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Dissociation and fractionation of heavy and light chains from IgG monoclonal antibodies

Pete Gagnon [a,*], George Rodriquez [b], Simin Zaidi [b]

[a] Bioprocessing Technology Institute, 20 Biopolis Way #06-01 Centros, 138668, Singapore
[b] Avid Bioservices, 14282 Franklin Avenue, Tustin, CA 92780, USA

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ABSTRACT

A basic method for dissociation and fractionation of monoclonal IgG heavy and light chain is described. It employs less noxious and hazardous reagents than the classical mercaptoethanol/propionic acid process and replaces size exclusion chromatography with cation exchange on a monolith to improve productivity. Significant scope remains to refine the conditions. The method can be applied to other disulfide bonded proteins with significant affinity for cation exchangers.

1. Introduction

At the dawn of molecular immunology, there was great interest in isolating light chain (LC) and heavy chain (HC) from IgG for individual characterization and to explore the dynamics of their association. A method was described in 1962 that employed reduction with mercaptoethanol and subsequent fractionation by size exclusion chromatography (SEC) in 1 M propionic acid [1]. The method required a full day at best and needed to be run in a fume hood. The strong denaturing environment was necessary to prevent spontaneous re-association of fragments. Otherwise, even when reduction was followed by alkylation to prevent reformation of disulfide bonds, other forces conspired to re-assemble "intact" functional IgG [2].

Interest persists in purification of heavy and light chain from monoclonal IgG, although for different reasons. For some applications, blocking of reduced sulfides can be tolerated. For others, residual iodoacetamide or *n*-ethylmaleimide may complicate interpretation of experimental results. In all cases, an alternative to the slow process of SEC is to be greatly desired, along with avoiding conditions that require a fume hood. We reasoned that urea might reduce reliance on acid, and since urea is nonionic, it might be possible to separate dissociated LC and HC by cation exchange (CX) chromatography. Most IgGs bind CX well at pH values of 4.5–6.0 and should bind even more strongly at lower pH values.

The mass of LC and HC are only about 25 and 50 kDa respectively. These low molecular weights correspond to reasonably rapid diffusion constants, and seem unlikely to impair fractionation performance even on porous particle-based media. 8 M urea however, increases viscosity by a factor of about 1.74 over water at 25 °C, and diffusivity diminishes in direct proportion to viscosity [3]. Flow rate could be reduced proportionally to conserve fractionation performance, but convective mass transfer should be unaffected by viscosity. This suggests application of perfusive particle-based media or monoliths. Whereas perfusive particles remain dominantly diffusive, monoliths are dominantly convective. In addition, they support flow rates of 5–10 column volumes (CV) per minute, which permit faster exploration of experimental conditions than either alternative. We chose a monolith for both reasons.

2. Materials and methods

All experiments were conducted on an AKTA™ Explorer 100 (GE Healthcare). Urea, dithiothreitol (DTT), buffers, and salts were obtained from Sigma. CIM® SO_3 monoliths (0.34 mL, axial flow) were obtained from BIA Separations (Klagenfurt, Austria). Chromatography experiments were run at 2 mL (6 CV)/min.

2.1. Reduction and dissociation of light and heavy chain

Freshly eluted protein A-purified monoclonal IgG at about 8 mg/mL in 100 mM acetic acid, 100 mM arginine, pH 3.8 was equilibrated to 8 M urea, 50 mM DTT by direct addition of dry urea and DTT. This approach was taken to conserve the highest possi-

* Corresponding author.
E-mail address: pete_gagnon@mac.com (P. Gagnon).

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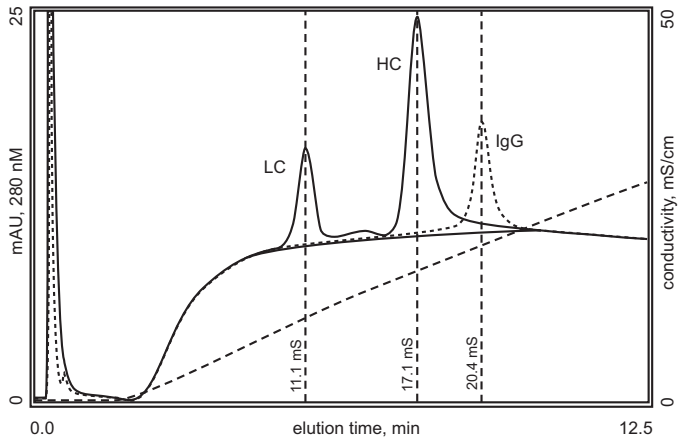

Fig. 1. Cation exchange fractionation of IgG light chain, heavy chain, and unreduced IgG.

