Development of a Non–Affinity Based Purification Platform for Neutral/Basic IgMs

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Antibody Purification

Abstract

The biological properties of IgM antibodies make them very effective vehicles for in vitro diagnostics and therapeutics. However, purification of IgM antibodies is far more difficult than that for the more common IgG antibodies due to their complex structural and biochemical characteristics. Furthermore, the commonly available affinity chromatography supports (Protein A and Protein G) have little or no binding capacity for IgM.

Here we propose a non–affinity based two-column platform protocol for IgM purification. The strategy utilizes the cation exchange (CEX) resin, Nuvia™ S, for capture and the mixed-mode media, CHT™ Ceramic Hydroxyapatite, for polish purification. This workflow is suitable for purification of neutral and basic IgMs. We optimized the protocol with three different IgMs and present reducing and nonreducing PAGE images of the purified samples. This strategy is simple, scalable, and efficient and thus well suited for purification of diagnostic IgMs.

Introduction

Immunoglobulin M (IgM) is the first antibody to be produced in an immune response. The use of IgM antibodies for in vitro diagnostics and therapy is on the rise, especially for cancer, infectious diseases, and stem cell therapies. Although IgM is composed of pentameric complexes of Ig monomers, purification strategies used for IgG cannot be translated directly for IgM purification because of the multiple differences between IgG and IgM. IgMs are very sensitive to pH conditions, making them highly susceptible to degradation or precipitation, are soluble in a narrower range of conditions, and are more heavily glycosylated than IgGs. Purification of IgMs is made even more complex by their labile nature and complex physicochemical properties. In addition, their large size (970 kD) decreases their diffusion constant relative to IgG. As a result, IgM purification generally requires slower flow rates to obtain maximum binding and separation in a packed bed. In addition, most affinity matrices show poor binding of IgM and IgM is generally unstable in the acidic conditions required for elution.

To overcome these challenges, we designed a scalable two-column non–affinity based platform process for IgM manufacturing (Figure 1). Typically, IgMs are more charged than IgGs and hence are retained more strongly on ion exchangers (IEX). IgMs also bind more strongly to hydroxyapatite-based media relative to IgGs and many contaminants. CHT also has the advantage of operating well at neutral pH and generally requiring mild salt concentrations for elution (Gagnon et al. 2014). The newly designed purification strategy exploited this binding of IgMs to IEX and hydroxyapatite-based media. We show that by optimizing buffer pH and conductivity we can purify neutral and basic pentameric IgMs from expression-related impurities, such as transferrin (TF) and serum albumin (SA), and product-related impurities, such as free IgM light chain (LC) and monomeric IgMs, using Nuvia S Resin and CHT Media.

Fig. 1. New two-column non–affinity based purification platform strategy for neutral/basic IgM purification.

Materials and Methods

General

IgMs were expressed in HEK cells via transient expression. Foresight™ Prepacked Columns containing Nuvia S (catalog #7324720) and CHT Type II, 40 μm (CHT-II-40, #7324736) were used and column chromatography was conducted on an NGC Discover™ 10 Pro Chromatography System (#7880011). Protein fractions were analyzed by SDS-PAGE using Mini-PROTEAN® TGX Stain-Free™ Protein Gels (4–20% linear gradient, #4568093), which were imaged on the ChemiDoc™ MP Imaging System (#17001402).

Capture of Neutral/Basic IgMs with Nuvia S Resin

Three different IgMs were used, each with a calculated pI ≥7 as shown in Table 1. IgM-containing cell culture supernatants were adjusted to the required pH with 0.2 M acetic acid before loading. Initial elution was performed at pH 5.0. Nuvia S Foresight Columns were equilibrated with 10 column volumes (10 CV) of 50 mM sodium acetate, pH 5.0 (buffer A). The adjusted IgM samples were applied to the columns at 120 cm/hr. The columns were then washed with 10 CV of buffer A. Elution was performed with a 20 CV gradient of buffer A to 50 mM sodium acetate, pH 5.0 + 0.5 M NaCl (buffer B). Following this, optimization of the capture protocol was performed at pH 1.5 units lower than the respective IgM pIs with the same linear gradient.

