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Nonspecific interactions of chromatin with immunoglobulin G and protein A, and their impact on purification performance

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ABSTRACT

Chromatin released from dead host cells during in vitro production of IgG monoclonal antibodies exists mostly in complex hetero-aggregates consisting of nucleosomal arrays (DNA + histone proteins), non-histone proteins, and aberrant forms of IgG. They bind immobilized protein A more aggressively than IgG, through their nucleosomal histone components, and hinder access of IgG to Fc-specific binding sites, thereby reducing dynamic binding capacity. The majority of host cell contaminants in eluted IgG are leachates from chromatin hetero-aggregates that remain bound to protein A. Formation of turbidity in eluted IgG during pH titration is caused by neutral-pH insolubility of chromatin hetero-aggregates. NaOH is required at 500 mM to remove accumulated chromatin. A chromatin-directed clarification method removed 99% of histones, 90% of non-histone proteins, achieved a 6 log reduction of DNA, 4 log reduction of lipid-enveloped virus, and 5 log reduction of non-enveloped retrovirus, while conserving 98% of the native IgG. This suspended most of performance compromises imposed on protein A. IgG binding capacity increased ~20%. Host protein contamination was reduced about 100-fold compared to protein A loaded with harvest clarified by centrifugation and microfiltration. Aggregates were reduced to less than 0.05%. Turbidity of eluted IgG upon pH neutralization was nearly eliminated. Column cleaning was facilitated by minimizing the accumulation of chromatin.

1. Introduction

Protein A affinity chromatography provides a worthy foundation for IgG purification but it has not supported the single-step purification performance that was expected when it was introduced. Investigators have since documented that interactions between feed stream contaminants and various components of the system contribute to the lower-than-hoped-for performance. Some have studied the contribution of nonspecific contaminant-base matrix interactions [1–3]. Some have studied the contribution of nonspecific contaminant-IgG interactions [3,4]. Some have studied nonspecific contaminant interactions with protein A itself [4,5]. Numerous pre-elution wash formulations have demonstrated the ability to reduce post-protein A host cell protein (HCP) levels [1–3]. More than two dozen approved IgG therapeutics purified with protein A and a pair of follow-on chromatography steps suggest that protein A performance is not a handicap. The persistent absence of licensed products purified by protein A and a single follow-on chromatography step suggests it is.

Gan et al. [6] recently reported that chromatin catabolites expelled from dead host cells formed non-immunospecific complexes and aggregates with IgM monoclonal antibodies that were so stable they survived exposure to 4 M guanidine. Chromatin-derived species included nucleosomal arrays and individual nucleosomes, histone proteins and DNA. This parallels the known pattern of chromatin catabolism for apoptotic cells [7–9]. The 147 base pair (bp) segment of DNA wrapped 1.65 times around the circumference of the 11 nm × 5.5 nm cylinder comprising each nucleosome creates a perimeter of negative charges, geometrically isolated from the alkaline hydrophobic surfaces of the core-octamer histones ($(H2A,H2B,H3,H4)_2$) presented at the cylinder top and bottom [10–13]. Individual nucleosomes are connected in linear arrays by 20–80 bp linker-DNA sequences associated with histone H1.

Limited data suggest that chromatin degradation products form strong associations with IgG as well as IgM [6]. Shukla and Hinckley [3] reported that 90% of the HCPs co-eluting with IgG from protein A do so because they form stable associations with IgG during cell culture production. Nogal et al. [4] concluded that antibodies

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associate with a particular subset of contaminants. These observations point to the possibility that the offending contaminant subset could be chromatin remnants. Shukla and Hinckley further observed that pre-elution washes containing various additives reduced aggregate levels in the eluted IgG. This hints that the multiple surface chemistries presented by nucleosomes could promote aggregation through nonspecific interactions, and might also be involved in turbidity formation during pH neutralization of IgG eluted from protein A columns.

Another finding by Gan et al. [6] was that chromatin and chromatin-IgM aggregates bound very strongly to a cation exchanger. Guanidine (3–4 M) was required to remove them after 2 M NaCl failed. They suggested that binding was mediated through nucleosomal histone proteins, based on experimental data and independent documentation that histones require 4 M guanidine for elution from a cation exchanger [14]. Immobilized protein A can be considered a cation exchanger, thanks to its carboxyl-rich-mediated isoelectric point (p*I*) of ~5.1 [15–18]. This suggests protein A could also embody a strong attraction for nucleosomes. The hydrophobicity of protein A [2,16–19], might enhance that interaction since histone proteins are documented to be strongly hydrophobic in addition to being alkaline [20].

Nonspecific chromatin binding to protein A might logically produce two negative outcomes: it might reduce accessibility to Fc-specific binding sites and thereby reduce IgG capacity, and it might reduce purification performance by co-eluting or leaching subsets of contaminants during IgG elution. Gan et al. [6] provided indirect support for the latter by documenting leaching of DNA from cation exchange-bound chromatin during IgM elution with NaCl. Gagnon et al. [21] showed previously that IgM-DNA complexes contained at least a dozen PAGE-coomassie-detectable contaminating proteins in addition to host histones. This warns that chromatin could serve as a vehicle for smuggling a broader diversity of contaminants through a purification procedure [6,21].

