed a vipers' nest of mechanisms that cause disulfide anomalies in antibodies. These include improper assembly in the endoplasmic reticulum [316], formation of trisulfides during cell culture production [317], disulfide cleavage by oxidation or beta elimination [318–320], enzymatic reduction of disulfide bonds during harvest and early downstream processing [321,322], conversion to thioether bonds [323], and promotion of disulfide scrambling, in some cases producing aberrant product conformations [317], in other cases forming covalent IgG aggregates, leading to formation of particulates [99], and potentially leading to formation of covalent complexes with sulfhydryl-bearing contaminants [321].

The most extensive reduction of disulfides has been encountered with excessive cell lysis during harvest, in one case causing 90% product loss [321,322]. This was traced to liberation of thioredoxin and/or thioredoxin-like enzymes [321]. The pentose-phosphate shunt enzymes glucose-6-phosphate dehydrogenase and hexokinase were also involved. Disulfide reduction was halted by a variety of agents that inhibited any of the three enzymes. Their effects were globally blocked by sparging with air to maintain an oxidizing redox environment, or through stabilization of disulfide bonds by diminishing pH to a value below 6.0. Lacking enzyme inhibition in the feed stream, disulfide reduction continued during sample loading on protein A. The enzymes did not bind protein A and were diverted to the flow-through, thereby preventing subsequent loss.

Brych et al. [300] reported that covalent IgG aggregates form at air–liquid interfaces, leading to eventual formation of particles. They blocked this pathway by adding the surfactant polysorbate-20.

Aono et al. [317] discovered that interchain trisulfides form spontaneously during cell culture due to the interposition of sulfur from hydrogen sulfide gas that evolves naturally from cell metabolism. Instability of trisulfides leaves sulfhydryl interactions in a fluid state that facilitates incorrect pairing. Inclusion of 1 mM cysteine in the protein A wash displaced the extra sulfur atoms. This reduced trisulfide content by a factor of 13 and increased recovery of correctly disulfide-paired IgG to greater than 95%.

5. Concluding remarks

The authors of the 2007 review *Future of Antibody Purification* predicted that industrial practice would not change much in the ensuing five years [5]. They were largely correct with respect to industrial practice, but far from it with new technology development, and the next five years will likely see significant changes even in manufacturing circles.

The transition has already begun, with many companies evaluating mixed modes as replacements for anion exchange chromatography. Hydrophobic anion exchangers have a head start, but hydrogen bond-enhanced anion exchangers seem likely to take over the momentum. Method development with the former has turned out to not to be intuitive, and often imposes a compromise between final product quality and recovery. The latter have the familiar feel of traditional anion exchangers, with the main difference being their ability to bind acidic contaminants over a much wider range of conditions. Their availability on membranes and monoliths offers radically higher throughput than porous particles. Productivity of displacement mode applications on these media could be extraordinary.

Aggregate removal will continue to influence choice of intermediate purification methods. Hydroxyapatite and hydrophobic anion exchangers stand out at present because of their coincident ability to reduce levels of DNA, virus, leached protein A, and acidic host proteins, but neither has proven entirely satisfactory. The continuing emergence of new mixed modes will probably keep this area fluid for some time.

Implementation of secondary washes seems likely to spread quickly, in part because of their ability to dissociate antibody-contaminant complexes, but equally because they do not require a fundamental change to established materials or practice. They are already used widely with protein A affinity. They can be added seamlessly to other bind-elute chromatography steps. Their ability to drive down contaminant levels is substantial. Their ability to restore correct disulfide pairing makes them even more valuable. It is fair to predict that their broader application will result in higher product quality, better reproducibility, and better long-term clinical performance.

Changes in capture technology appear to be farther off but likely to follow a similar path of evolution rather than revolution. The crucial limitation with protein A is productivity, not cost, and that limitation derives from porous particles in single fixed-bed columns. Re-engineering the channel architecture of protein A monoliths to optimize capacity for IgG could increase productivity to a level that would make their implementation obligatory. Simulated moving bed chromatography systems have already demonstrated the ability to compound productivity of protein A on porous particles, and could elevate it to a much higher level with monoliths.

Capture by cation exchange chromatography or ultrafiltration with positively charged membranes could make inroads with highly alkaline antibodies, but if they were going to seriously challenge protein A, it would already have happened.

Technologies with revolutionary potential await enabling developments. Aqueous two-phase extraction offers competitive capacity, host protein removal, and IgG recovery, but it remains unclear if it can offer sufficient overall benefit to compel its implementation. Second-generation fluidized beds employing mixed mode ligands offer promising results, but capacity and purification performance must improve to make the system a credible contender. Magnetic nanoparticle applications promise capacity and product concentration beyond any method except precipitation, in addition to bypassing clarification, but they await introduction of cost-effective media and scalable hardware systems. Ultramacroporous monoliths also bypass clarification, and support faster throughput than any other option, but they need to demonstrate compelling capacity.

The real unknown is what might be lurking beyond the borders of the map, or more likely lying dormant among the knowledge gains of the past. Protein A was known for many years before that magic moment when its potential was recognized and it transformed the field of antibody purification. It could happen again.

Acknowledgement

Thanks to Dr. Kornelia Schriebl for assistance with developing the section on disulfide anomalies.

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