Table 1. Respective pIs of the three purified IgMs.

| IgM	pI
IgM-1	7.5
IgM-2	7.2
IgM-3	7.0

Polish of Neutral/Basic IgMs with CHT-II-40 Media

CHT-II-40 Foresight Columns were equilibrated with 10 CV of 20 mM sodium phosphate, pH 7.0, 1 M NaCl (buffer E). Samples from the capture step were pretreated with buffer E and then loaded at 120 cm/hr onto the CHT Column. The columns were then washed with 15 CV of buffer E. The purified IgMs were eluted with a 20 CV linear gradient of buffer E to 500 mM sodium phosphate, pH 7.0 + 1 M NaCl (buffer F). Various purified fractions were analyzed by SDS-PAGE gel electrophoresis.

Results and Discussion

Capture of Neutral/Basic IgMs on Nuvia S Resin

Nuvia S is a high-capacity strong cation exchanger that has been used to successfully purify monoclonal antibodies (He et al. 2012, Ng and Snyder 2012) and other biomolecules (see Bio-Rad bulletin 6869 for a list of references). Since the calculated pIs of the three IgMs were ≥7, they are expected to carry a positive charge at pHs lower than their pIs and therefore would be captured by a cationic exchanger. The use of Nuvia S Resin eliminated the need for potential purification of any leached Protein A from downstream samples, which is a limitation of using a Protein A affinity resin for capture. The three IgM antibodies were initially purified with a buffer at pH 5.0 and a linear salt gradient to 500 mM NaCl. At this pH, all the impurities (TF, SA, LC, and monomeric IgMs) were seen in the same fractions as the multimeric IgMs. IgM-3 fractions were relatively purer (Figure 2) than the IgM-1 (Figure 3) and IgM-2 (data not shown) fractions. We hypothesized that a pH closer to the actual pI of the IgMs would lead to cleaner

samples. Accordingly, we chose a pH 1.5 units lower than the pIs of each IgM, and continued the elution with a linear gradient of 500 mM NaCl. This led to partial removal of the impurities (TF, SA, and LC) for the three IgMs as shown by the representative IgM-1 data (from purification at pH 6.0, Figure 4). Not only is this a relatively mild pH, but less acidic conditions also generally require lower salt concentrations during elution. Lower salt concentrations can often eliminate the need for downstream dilution before loading onto the next column. This goal was achieved by the optimized protocol.

A
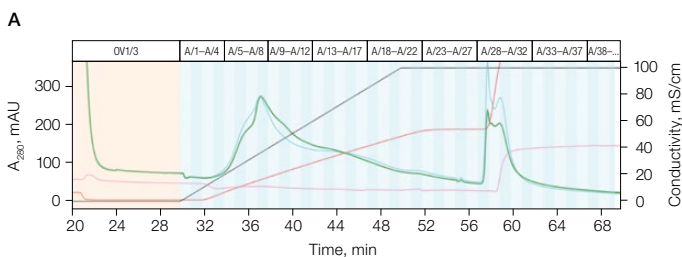
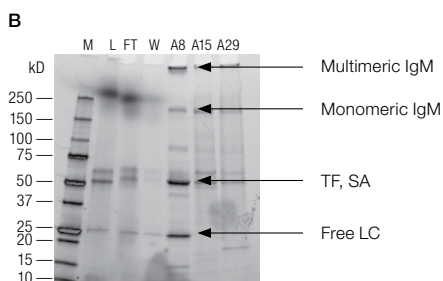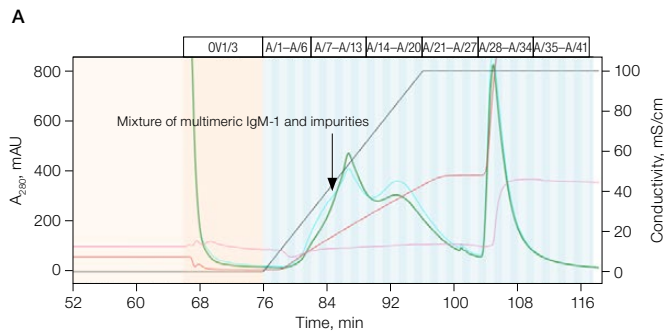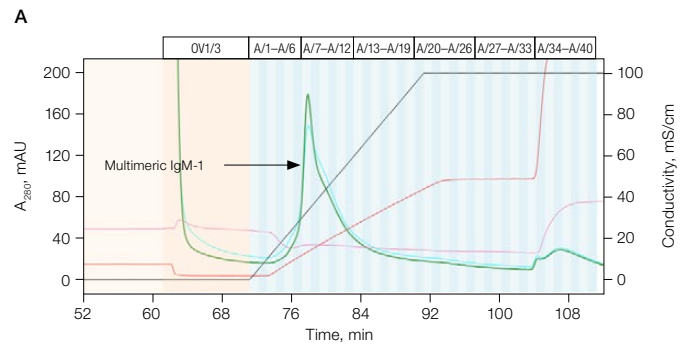| Column:	Nuvia S, 1 ml Foresight™
Sample:	IgM-3 cell culture supernatant, adjusted to pH 5.0 with 0.2 M acetic acid, 3 mg IgM-1 in 66 ml
Flow rate:	120 cm/hr
Equilibration:	50 mM sodium acetate, pH 5.0, 10 CV
Postloading wash:	50 mM sodium acetate, pH 5.0, 10 CV
Elution:	Buffer A: 50 mM sodium acetate, pH 5.0
	Buffer B: 50 mM sodium acetate, pH 5.0, 0.5 M NaCl
	A to B linear gradient, 0–100%B, 20 CV

