Preliminary Results Towards Development of a Simple Rapid Method for Dissociation and Fractionation of Heavy and Light Chain from IgG Monoclonal Antibodies

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Introduction

At the dawn of molecular immunology, there was great interest in isolating quantities of light chain (LC) and heavy chain (HC) from IgG for individual characterization, and to explore the dynamics of their association (Figure 1). By 1962, a method had emerged that employed reduction of polyclonal IgG 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 reassociation of fragments. Otherwise, even when reduction was followed by alkylation to prevent reformation of disulfide bonds, other molecular 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. In addition, recently published results indicate that IgG-derived Fab fragments in general are more heavily carboxylated than Fc, while Fc is more aminated than Fab.[3] This suggested that LC might elute first from CX, followed by HC, and probably thereafter by intact IgG, if any remained. The mass of LC and HC are only about 25 and 50 kDa respectively, which correspond to reasonably rapid diffusion constants, unlikely to impair chromatographic fractionation even on porous particle-based media. High concentrations of urea however are viscous, and diffusivity diminishes in direct proportion to viscosity. Convection on the other hand should be unaffected by viscosity. We therefore chose to explore fractionation on monoliths.

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.

Protein A-purified monoclonal IgG at about 8 mg/mL 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 possible protein concentration. The solution was warmed to room temperature, titrated to pH 7.5 with untitrated 1 M Tris (pH ~10.3), and incubated for at least 60 minutes.

The CX monolith was equilibrated to 200 mM acetic acid, 8 M urea, pH ~4. No pH titration was required; this was the native pH of the buffer upon formulation. The high acetate concentration was intended to overwhelm the buffering effects of residual Tris in the reduced IgG sample. Conductivity as measured by the AKTA was 0.75 mS/cm. 0.34 mL monoliths are typically run at 4 mL/min (12 column volumes (CV)/min) but we used 2 mL /min as a precaution to avoid excess pressure. Just prior to injection, 1 part reduced IgG was diluted with 3 parts equilibration buffer. After injection, the column was washed with equilibration buffer and eluted with a linear gradient (LG) to 200 mM acetate, 8 M urea, 0.5 M NaCl, pH ~3.8. As with the equilibration buffer, no pH titration was required. The column was then cleaned with 200 mM acetate, 8 M urea, 1 M NaCl, pH 3.8. AKTA conductivity was 49 mS/cm.

Results and Discussion

Figure 2 illustrates results obtained with 100 μL injections of native IgG (red) and reduced IgG (blue). These separations were conducted with a 20 mL (60 CV, 10 min) LG. LC and HC should be 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. Relative peak size thus suggested that LC eluted first, followed by HC, then intact molecule as expected. We collected HC and LC peaks from a 10 mL injection (2.5 mL of reduced sample, ~20 mg of protein, Figure 3) and applied them to a non-reduced SDS-PAGE gel. Results confirmed the identity of the LC and HC peaks.

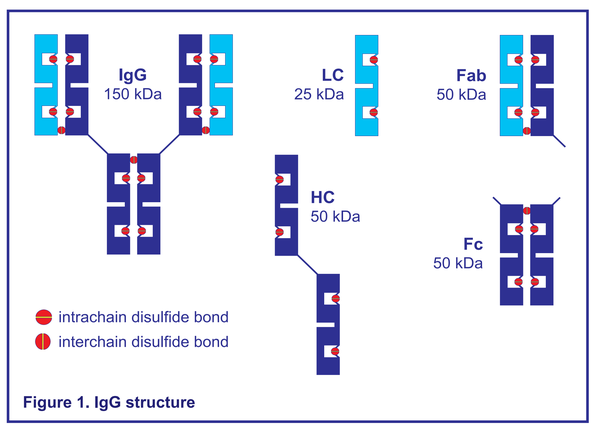

Figure 1. IgG structure

PAGE 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. Conductivity of the 200 mM acetate equilibration buffer was suspect to begin with (0.75 mS), 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 92 mS. 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 suggested that urea suppressed only the ability of the conductivity probe to measure accurately; the ability of dissolved ions to elute ion exchange interactions was apparently conserved. This revealed several opportunities to improve performance of the system overall.

Application Scope and Adaptability

This basic experimental format can be applied to all IgGs, as well as other immunoglobulins and non-antibody disulfide-bonded proteins with a significant affinity for CX —with the understanding that each application will need to be optimized individually. The species and concentration of the primary buffering component obviously require attention. The lower the concentration, the lower the conductivity, and the more broadly applicable the system. For lower pH values, it will probably be necessary to replace acetate with citric or phosphoric acid. Lower pH will favor more effective protein binding, and perhaps more effective chain dissociation, but whether a given protein will tolerate very low pH —and whether it matters— will need to be considered on a case by case basis. It may be possible to reduce the urea concentration, but this may compromise effectiveness of the method overall, with lower viscosity the only potential benefit, which is moot for monolithic separations. Lower DTT concentrations may also be explored, along with shorter incubation times, but also at the risk of diminishing dissociation efficiency.

It should be helpful to equilibrate the sample to low conductivity before reduction. Sample preparation following reduction also needs to be evaluated more thoroughly. As with column equilibration, the lower the conductivity and pH, the stronger the protein binding to CX. Such conditions are normally not tolerated by IgGs; they precipitate, but the solubilizing influence of 8 M urea apparently suspends this limitation. The diluent should have the same urea concentration as the CX buffers, but the concentration of the buffering species can possibly be reduced. Beyond these variations, IgG monoclonal antibodies exhibit substantial diversity in their individual CX binding characteristics, so it is likely that the elution behaviors of its HC and LC chain components will do the same. Gradient configuration will need to be optimized for each application.

Literature Cited

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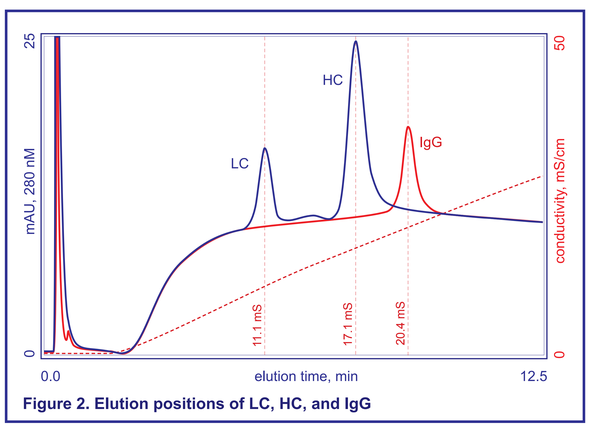

Figure 2. Elution positions of LC, HC, and IgG

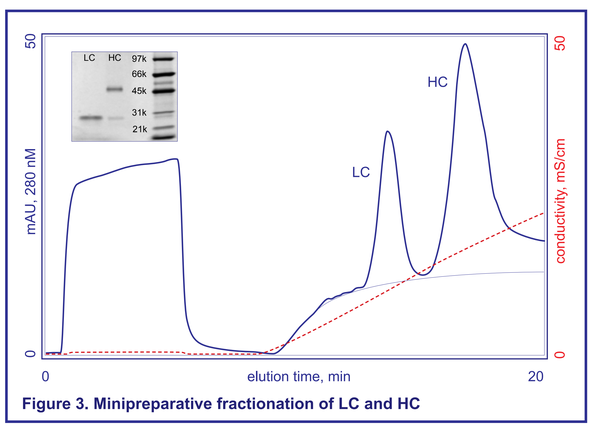

Figure 3. Minipreparative fractionation of LC and HC