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# A Non-Chromatographic Method for the Purification of a Bivalently Active Monoclonal IgG Antibody from Biological Fluids

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***Abstract:*** This paper describes a method for the purification of monoclonal antibodies (rat anti-2,4-dinitrophenyl IgG: $\mathrm{IgG}^{\mathrm{DNP}}$; and mouse antidigoxin IgG: $\mathrm{IgG}^{\mathrm{Dgn}}$) from ascites fluid. This procedure (for $\mathrm{IgG}^{\mathrm{DNP}}$) has three steps: (i) precipitation of proteins heavier than immunoglobulins with ammonium sulfate; (ii) formation of cyclic complexes of $\mathrm{IgG}^{\mathrm{DNP}}$ by causing it to bind to synthetic multivalent haptens containing multiple DNP groups; (iii) selective precipitation of these dimers, trimers, and higher oligomers of the target antibody, followed by regeneration of the free antibody. This procedure separates the targeted antibody from a mixture of antibodies, as well as from other proteins and globulins in a biological fluid. This method is applicable to antibodies with a wide range of monovalent binding constants (0.1 $\mu$M to 0.1 nM). The multivalent ligands we used (derivatives of DNP and digoxin) isolated $\mathrm{IgG}^{\mathrm{DNP}}$ and $\mathrm{IgG}^{\mathrm{Dgn}}$ from ascites fluid in yields of $>$80% and with $>$95% purity. This technique has two advantages over conventional chromatographic methods for purifying antibodies: (i) it is selective for antibodies with two active Fab binding sites (both sites are required to form the cyclic complexes) over antibodies with one or zero active Fab binding sites; (ii) it does not require chromatographic separation. It has the disadvantage that the structure of the hapten must be compatible with the synthesis of bi- and/or trivalent analogues.

## Introduction

This paper describes a non-chromatographic procedure for purifying monoclonal IgG antibodies (mAbs) from a biological fluid. This procedure is based on selective precipitation of cyclic complexes of the targeted antibody and multivalent haptens with ammonium sulfate (AMS) from a biological fluid (e.g., acites fluid or a cell lysate). Because the cyclic oligomers of $[\mathrm{IgG}]_n$ ($n = 2, 3$) have molecular weights that are two or three times that of the monomeric IgG (150 kDa), the complexes precipitate at lower concentrations of AMS than does monomeric IgG. The key step in this procedure is the precipitation that separates the oligomeric $[\mathrm{IgG}]_n$ complexes from other monomeric antibodies that do not form complexes (including IgGs that are not bivalently active), and from other proteins in the ascites fluid. We used two commercial IgGs (rat anti-2,4-dinitrophenyl, $\mathrm{IgG}^{\mathrm{DNP}}$ and mouse antidigoxin, $\mathrm{IgG}^{\mathrm{Dgn}}$) as model systems in developing this method. To the best of our knowledge, this procedure is the first for purifying monoclonal IgGs that selects for *bivalently* active antibodies. We have successfully precipitated complexes of $\mathrm{IgG}^{\mathrm{DNP}}$ using bi- and trivalent DNP (2,4-dinitrophenyl) haptens ($K_{\mathrm{d}}^{\mathrm{affinity}} \approx 0.8$ nM), and bi- and trivalent 4-NP (4-nitrophenyl, $K_{\mathrm{d}}^{\mathrm{affinity}} \approx 0.5$ $\mu$M), and complexes of $\mathrm{IgG}^{\mathrm{Dgn}}$ using bivalent digoxin ($K_{\mathrm{d}}^{\mathrm{affinity}} \approx 0.1$ nM). We believe, based on these results, it should be possible to apply this technique to antibody-ligand systems that have monovalent dissociation constants ranging from micro- to nanomolar (provided that the bi- and/or trivalent analogues of these haptens are synthetically accessible).

Monoclonal antibodies are important in biomedical research, and antibody-based therapies have become increasingly important in the past decade:[1−7] 55% of the drugs that are under development currently are mAbs.[8] The pharmaceutical industry is considering a target for production of mAbs of 10 tons per year.[9] Bioreactors with a capacity from 15 000 to 25 000 L are becoming more common, and an expression rate of 5 g of antibody/L is standard.[9] Purification of mAbs—both at process scales, and for research—remains an expense and inconvenience. The current processes for purifying mAbs use multiple steps that may have detrimental effects on the specific activity of isolated mAbs. Methods for purification that are faster, less expensive, more convenient at research scales, and result in greater yields (of specifically active product) would be useful.[10−12]

Immunoglobulin (e.g., IgD, IgE, and IgG antibodies) consists of two antigen recognition sites and is bivalent (Figure 1a). The bivalency of IgG increases its avidity for antigens displayed on

