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Affinity-Based Precipitation via a Bivalent Peptidic Hapten for the Purification of Monoclonal Antibodies†

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Abstract

In a previous study, we demonstrated a non-chromagraphic affinity-based precipitation method, using trivalent haptens, for the purification of mAbs. In this study, we significantly improved this process by using a simplified bivalent peptidic hapten (BPH) design, which enables facile and rapid purification of mAbs while overcoming limitations of the previous trivalent design. The improved affinity-based precipitation method (ABP^{BPH}) combines the simplicity of salt-induced precipitation with the selectivity of affinity chromatography for the purification of mAbs. The ABP^{BPH} method involves 3 steps: (i) precipitation and separation of protein contaminants larger than immunoglobulins with ammonium sulfate; (ii) selective precipitation of the target-antibody via BPH by inducing antibody-complex formation; (iii) solubilization of the antibody pellet and removal of BPH with membrane filtration resulting in pure antibody. The ABP^{BPH} method was evaluated by purifying the pharmaceutical antibody trastuzumab from common contaminants including CHO cell conditioned media, DNA, ascites fluid, other antibodies, and denatured antibody with >85% yield and >97% purity. Importantly, the purified antibody demonstrated native binding activity to cell lines expressing the target protein, HER2. Combined, the ABP^{BPH} method is a rapid and scalable process for the purification of antibodies with the potential to improve product quality while decreasing purification costs.

Introduction

Monoclonal antibodies (mAb) have been successfully designed to target a wide range of extracellular targets with high specificity. Accordingly, antibodies have rapidly become a clinically effective class of therapeutics for a wide range of diseases including cancers, autoimmunity and inflammation, organ transplantation, cardiovascular disease, infectious diseases, and ophthalmological diseases.[1] However, a serious concern in the administration of mAbs as therapeutics is the possible contamination of the final product with host cell proteins (HCP), DNA, or contaminants inherent to the purification process.[2-4] Due to the difficultly in removing these contaminants and the high purity requirements in the pharmaceutical industry, the downstream processing and purification of mAbs accounts for 50-80% of the total manufacturing costs and significantly contributes to the exceptionally high price of these therapeutics.[5-7]

The current standard for the purification of mAbs is based on affinity chromatography using immobilized protein A.[8] Protein A binds to the Fc domain of IgGs, retaining the antibody in the column while contaminants flow through. Decreasing the pH to 2-3 elutes the antibody from the column, consistently

delivering >95% mAb purity after a single step.[5] However, this is an expensive process that is not without complications. The acidic pH required to elute the mAb can cause the antibody to aggregate, denature and lose activity.[9-14] Additionally, hydrolysis and proteolysis of protein A limits the lifetime of the column and can contaminate the product.[9-14] Therefore, additional polishing steps including ion exchange chromatography and ultrafiltration are required to achieve the necessary high degree of purity.[4-6] Due to the limitations of the industry standard protein A affinity chromatography, new methods that enable rapid antibody purification with a high yield and high quality, while reducing the overall cost are urgently needed.

Relative to protein A affinity chromatography, the salt-induced precipitation of proteins is an inexpensive, simple, and rapid purification technique.[15] This process is based on selectively precipitating proteins by increasing the concentration of ammonium sulfate (AMS) salt. The solubilities of proteins decrease as the concentration of AMS increases with larger proteins typically precipitating first.[16, 17] However, applications of protein purification using this method are limited as many proteins have similar solubilities in aqueous solutions of AMS and precipitate simultaneously resulting in products with low purity.[18, 19] An ideal method for antibody purification will combine the high purity obtained using affinity chromatography with the simplicity and low cost benefits of salt-induced precipitation.

In this article, we describe an affinity-based precipitation method via a bivalent hapten (ABP^{BPH}) for the purification of mAbs from complex biological solutions. This technique is based on converting the target antibody from a soluble monomer to an insoluble complex using a peptidic bivalent hapten in aqueous solutions of AMS. The ABP^{BPH} method has 3 steps as described in Figure 1. First, the concentration of AMS in the crude mixture of antibody and other contaminant molecules is increased to a concentration just below that required to precipitate monomeric antibody. This step ensures that any larger proteins or protein complexes that could potentially precipitate with the antibody complexes and contaminate the product are removed. Second, a peptidic bivalent hapten is added to the supernatant from step 1 causing the formation of antibody complexes that immediately precipitate from the solution. The antibody complexes have increased molecular mass and decreased solubility such that upon formation, the complexes selectively precipitate from the AMS solution. The concentration of AMS is low enough that monomeric antibody remains in solution, yet high enough that the antibody complexes are insoluble and precipitate. Antibodies that cannot bind to the bivalent hapten (damaged, denatured, or other specificity) will not be able to form complexes and will remain in solution. The precipitated antibody complexes are easily separated from the solution using centrifugation. Third, the separated