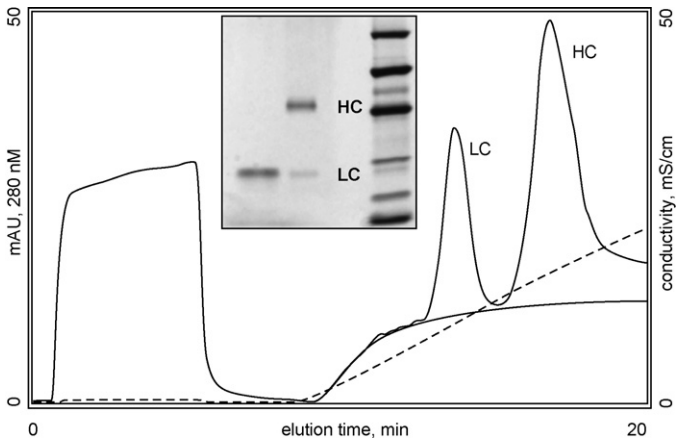

Fig. 2. Mini-preparative cation exchange fractionation of reduced light and heavy chain. Inset non-reduced SDS PAGE. Lane 1: elution peak 1 (light chain). Lane 2: elution peak 2 (heavy chain, with light chain contaminant). Lane 3: molecular weight standards.

ble protein concentration. The solution was titrated to pH 7.5 with untitrated 1 M Tris (pH ~10.3, ~2% v:v) and incubated for at least 60 min. Titration with Tris instead of NaOH was precautionary. We had observed recent examples where titration with NaOH induced aggregate formation.

2.2. Fractionation of reduced light and heavy chain

The CX monolith was equilibrated to 200 mM acetic acid, 8 M urea. The conductivity of this buffer as measured by the AKTA was 0.75 mS/cm. No pH titration was required; 3.8 was the native pH of the buffer upon formulation. Just prior to injection, 1 part reduced IgG was diluted with 3 parts equilibration buffer. We note that the high acetate concentration was intended to overwhelm the buffering effects of residual Tris in the reduced IgG sample. This approach was intended to minimize spontaneous re-association of LC and HC. After injection, the column was washed with equilibration buffer and eluted with a 20 mL (60 CV, 10 min) linear gradient to 200 mM acetic acid, 8 M urea, 0.5 M NaCl, pH ~3.8. As with the equilibration buffer, no pH titration was required. The column was cleaned after each run with 200 mM acetate, 8 M urea, 1 M NaCl, pH 3.8. AKTA conductivity of this buffer was 49 mS/cm.

3. Results and discussion

3.1. Initial screening

Fig. 1 illustrates results obtained with 100 μL injections of native and reduced IgG. If fully dissociated, LC and HC should have been present in equimolar concentrations in the sample, but the LC peak should be only half the size of the HC peak due to its relative mass. These proportions conformed well to the chromatogram, suggesting that LC eluted first, followed by HC, then intact molecule. The absence of a peak in the elution position for intact molecule suggested that sample reduction and dissociation was complete and spontaneous re-association was nil.

Backpressure was conspicuously high with this system. We initially attempted a flow rate of 4 mL/min but observed backpressures approaching 4 MPa. Rather than risk system shutdown, we adopted a flow rate of 2 mL/min throughout.

3.2. Minipreparative fractionation

We collected HC and LC peaks from a 10 mL injection (2.5 mL of reduced sample, ~20 mg of protein, Fig. 2) and applied them to a non-reduced SDS-PAGE gel. Results confirmed the identity of the LC and HC peak and indicated that the LC peak was essentially pure (see inset). The HC peak contained about 10% LC. PAGE results also indicated the presence of HC and LC in the flow-through upon injection. This was inconsistent with the apparently low conductivity of the injected sample so we investigated further.

3.3. Conductivity masking by urea

Conductivity of the 200 mM acetate equilibration buffer was suspect to begin with (0.75 mS/cm), and the value of the 1 M NaCl elution buffer (49 mS) even more doubtful, since 1 M NaCl typically gives a value of about 93 mS (AKTA). This suggested that urea depressed either the actual or apparent conductivity of the system. We diluted some of the flow-through fraction with water, reinjected it, and found that significantly more LC and HC bound to the column. This revealed that urea suppressed only the ability of the probe to measure conductivity accurately; the ability of dissolved ions to elute ion exchange interactions was conserved.

3.4. Opportunities for improvement

These preliminary results reveal several opportunities to improve performance of the system overall, the most obvious being further reduction of sample conductivity to improve binding efficiency. Work will be required to determine how dilute the buffering acid can be without permitting re-assembly of IgG. Replacement of acetic acid with citric or phosphoric acid will permit effective buffering at pH values in the range of 2.0–3.0 and further restrict potential for re-association in the CX buffer. Such conditions normally cause IgGs to precipitate, but the solubilizing influence of 8 M urea apparently suspends this problem. Lower operating pH should also tolerate higher sample conductivity, and further improve binding efficiency. Beyond these variations, IgG monoclonal antibodies exhibit substantial diversity in their individual CX binding characteristics, so it seems likely that the retention behaviors of their HC and LC chain components will do the same. Gradient configuration will need to be optimized for each application.

4. Conclusions

A basic method for reduction, dissociation, and CX fractionation of IgG heavy and light chain was developed. This basic operational format can be applied to dissociate and fractionate LC and HC from other IgGs, as well as other immunoglobulin classes and non-antibody disulfide-bonded proteins with a sig-

nificant affinity for CX. Future comparison of monoliths with diffusive and perfusive particle media may offer insights into the relative effects of viscosity on fractionation performance and productivity.

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