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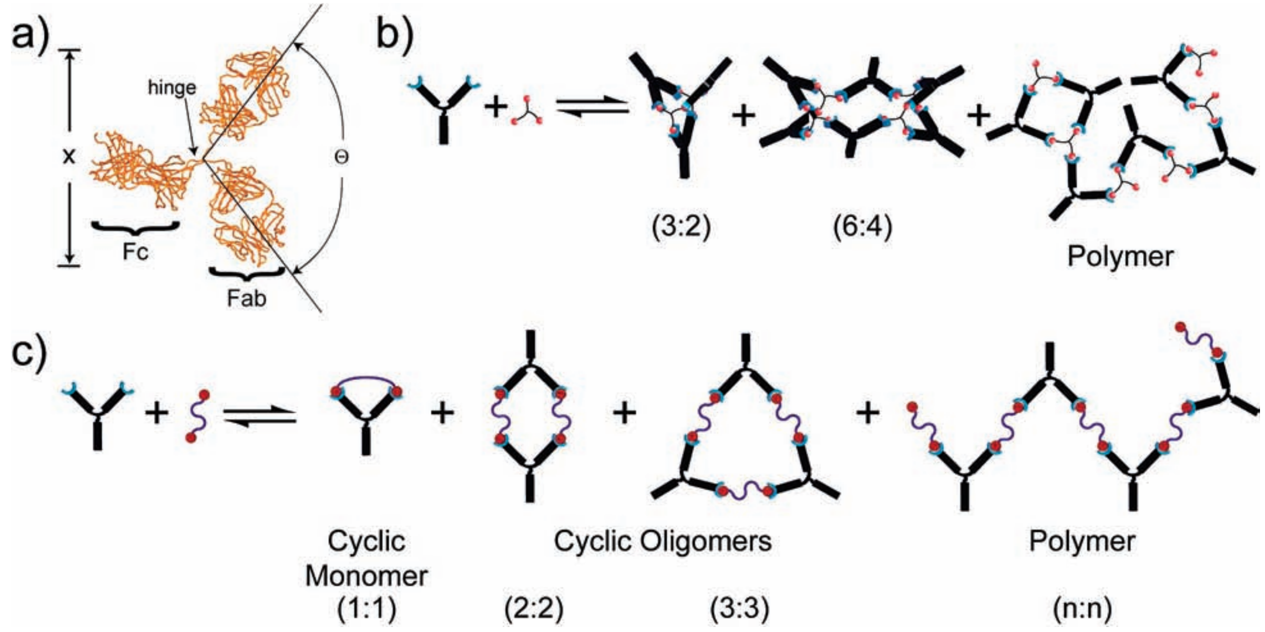

***Figure 1.*** (a) Crystal structure of an antibody with the dimensions labeled (PDB: 1HZH). The flexibility of the hinge region gives rise to a range of values for Θ and $x$ (distance between binding sites). The complexes of antibodies (IgG) that can be formed by incubation with (b) trivalent hapten include bicyclic antibody trimer, tricyclic antibody hexamer, and branched polymer; (c) bivalent hapten include cyclic dimer, cyclic trimer, and linear polymer.

cell surfaces and also allows it to form high-molecular-weight complexes with soluble, multivalent antigens and allergens.[13]

During purification, several processes may yield monoclonal antibodies with only one active Fab binding site; examples include (i) protein unfolding, misfolding, and aggregation; (ii) covalent modification (e.g., oxidation); (iii) enzymatic proteolysis; and (iv) the "scrambling" of light chains.[12] The biological significance of bivalently active (as opposed to monovalently active) immunoglobulins is presently unclear because there has been no consistent route for preparing either.[14−16]

Procedures for purifying antibodies must remove a number of contaminants that are associated with their expression, such as host cell proteins, DNA, endotoxins, and cell culture media additives. In addition, antibody-derived impurities, such as high-molecular-weight aggregates and proteolytic fragments of immunoglobulin, can also contaminate the desired product. Current procedures for purifying therapeutic antibodies typically rely on protein A (proA) chromatography (proA binds to the Fc domain of IgGs), where the elution of the antibody is achieved by decreasing the pH to 2−3.[9] Although proA affinity chromatography can yield products with 95% purity after a single chromatographic step,[17] it introduces a number of additional challenges and new routes for contamination: hydrolysis and proteolysis can contaminate the isolated product with cleaved proA and its truncated derivatives, and leached proA can adhere to the eluting product.[18−20] Furthermore, the acidic pH of the mobile phase can cause the mAb to unfold and lose activity, and/or aggregate nonspecifically and precipitate.

Because chromatographic procedures are labor-intensive, expensive, and operationally demanding at large scales, there is substantial effort directed toward developing new methods of purification of mAbs. The Cohn fractionation process, which currently provides yields of 80 tons per year for IgIV purification, is a potential candidate for the ultimate goal for producing 10 tons per year of mAbs. The Cohn fractionation process employs selective precipitation steps through control of pH, temperature, concentration of ethanol, and ionic strength but does not employ any chromatographic steps;[21,22] additional unit operations include microfiltration, ultrafiltration, and centrifugation. Nevertheless, the Cohn process is not yet a viable method for the production of mAbs, and neither the Cohn process nor the chromatographic techniques under development explicitly select for bivalently active IgG.