antibody pellet from step 2 is redissolved and the bivalent hapten is removed via membrane filtration. It is noteworthy that the ABP^{BPH} method described in this article is based on our previous work where we describe the purification of antibodies using a trivalent hapten specific for the target antibody.[20] A shortcoming of this previous method was that the trivalent hapten required a complex synthetic strategy with a low overall yield. This would make the method prohibitively expensive to produce on the large scale required in the pharmaceutical industry. In addition, the trivalent hapten was difficult to separate from the target antibody via membrane filtration at the end of the purification process. The herein described ABP^{BPH} approach addressed the shortcoming of the previous method through the design of a simplified bivalent peptidic hapten, which enables facile synthesis on a solid support. In addition the bivalent hapten can be recombinantly expressed thereby enabling easy scale up in industrial applications. Finally, the bivalent peptidic hapten is more readily separated from the antibody via membrane filtration, further simplifying the purification procedure. Taken together, the ABP^{BPH} provides a rapid and facile method for mAb purification. In this study, we specifically demonstrate the efficiency of the ABP^{BPH} method by purifying the pharmaceutical antibody trastuzumab from common contamination sources including CHO cell conditioned media and ascites fluid with >85% yield and >97% purity.

Materials and Methods

Synthesis of Bivalent Hapten. The bivalent hapten was synthesized using standard Fmoc chemistry on a rink amide resin (EMD Biosciences). Residues were activated with 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) in DMF with N,N-Diisopropylethylamine (DIEA) for 3 minutes and coupling completion was monitored with Kaiser tests. The Fmoc protected residues were deprotected using three exposures to 20% piperidine in DMF for 3 minutes. The peptides were cleaved from the solid support using two exposures to 94/3/3 Trifluoroacetic acid (TFA)/H_2O/Triisopropylsilane (TIS) for 30 minutes. The peptides were purified using RP-HPLC on an Agilent 1200 series system with a semi-preparative Zorbax C18 column (9.4 mm x 250 mm), using linear solvent gradients of 2.5% min^{-1} increments in ACN concentration at 4.0 mL/min flow rate. We monitored the column eluent with a diode array detector allowing a spectrum from 200 to 400 nm to be analyzed. The purity of the bivalent haptens was estimated to be >97% by an analytical injection using the above-described HPLC with an analytical Agilent Poroshell 300SB-C8 column (2.1 mm x 75 mm). The purified product was characterized using a Bruker micrOTOF II mass spectrometer. The calculated exact mass of the bivalent hapten ($C_{185}H_{272}N_{44}O_{47}$) was 3862.025 Da; found 3863.084 Da. The calculated exact mass of the DNP labeled

bivalent hapten ($C_{204}H_{299}N_{49}O_{55}$) was 4315.211 Da; found 4316.248 Da. HPLC traces and mass spec chromatograms are in the Supporting Information as Fig. S1† and Fig. S2† for the bivalent hapten and DNP labeled bivalent hapten respectively.

Fluorescence Quenching Assay for the Determination of Antibody-Bivalent Hapten Binding Affinity. A DNP labeled bivalent hapten was synthesized using Fmoc chemistry by adding an ethylene glycol (EG_2) spacer, lysine, and then DNP to the N terminus of the bivalent hapten. The labeled bivalent hapten was then used in a fluorescence quenching assay to determine the binding affinity for trastuzumab as previously described in detail.[21] Briefly, DNP quenches the fluorescence of tryptophan residues in the antibody, occurring at 335 nm when the two molecules are in close proximately to each other (<10 nm). The bivalent hapten was titrated into a 96-well plate containing a 200 µL solution of 15 nM trastuzumab in PBS.

Trastuzumab Complex Formation. The sizes of trastuzumab complexes that formed upon the addition of the bivalent hapten were measured using a Malvern Zetasizer Nano S. To measure the size of trastuzumab, 6.7 µM trastuzumab was prepared in PBS, and the particle size was measured. To measure trastuzumab complex formation, 20 µM of the bivalent hapten was mixed with 6.7 µM trastuzumab, and the complex size was measured using an estimated refractive index of 1.45.