B

Fig. 2. Purification of IgM-3 on Nuvia S at pH 5.0. **A**, chromatography run of IgM-3 on Nuvia S at pH 5.0 with a constant linear gradient of 500 mM NaCl. A₂₆₀ (—); A₂₈₀ (—); conductivity (—); %B (—); pH (—). **B**, collected fractions were analyzed by nonreducing PAGE. Equal volumes (22.5 µl) of each fraction were loaded on the gel. M, prestained protein standards; L, load; FT, flowthrough; W, wash; A8–A29, elution fractions from 2A.

Polish Purification of Neutral/Basic IgMs on CHT-II-40 Media

CHT Ceramic Hydroxyapatite is a mixed-mode media widely used in monoclonal antibody polishing (see Bio-Rad bulletin 6870 for a list of references) to remove aggregates, endotoxins, host cell proteins (HCPs), and DNA (Gagnon et al. 2006). CHT Type II has a very low affinity for albumin and is particularly well-suited for the purification of many species and classes of immunoglobulins. Biomolecules can bind to

A

Column:	Nuvia S, 1 ml Foresight
Sample:	IgM-1 cell culture supernatant, adjusted to pH 5.0 with 0.2 M acetic acid, 3 mg IgM-1 in 66 ml
Flow rate:	120 cm/hr
Equilibration:	50 mM sodium acetate, pH 5.0, 10 CV
Postloading wash:	50 mM sodium acetate, pH 5.0, 10 CV
Elution:	Buffer A: 50 mM sodium acetate, pH 5.0
	Buffer B: 50 mM sodium acetate, pH 5.0, 0.5 M NaCl
	A to B linear gradient, 0–100%B, 20 CV

B
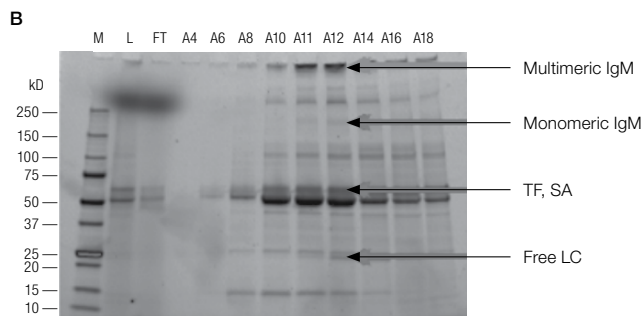
Fig. 3. Purification of IgM-1 on Nuvia S at pH 5.0. A, chromatography run of IgM-1 on Nuvia S at pH 5.0 with a constant linear gradient of 500 mM NaCl. A_{260} (—); A_{280} (—); conductivity (—); %B (—); pH (—). **B**, fractions collected from the chromatography run in 3A were analyzed by nonreducing PAGE. Equal volumes (22.5 µl) of each of the fractions were loaded on the gel. M, prestained protein standards; L, load; FT, flowthrough; A4–A18, elution fractions from 3A.