The technique we introduce here is based on the formation of discrete, cyclic complexes of antibodies, and therefore can avoid many of these disadvantages when the appropriate oligovalent ligand can be prepared. This procedure is an affinity-based method, with the strengths and weaknesses of such methods. In particular, it is specific for a hapten but requires that that hapten be accessible and amenable to synthetic manipulation. Importantly, this procedure differs from conventional techniques in that the formation of the complexes *requires* both Fab sites of an antibody to have binding activity.

Early in the development of molecular immunology, Pecht, Baird, Posner, and others described the formation of discrete,

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cyclic dimers and trimers resulting from the interaction of IgEs and IgGs with bivalent haptens (Figure 1c).[23−28] On the basis of analytical modeling of the assembly of antibody complexes, Dembo and Goldstein predicted that the concentration of bivalent hapten ($C_{\mathrm{total}}$) at which maximum conversion ($C_{\mathrm{Tmax}}$) took place would depend on the monovalent dissociation constant ($K_{\mathrm{d}}^{\mathrm{affinity}}$) and the total concentration of antibody ($[\mathrm{IgG}]_{\mathrm{total}}$) according to eq 1.[28] Hence, in order to achieve maximum conversion to cyclic complexes, the dissociation constant ($K_{\mathrm{d}}^{\mathrm{affinity}}$) should be lower than the concentration of antibody, and the ratio of the bivalent ligand to antibody should be 1.

$$C_{\mathrm{Tmax}} = K_{\mathrm{d}}^{\mathrm{affinity}}/2 + [\mathrm{IgG}]_{\mathrm{total}} \qquad (1)$$

## Results

**Summary of Purification.** Using this purification procedure, we isolated pure, bivalently active anti-2,4-DNP and antidigoxin from rat and mouse ascites fluids; ascites fluid and the supernatant from hybridoma bioreactors are the two most common biological sources for monoclonal antibodies for both small and large scales.[10,26] Ascites fluid contains 1−10 mg/mL of globulins, and 10−30 mg/mL of other serum proteins including albumin (MW ≈ 66 kDa) and transferrin (MW ≈ 80 kDa).

The procedure consists of three steps: (i) Addition of ammonium sulfate (AMS) to a final concentration of 35% of the saturated concentration precipitated of all proteins and complexes heavier than an IgG (150 kDa). (ii) After removing the precipitate, addition of bi- or trivalent hapten formed cyclic, higher-molecular-weight complexes (described in Figure 1) of the IgG of interest (here, anti-2,4-DNP or antidigoxin) (Figure 2).[13,27−29] These complexes—with molecular weights of 300, 450, or 600 kDa—precipitated from the 35% AMS solution immediately. (iii) Centrifugation separated the precipitated complexes from the supernatant, which retained IgG molecules incapable of forming complexes with the multivalent haptens (i.e., those with different specificity, or with one or no active Fab sites), as well as other proteins with MW ≤ 150 kDa. We finally solubilized and dissociated the complexes by incubation with a large excess of monovalent hapten and removed the haptens by dialysis.

Anti-2,4-DNP rat monoclonal IgG1$\kappa$ (from clone LO-DNP-2) and antidigoxin mouse monoclonal IgG1 (from clone DI-22) antibodies are appropriate for proof-of-principle demonstrations for five reasons: (i) The purified antibodies and the ascites fluids are both commercially available. (ii) Both antibodies have high affinity ($K_{\mathrm{d}}^{\mathrm{DNP}} \approx 0.8$ nM and $K_{\mathrm{d}}^{\mathrm{Dgn}} \approx 0.1$ nM) for their monovalent haptens (a requirement to observe and isolate the complexes by SE-HPLC). (iii) $\mathrm{IgG}^{\mathrm{DNP}}$ has a substantially lower affinity ($K_{\mathrm{d}} \approx 0.5\ \mu$M) for monovalent 4-NP than for 2,4-DNP; we used this low-affinity interaction to demonstrate the range of values of $K_{\mathrm{d}}$ for which this procedure is applicable. (iv) The syntheses of bi- and trivalent haptens are straightforward. (v) The lifetime of the IgG-DNP complex is sufficiently long to allow the use of chromatography to separate the aggregates relevant to this work for analysis and to understand the mechanisms underlying the process.