A chromatin-directed clarification method removed 99% of the chromatin and the majority of aggregates from IgM [6]. Supersaturated allantoin, with a crystal density of 1.45 g/mL, was suggested to scavenge and sediment large molecular assemblages. Subsequent work has shown that allantoin crystal binds such assemblages by multi-point hydrogen bonding [22,23]. Non-precipitating concentrations of ethacridine were suggested to weaken protein-DNA interactions, with the effect of facilitating aggregate dissociation [6]. This is consistent with the action of related intercalating structures such as doxorubicin and daunomycin that conformationally disrupt DNA and induce partial unwinding with the effect of dissociating it from histones [24–27]. Addition of multimodal particles enhanced removal of aggregates and residual additives. Antibody recovery was 98–99%. Here, we exploit a variation of this treatment as an experimental control to investigate the effects of chromatin on IgG purification with protein A affinity chromatography.

2. Materials and Methods

2.1. Reagents and Equipment

Buffers, salts, and reagents were obtained from Sigma-Aldrich (St. Louis, MO), except allantoin, which was obtained from Merck Millipore (Darmstadt, Germany). UNOsphere™ Q, UNOsphere SUPrA™, Chelex™-100 (Chelex), MacroPrep™ High-Q (MPHQ), MacroPrep High-S (MPHS), and MacroPrep t-Butyl (MPtB) were obtained from Bio-Rad Laboratories (Hercules, CA). Toyopearl AF-rProtein A-650 was purchased from Tosoh Biosience (Tokyo, Japan). MabSelect SuRe™ and Capto™ Adhere were purchased from GE Healthcare (Uppsala, Sweden). ProSep® vA was purchased from Merck-Millipore (Billerica, MA, USA). Chromatography media were packed in XK or Tricorn™ series columns (GE Healthcare). Chromatography experiments were conducted on an ÅKTA™ Explorer 100 or Avant 25 (GE Healthcare).

2.2. Experimental Methods

An IgG monoclonal antibody immunospecific for human epidermal growth factor receptor 2 was produced by mammalian cell culture using a tricistronic vector developed by Ho et al. [28]. The amino acid sequence of IgG produced by the clone is identical to Trastuzumab (Herceptin®, Roche, South San Francisco, CA, USA). Antibody was produced in 15–30 day fed-batch cultures harvested at 20–50% cell viability.

IgM clone 85 was produced in serum-free media by mammalian cell culture and harvested at less than 20% cell viability to maximize productivity. Cells were removed by centrifugation and the supernatant was filtered through a 0.22 μm membrane (Nalgene® Rapid-Flow Filters, Thermo Scientific, Waltham, MA), then stored at 2–8°C.

"Traditional" harvest clarification was performed by centrifugation at 4000×*g* for 20 min at room temperature, followed by filtration through 0.22 μm membrane. We alternatively conducted IgG clarification based on a method described for IgM cell cultures [6]. In brief, an equal mixture was made of MPHQ, MPHS, MPtB, and Chelex, and equilibrated by washing with PBS (50 mM phosphate, 150 mM NaCl, pH 7.2). Allantoin was added to cell-containing harvest or harvest prepared as above at a final concentration of 1%, then 0.025% ethacridine. The suspension was mixed for 60 min, then equilibrated mixed particles were added at a proportion of 5% (v/v). The suspension was mixed for 4 h up to overnight at 4 °C, solids were removed by centrifugation, and the supernatant microfiltered or passed through a Sartoclear® PC1 Cap depth filter capsule (Sartorius-Stedim, Göttingen, Germany).

Dynamic binding capacity was determined on 1 mL (5 × 50 mm) Toyopearl protein A columns packed and run at a linear flow rate of 150 cm/h (0.5 mL/min). This gave a flow-through residence time of 2 min. The columns were equilibrated with PBS then put off line. The UV monitor was zeroed. Sample at physiological conditions was pumped through the inlet line until the UV signal indicated that antibody concentration at the entrance of the UV monitor was the same as concentration of the feed. This UV value was taken to represent 100% breakthrough. The column was put in line and monitored until UV signal indicated at least 20% breakthrough (BT). In cases where the column was loaded with unpurified harvest, baseline (0% BT) was taken to be the level at which the flow-through reached a reasonably stable equilibrium.

IgG aggregates remaining after protein A affinity chromatography were removed in some cases with Capto adhere. The sample and column were equilibrated to 50 mM Tris, 1 M NaCl, pH 8.0. 10 mg IgG was loaded per mL of gel, the column was washed with equilibration buffer, then the IgG was eluted by reducing NaCl concentration to 0.3 M. The column was cleaned with 4 M guanidine. In other cases, aggregates were removed by anion exchange chromatography in void exclusion mode as described by Nian et al. [29], using UNOsphere Q equilibrated to 50 mM Tris, pH 8.2.

2.3. Analytical methods

Reduced urea–SDS–PAGE was performed on 12% Mini-PROTEAN® TGX™ precast gels (Bio-Rad). Protein bands were with visualized with coomassie to guide band cutting for mass spectrophotometric analysis (see below), or silver to detect low-level proteins. Western blotting was performed to identify IgG-Fc-containing bands with Fc-specific anti-human IgG1-HRP (Sigma-Aldrich), or histones with anti-Histone H3-HRP antibody