A

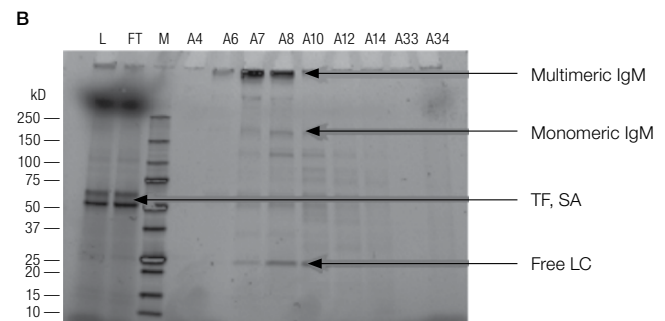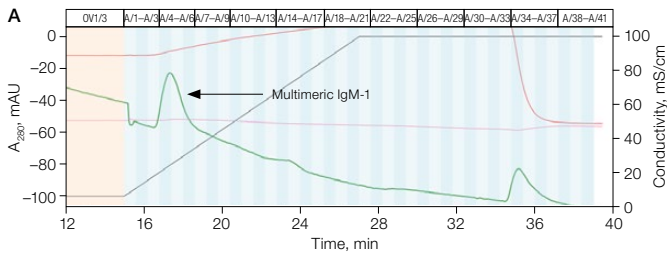| Column: | Nuvia S, 1 ml Foresight |
Sample:	IgM-1 cell culture supernatant, adjusted to pH 6.0 with 0.2 M acetic acid, 3 mg IgM-1 in 62 ml
Flow rate:	120 cm/hr
Equilibration:	50 mM sodium acetate, pH 6.0, 10 CV
Postloading wash:	50 mM sodium acetate, pH 6.0, 10 CV
Elution:	Buffer C: 50 mM sodium acetate, pH 6.0
	Buffer D: 50 mM sodium acetate, pH 6.0, 0.5 M NaCl
	C to D linear gradient, 0–100%D, 20 CV

B

Fig. 4. Purification of IgM-1 on Nuvia S at pH 6.0. A, chromatography run of IgM-1 on Nuvia S at pH 6.0 with a constant linear gradient of 500 mM NaCl. A_{260} (—); A_{280} (—); conductivity (—); %B (—); pH (—). **B**, fractions collected from the chromatography run in 4A were analyzed by nonreducing PAGE. Equal volumes (22.5 µl) of the fractions were loaded on the gel. L, load; FT, flowthrough; M, prestained protein standards; A4–A34, elution fractions from 4A.

it by calcium affinity and/or CEX interactions (see Bio-Rad bulletin 6902 for an overview of CHT chemical interactions). The carboxyl or phosphate groups on biomolecules bind to the calcium in CHT, while amino groups or other positively charged moieties bind via CEX to the phosphate groups. Since hydroxyapatite binds IgM strongly at physiological pH and conductivity, mild, nondenaturing conditions can be used for elution. We used a phosphate gradient for IgM elution. This resulted in monomeric and multimeric IgM species in the same fraction (data not shown). We then introduced a 1.5 M NaCl salt wash prior to the phosphate gradient, which resulted in the separation of the multimeric IgM from all impurities (data not shown). The requirement of both a salt and a phosphate gradient indicates that the IgMs are interacting with CHT by both affinity and CEX interactions. These dual interactions

allowed for optimal polishing in a single step. In order to optimize the salt gradient further, similar washes with 0.4 and 1 M NaCl were performed prior to the elution. The multimeric IgM eluted in a separate fraction with minimal contaminating proteins at 1 M NaCl (Figure 5).