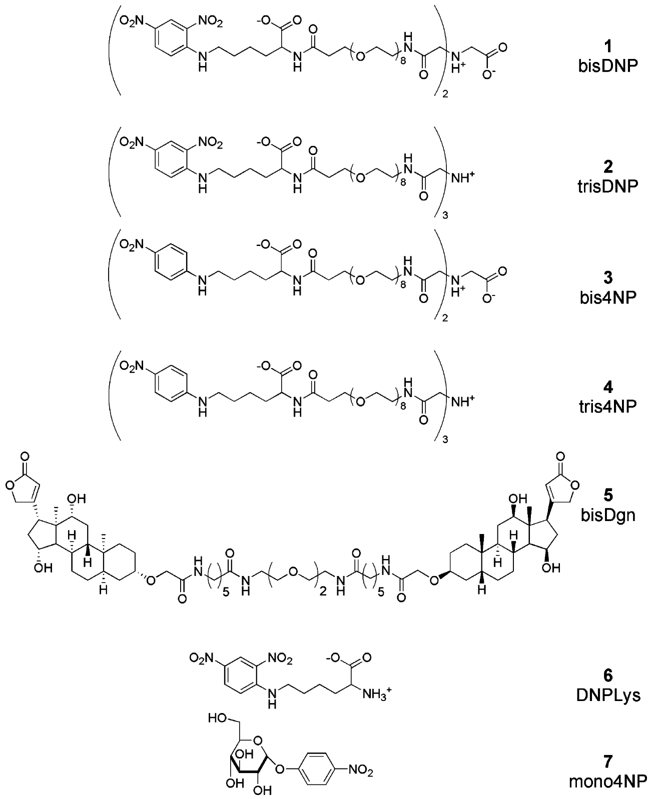

***Figure 2.*** Structures of the bi- and trivalent DNP and 4-NP haptens (**1**, **2**, **3**, and **4**), bivalent digoxin hapten (**5**), monovalent 2,4-dinitrophenyl lysine (**6**), and 4-nitrophenylglucose (**7**).

**Analytical Methods.** We determined the efficiency of the purification with size-exclusion chromatography (SE-HPLC).[30,31] This analytical technique can resolve antibody complexes of different molecular weights, provided that these complexes do not dissociate over the time required to carry out a separation (~20 min).[13] Using this technique, we separated the complexes formed by mixing commercially available $\mathrm{IgG}^{\mathrm{DNP}}$ with bi- and trivalent derivatives of DNP, as well as the complexes that formed by mixing commercially available $\mathrm{IgG}^{\mathrm{Dgn}}$ with bivalent digoxin (Figure 3). The procedure developed with these compounds also works with ligands that bind less tightly than DNP and dissociate more rapidly (as we show using 4-nitrophenyl hapten, $K_{\mathrm{d}}^{\mathrm{affinity}} \approx 0.5\ \mu$M), but the ability to resolve aggregates was very important in understanding the mechanism of the purification. SE-HPLC was an effective tool for measuring the amount of $\mathrm{IgG}^{\mathrm{DNP}}$ at each step of the purification procedure (except in the starting ascites fluid), as well as determining the mole fraction of each complex.

By contrast, although this procedure was applicable to the purification of IgG using the much more weakly binding 4-NP as a hapten, SE-HPLC could not resolve complexes formed by bi- and trivalent 4-NP ligands (the chromatograms showed only

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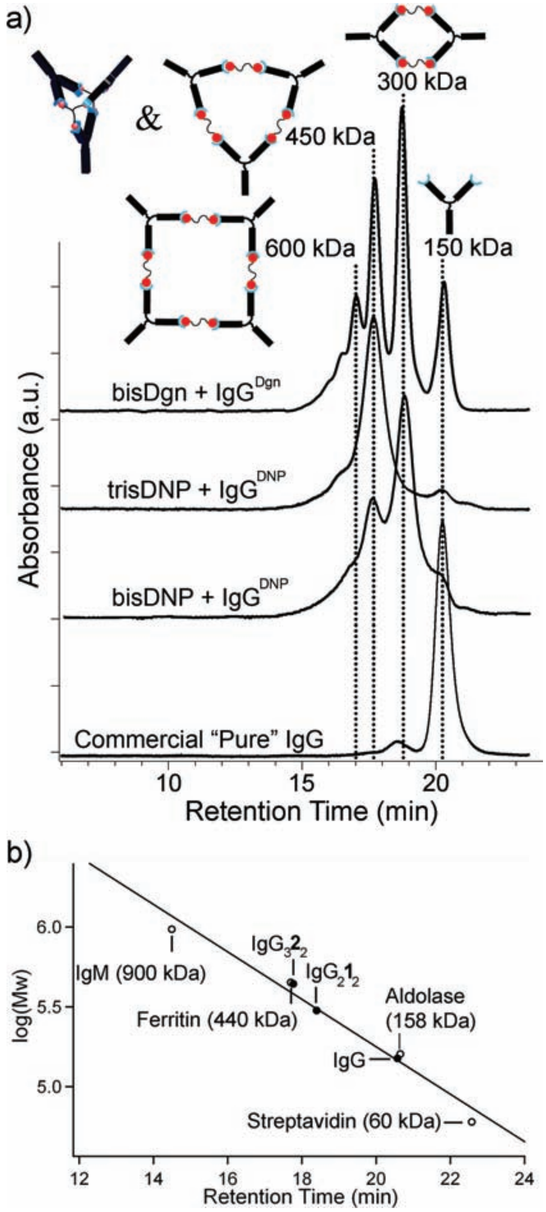

***Figure 3.*** (a) SE-HPLC chromatograms of IgG$^{\mathrm{Dgn}}$ and IgG$^{\mathrm{DNP}}$ complexes formed upon binding to multivalent ligands. Each antibody-ligand complex is labeled by the chromatogram. The schematic structures show the aggregates expected to be formed from IgG; other proteins with the same MW also seem to be present in small quantities. The products in a mixture of bis-DNP ligand **1** and IgG$^{\mathrm{DNP}}$ showed the cyclic antibody dimer $(\mathrm{IgG})_2\mathbf{1}_2$ (MW = 300 kDa and trimer $(\mathrm{IgG})_3\mathbf{1}_3$ (MW = 450 kDa) (Figure 3a). The product of a mixture of tris-DNP ligand **2** to IgG$^{\mathrm{DNP}}$, as expected from previous work, was a bicyclic trimer complex.[13] Mixing bis-Dgn ligand **5** with IgG$^{\mathrm{Dgn}}$ yielded multiple peaks that corresponded to cyclic dimer, trimer, and tetramer, as well as monomeric antibody. (b) Calibration of the size-exclusion column using proteins with known molecular masses.

a monomeric IgG peak) as a result of the kinetic instability of these complexes. All IgG$^{\mathrm{DNP}}$ complexes dissociated rapidly (<1 min) in the presence of excess monovalent hapten (**6**).

The presence of many other proteins precluded the use of SE-HPLC for determining the amount of IgG$^{\mathrm{DNP}}$ in the ascites, as many proteins eluted together with the antibody. Therefore, we used an enzyme-linked immunosorbent assay (ELISA) to quantify the concentration of IgG$^{\mathrm{DNP}}$ in the ascites fluid. We calculated the percentage of IgG$^{\mathrm{DNP}}$ separated after each step of the protocol by comparing the intensity of fluorescence from the sample to the intensity of fluorescence from the original ascites fluid. We did not carry out the ELISA procedure for IgG$^{\mathrm{Dgn}}$. The chromatograms in Figure 4 are of IgG$^{\mathrm{Dgn}}$-containing ascites with and without the bivalent Dgn ligand, and after purification. The concentration of IgG$^{\mathrm{Dgn}}$ was the same in all three injections and the peak intensities demonstrate the relative concentration of IgG$^{\mathrm{Dgn}}$ to the rest of the proteins in the ascites fluid.

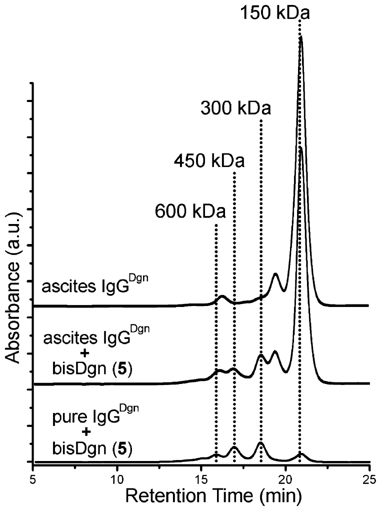

***Figure 4.*** Size-exclusion chromatograms of antidigoxin ascites fluid. Top SE-HPLC trace labeled "ascites IgG$^{\mathrm{Dgn}}$" is untreated ascites fluid. Antidigoxin ascites fluid after incubation with bis-Dgn (**5**) (2 $\mu$M) is labeled "ascites IgG$^{\mathrm{Dgn}}$ + bis-Dgn (**5**)". The bottom trace is purified IgG$^{\mathrm{Dgn}}$ mixed with bis-Dgn (this sample was prepared to match the original IgG$^{\mathrm{Dgn}}$ concentration in the ascites). The peaks at 300, 450, and 600 kDa correspond to cyclic dimer, trimer, and tetramer (Figure 3). The differences in the absorbance illustrate that the concentration of the IgG in the ascites was low relative to the other proteins.

**Step 1. Removal of High-Molecular-Weight Impurities.** The first step consisted of filtering the ascites fluid (0.5 mL) through glass wool to remove the majority of the liposaccharides. We rinsed the glass wool with an additional 0.5 mL of phosphate-buffered saline (PBS, pH 7.4, 10 mM phosphate, 150 mM NaCl) for a final volume of 1 mL (diluting the ascites fluid 2-fold). Addition of saturated AMS solution (540 $\mu$L; to a final concentration of 1.4 M (35%)) to the filtered ascites fluid, followed by centrifugation, separated the proteins having molecular weights >150 kDa (Figure 5). The proteins that precipitated have retention times similar to that of the cyclic complexes that form upon addition of multivalent ligands. Using an ELISA, we determined that this initial precipitation step resulted in the loss of only ~4% of the IgG$^{\mathrm{DNP}}$.