Application of the Optimized Protocol to the Neutral/Basic IgM Purifications

The final optimized protocol included capture with Nuvia S at a pH value 1.5 units lower than the IgM's pI with a constant linear gradient of 500 mM NaCl and polish with CHT using 1 M NaCl in a 20–500 mM $NaPO_4$ gradient. The optimized protocol yielded purified fractions of all three IgMs tested (Figure 6), with minimal product- and process-related impurities, making this an ideal platform for the non–affinity based purification of neutral/basic IgMs.

A

Column: CHT Type II, 40 µm, 1 ml Foresight
Sample: Nuvia S elution pool, buffer exchanged to buffer E
Flow rate: 120 cm/hr
Equilibration: 20 mM sodium phosphate, pH 7.0, 1 M NaCl, 10 CV
Postloading wash: 20 mM sodium phosphate, pH 7.0, 1 M NaCl, 15 CV
Elution: Buffer E: 20 mM sodium phosphate, pH 7.0, 1 M NaCl
Buffer F: 500 mM sodium phosphate, pH 7.0, 1 M NaCl
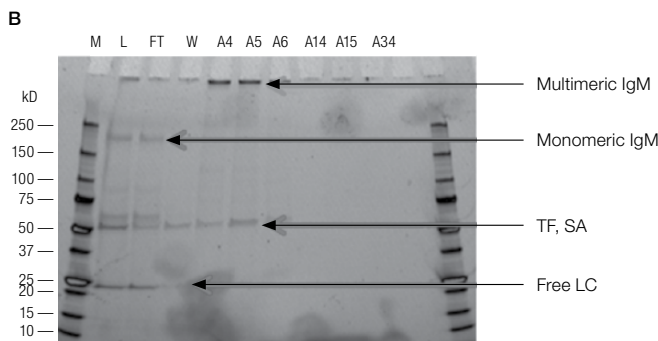E to F linear gradient, 0–100%F, 20 CV

B

Fig. 5. Purification of IgM-1 on CHT-II-40. A, chromatography run of the IgM-1 on CHT-II-40 with a 20–500 mM phosphate and 1 M NaCl gradient. A_{260} (—); A_{280} (—); conductivity (—); %B (—); pH (—). B, fractions collected from the chromatography run in 5A were analyzed by nonreducing PAGE. Equal volumes (22.5 µl) of each of the fractions were loaded on the gel. M, prestained protein standards; L, load; FT, flowthrough; W, wash; A4–A34, elution fractions from 5A.

Conclusions

The non–affinity based purification protocol proposed here constitutes a new platform process for diagnostic IgM purification. It involves only two steps and is suitable for scalable purification. While the two-step purification is designed to meet purity requirements for manufacture of diagnostic IgMs, therapeutic targets generally require significantly lower levels of process- and product-related impurities. For such purposes, this platform could be extended with a suitable third purification step. Using

these state-of-the-art media with high capacity and selectivity also helped overcome the affinity-based challenges of low binding and/or recovery during IgM purification. Pending further testing, this purification platform could also be used for purification of acidic IgMs, Fab fragments, diabodies, and bispecific antibodies.
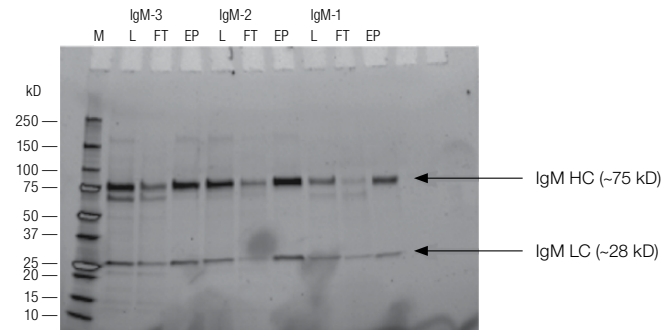

Fig. 6. Analysis of the neutral/basic IgMs purified on Nuvia S and CHT-II-40 Columns. Fractions collected during the chromatography run using the optimized protocol were analyzed by SDS-PAGE. Equal volumes (22.5 µl) of each fraction were loaded on the gel. M, prestained protein standards; L, load; FT, flowthrough; EP, eluted protein; HC, heavy chain; LC, light chain.

Acknowledgments

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