**Step 2. Isolation of Bivalently Active IgG$^{\mathrm{DNP}}$ as Complexes.** The supernatant from step 1 contained active IgG, inactive IgG, and serum proteins with molecular weights equal to or lower than that of IgG. Adding multivalent haptens **1**−**4** (to a final concentration of 5 $\mu$M) to the supernatant induced the aggregation of bivalently active IgG; these complexes immediately formed a precipitate. To ensure maximum recovery, we incubated the sample overnight at 4 °C. We then centrifuged (16 000 $g$, 30 min) these samples, isolated the pellets, and redissolved them in PBS for analysis by SE-HPLC (Supporting Information, Figure S.1).

SE-HPLC chromatograms of the pellets isolated using bis- and tris-DNP haptens **1** and **2** had peaks that corresponded to the IgG monomer and the cyclic antibody complexes (dimer and trimer). The presence of antibody monomers in the chromatogram suggests two possibilities: (i) a fraction of the

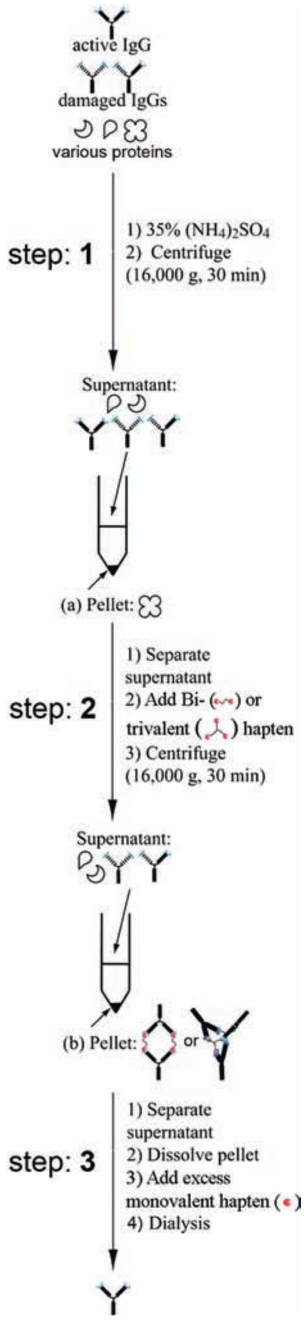

***Figure 5.*** Schematic representation of the three steps (1−3) used to purify bivalently active monoclonal $IgG^{DNP}$ from ascites fluid using AMS precipitation. The starting material was ascites fluid, which contained a mixture of $IgG^{DNP}$ with two active Fab binding sites that recognize 2,4-DNP (active IgG), improperly folded or denatured $IgG^{DNP}$ (damaged IgG), and IgG fragments (heavy or light chain), as well as other proteins present in ascites with a range of molecular weights. (1) A low concentration of AMS (35%) precipitated high molecular weight (≥300 kDa) proteins. These proteins (as a precipitate) were separated by centrifugation as a pellet (a). (We carried the supernatant, which contained all IgG and low molecular weight serum proteins, to the next step.) (2) The addition of bi or trivalent hapten molecules to the supernatant formed complexes of $IgG^{DNP}$ (represented here as the cyclic dimer and bicyclic trimer), which immediately precipitated from the solution. This precipitate (b) was isolated by centrifugation. The supernatant, which now contained any damaged $IgG^{DNP}$, immunoglobulins against other antigens, and other serum proteins, was discarded. (3) The pellet (b) was dissolved in PBS, and the $IgG^{DNP}$ complexes were dissociated by the addition of excess monovalent 4-NP **7** (∼1 mM). Dialysis of this solution against phosphate buffered saline (pH 7.4) removed both the monovalent and multivalent haptens, and gave the final product as monomeric, bivalently active, $IgG^{DNP}$.

multivalent ligand did not precipitate with the antibody or (ii) an excess of ligand precipitated and, as a result, a fraction of the complexes dissociated. Regarding the first possibility: the addition of more ligand would have driven the formation of more dimeric and trimeric complexes (but this was not observed; data not shown). This distribution of antibody complexes suggests, therefore, that an excess of multivalent ligand precipitated with the complexes as a result of nonspecific binding. This type of precipitation, if it did occur, did not interfere with the rest of the purification.

Analysis of pellets from AMS precipitation with bi- and trivalent 4-NP haptens **3** and **4** with SE-HPLC showed a single peak at a retention time that corresponded to monomeric $IgG^{DNP}$; neither chromatogram showed peaks corresponding to higher aggregates. This observation indicated that, although the complexes that formed upon the interaction of antibody with these haptens were sufficiently stable to facilitate precipitation with AMS, the complexes were not sufficiently stable kinetically to survive a 20-min SE-HPLC separation. The absence of peak broadening indicates that the rate constant for dissociation must be $>2.5 \times 10^3$ $s^{-1}$.

According to the results of the ELISA experiments, the yields of purified IgG (after step 2) with the multivalent DNP haptens appear to be much lower than the yields with the multivalent 4-NP haptens: 16% ± 2% from bis-DNP, 11% ± 4% from tris-DNP, 78% ± 12% from bis-4NP, and 82% ± 20% from tris-4NP. SE-HPLC experiments with the purified antibodies, however, indicated that the actual yield obtained by precipitation with bis-DNP **1** was 66% (the peak area was ∼80% of the peak area of the pellet generated using tris-4-NP). We hypothesized that the reason for the low apparent yield of antibody purified by multivalent DNP ligands, when determined with ELISA, was that those aggregates remained stable when redissolved in PBS, and were not accessible (either kinetically or thermodynamically, we have not determined which) to the DNP on the surface of the ELISA plate. To test this hypothesis, we reacted commercially available, affinity purified $IgG^{DNP}$ with tris-DNP (ligand **2**) to form the bicyclic complex ($IgG_3\mathbf{2}_2$). An ELISA of this sample detected a concentration of $IgG^{DNP}$ that was only ∼6% of its actual concentration. Therefore, the $IgG^{DNP}$ purified using the multivalent DNP haptens required purification step 3: dissociation of the complexes by the addition of monovalent DNP (ligand **6**), and removal of the monovalent and multivalent ligands from the antibody by dialysis.

**Step 3. Dissociation of the Cyclic Complexes with Monovalent DNP.** We solubilized the pellets from step 2 in PBS buffer and added excess (∼1 mM) DNP-Lysine (**6**); this monovalent ligand completely dissociated the cyclic IgG complexes (as confirmed by SE-HPLC). We dialyzed (10 kDa MWCO membrane, 4 °C) the sample against monovalent 4-NP **7** (in order to prevent the reformation of IgG complexes) and eliminated all the multivalent ligands from the dialysis chamber. Upon the completion of dialysis, the chamber contained monovalent ligand **7** together with $IgG^{DNP}$. A second dialysis step against PBS at 4 °C removed the low molecular weight monovalent ligand **7** (as monitored by UV absorbance, $\lambda = 360$ nm). The final product was ∼ 80% of the total amount of the $IgG^{DNP}$ in the starting ascites fluid (as estimated by ELISA) and yielded a clean single peak corresponding to 150 kDa on the SE-HPLC (Figure 6).

This procedure yielded a final product that was >95% pure by HPLC and, we believe, has two active Fab binding sites. The most significant experiment to establish the bivalency of

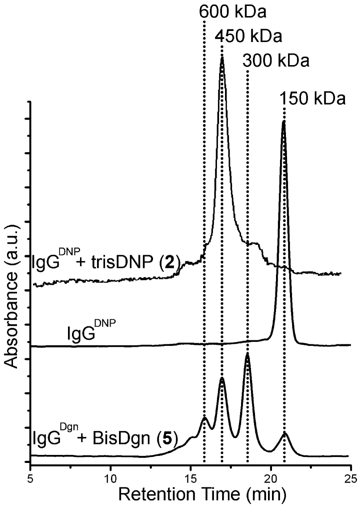

***Figure 6.*** (Bottom) SE-HPLC trace of the IgG$^{Dgn}$ purified using the described procedure in Figure 5. IgG$^{Dgn}$ was present as a mixture of cyclic complexes (monomer, dimer, trimer, and tetramer), as we did not have a weaker-binding monomeric ligand to dissociate its complexes. (Middle) SE-HPLC trace of the IgG$^{DNP}$ purified using our protocol and (Top) trace after mixing purified IgG$^{DNP}$ with trisDNP (ligand **2**).

the purified IgG$^{DNP}$ was to affirm its capacity to form the kinetically stable bicyclic complex ((IgG$^{DNP}$)$_3$**2**$_2$) upon addition of tris-DNP **2** (Figure 6b). Conversion of the monomeric IgG$^{DNP}$ purified with this method to the complex proceeded with >95% yield. This value was slightly greater than that observed for the commercially available, affinity-purified IgG$^{DNP}$ (>90%).

This procedure can selectively isolate one IgG from a mixture of ascites fluids—one containing IgG$^{DNP}$, and the second IgG$^{Dgn}$. The procedure described above separated IgG$^{DNP}$ from this mixture and gave results similar to those we have described in detail in the preceding section. Bivalent digoxin ligand **5** could also purify IgG$^{Dgn}$ from this mixture. We conclude that the procedure is capable of selective precipitation of a target IgG from a mixture containing multiple IgG molecules with different specificities. (The experiment summarized in Figure 4 implies the same conclusion).

## Discussion

The thermodynamic stability of the complexes, rather than their kinetic stability, is critical to the effectiveness of this protocol. Theoretical studies predict that this stability is directly related to the monovalent affinity of the antibody for the hapten and the concentration of antibody.[28,32,33] SE-HPLC established that isolation of IgG$^{DNP}$ using multivalent ligands of 4-NP ($K_d^{affinity} \approx 0.5\ \mu$M) or of DNP ($K_d^{affinity} \approx 0.8$ nM) gave comparable yields. We believe, based on the results, that this procedure is applicable for the purification of monoclonal antibodies with affinities in the range from $\mu$M to nM for their haptens/antigens, provided that bi- and/or trivalent analogues of these haptens/antigens are synthetically available.

We have not explored the application of this technique to other antibody isotypes. The majority of previous studies of cyclic complexes have used bivalent (IgG or IgE) antibodies.[13,23,25,29] Further study of the aggregating behavior of IgAs and IgMs is required before this procedure can be applied to their purification. We believe, however, that the bivalent IgE class of antibodies will be amenable to the purification approach described here because the only difference between IgG and IgE antibodies is in the Fc region.

AMS precipitation of cyclic complexes of antibodies provides a convenient, nonchromatographic method to purify antibodies from complex solutions and to purify bivalently active antibody from inactive antibody and/or monovalently active antibody. This method is, to our knowledge, the only purification procedure for monoclonal antibodies that selectively isolates monoclonal IgGs with two active Fab binding sites and is able to start from a crude biological source of antibodies.

The logic of the method is straightforward, and the procedures are easy to execute experimentally. They can be applied to small quantities of solutions and antibodies; although we have not worked with large volumes or quantities, this procedure should be scalable to large quantities. We believe that the antibodies isolated using this procedure will exclusively have two fully active Fab binding sites, since both sites are required to form the cyclic complexes.

The primary limitation of this technique is its requirement that appropriate bi- and trivalent haptens be synthetically (or naturally) accessible. The antibodies that we used in this study were directed against small-molecule haptens. The requirement for a synthetically accessible bivalent derivative of the hapten may limit the application of this technique to purify antibodies directed toward a recognition site created by the tertiary structure of a protein, although oligopeptides sometimes can be developed that bind such proteins (by random combinatorial methods, if necessary).[34−36] For some antibodies directed against proteins, the bivalent hapten could, in principle, be a dimer of the antigenic protein. For antibodies raised against large or membrane bound proteins, mimotopes (short peptide sequences that mimic the binding site), or peptidomimetics (organic molecules that mimic the function of mimotopes) could serve as multivalent molecules.[37−39] Discovery of mimotopes usually requires high throughput screening of peptide libraries, or phage display.[40−42]

We believe that this technique has the potential to be useful in many applications that require purifying substantial quantities of antibodies for common biological and clinical analyses, and perhaps for human therapeutics.[43] This technique may also be useful for fractionating mixtures of polyclonal antibodies from serum on the basis of their affinity for a given hapten and/or their specificity.

## Experimental Section

**Synthesis and Purification of Multivalent Ligands.** We used straightforward synthetic strategies (see Supporting Information)

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based on previously reported syntheses to prepare and purify multivalent ligands **1**−**5**.[13]

**SE-HPLC.** SE- HPLC measurements were carried out on Tosoh TSK-GEL G3000SWXL and Tosoh TSK-GEL G4000SWXL size-exclusion columns using a Varian ProStar 400 HPLC system with autosampler. HPLC runs were performed with an isocratic solvent system that was 50 mM phosphate buffer and 370 mM NaCl (to adjust the ionic strength to 0.475 M) at pH 6.8, with a 0.5 mL/min flow rate. The sample peaks were analyzed with a UV−vis detector, as monitored at $\lambda = 214$ nm. The chromatograms of the cyclic complexes of commercial IgG were obtained from injections where we kept the concentration of antibody constant while carrying out serial dilutions of the bi- and trivalent DNP haptens **1** and **2**. We determined the concentrations of our samples using the reported extinction coefficients for IgGs and DNP. We incubated all samples for 12 h at 4 °C prior to injection onto the SE-HPLC column. Samples from purified IgGs from ascites fluid were run after 1/3 dilution into running buffer.

**ELISA.** The wells of a 96-well ELISA plate were incubated with DNP-BSA conjugate to adsorb it on the well surface. After treating the plate with the sample to be assayed and washing, we incubated the plate with a secondary antibody (antirat IgG from goat) conjugated to horseradish peroxidase (HRP). We treated the wells with Amplex Red, and monitored the HRP-catalyzed hydrolysis of this substrate using fluorescence (ex, 545 nm; em, 590 nm). To determine the concentration of $\text{IgG}^{\text{DNP}}$ in each well, we compared the fluorescence results from the wells that contain samples to that of a well that contains known concentration of pure $\text{IgG}^{\text{DNP}}$. We averaged four independent sets of ELISA results obtained from purified antibodies using our purification procedure. We also measured the enzymatic activity of a known concentration of commercially available $\text{IgG}^{\text{DNP}}$ in parallel to quantify the amount of antibody obtained from precipitation using each multivalent ligand listed in Figure 2.

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**Supporting Information Available:** Additional experimental details, including the synthesis of the multivalent ligands, and SE-HPLC chromatograms of redissolved pellets after step 2 of the purification. This material is available free of charge via the Internet at http://pubs.acs.org.

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