Reverse-Phase HPLC Analysis of Antibody Solutions. Reverse-phase HPLC was used for the rapid analysis of pure antibody and mixtures of antibody with contaminants using the above described Agilent 1200 series system with an analytical Agilent Poroshell 300SB-C8 column (2.1 mm x 75 mm). Typical injection volumes were 2 µL from a 10 fold dilution from the sample and the analysis was carried out at 70˚C with a flow rate of 2 mL/min and a rapid gradient from 5% to 100% ACN in 5 minutes. The column eluent was monitored with a diode array detector allowing a spectrum from 200 to 300 nm to be analyzed.

Residual Host Cell Protein Quantification. Trastuzumab was spiked into CHO cell conditioned media at a concentration of 6.7 µM. A small fraction of the sample was removed to determine the initial concentration of HCP while the ABP^{BPH} method was used to purify trastuzumab as described in the text. The HCP content in the initial antibody/CHO conditioned media and purified product were quantified using the third-generation CHO HCP ELISA kit from Cygnus Technologies following the manufacturer recommended protocol.

Residual DNA Quantification. Trastuzumab was spiked into CHO cell conditioned media and ascites fluid at a concentration of 6.7 µM. A small fraction of each sample was removed to determine the initial concentration of DNA while the ABP^{BPH} method was used to purify trastuzumab as described in the text. The DNA content in the initial antibody solutions and purified products were then quantified using the Quant-iT PicoGreen dsDNA high-sensitivity assay kit from Invitrogen following the manufacturer recommended protocol.

Cell culture. SK-BR-3 cells were obtained from American Type Culture Collection (Rockville, MD) and BT-474 cells were a generous gift from John Park at the University of California San Francisco. BT-474 cells were cultured in RPMI 1640 media (Cellgro, Manassas, VA) and SK-BR-3 cells were cultured in McCoy's 5A media (ATCC). Both cell lines were supplemented with 10% fetal bovine serum (FBS), 2 mM l-glutamine (Gibco, Carlsbad, CA), 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco).

Trastuzumab binding activity *in vitro*. HER2 expressing BT-474 and SK-BR-3 adherent cell lines were scraped from the surface of a confluent cell culture dish and placed in binding buffer (1.5% BSA in PBS pH 7.4) for 30 min on ice. Variable concentrations of trastuzumab (anti-HER2) were incubated with the cells on ice for 1 hr and then washed twice with the binding buffer. Secondary anti-human fluorescein conjugated antibody (Jackson ImmunoResearch, West Grove, PA) was added at a 15 µg/mL concentration for 1 hr on ice. Samples were washed twice with binding buffer and analyzed on Guava easyCyte 8HT flow cytometer (Millipore).

Results

Characterization of Bivalent Hapten-Antibody Complex Formation

The ABP^{BPH} method requires a bivalent hapten specific for the antibody of interest. Several peptides have recently been identified that bind to pharmaceutical antibodies for the use of vaccine development. We previously synthesized several of these short peptides and found that the peptide, LLGPYELWELSH, has a K_d of 1.4 µM for trastuzumab and was therefore selected for use as a hapten.[20, 22, 23] A bivalent version of this hapten was synthesized by linking two repeats of the hapten sequence with the semi-rigid hydrophilic linker, GPGPKPKPGPG, as described in the Experimental Procedures. This linker was long enough to allow bivalent binding to two antibodies without steric constraints, yet short enough to prevent bivalent binding to a single antibody.[20, 24-27] The length of the linker was a critical

factor in our design, if the bivalent hapten binds bivalently to a single mAb, complexes will not form preventing the AMS induced precipitation of the antibody.

Next, we characterized the interactions of the bivalent hapten with trastuzumab. First, we determined the affinity of the bivalent hapten for trastuzumab using a DNP labeled version of the peptide in a fluorescence quenching technique as described in the Experimental Procedures. Using this method we determined that the bivalent hapten has a K_d of 460 nM for trastuzumab (Figure 2A). The enhanced affinity of the bivalent hapten for trastuzumab (K_d = 460 nM) compared to the monovalent hapten (K_d = 1.4 µM) indicates multivalent binding between the peptide and trastuzumab, a requirement for the ABP^{BPH} method.[20, 23] Next, we analyzed the complexes of trastuzumab that formed upon the addition of the bivalent hapten using dynamic light scattering (DLS). Monomeric trastuzumab had a hydrodynamic diameter of 9.7 nm that increased to 17.4 nm upon the addition of the bivalent hapten (Figure 2B,C). This result indicated that the dominant product formed with the addition of the bivalent hapten was a cyclic dimer consisting of two antibody molecules with two bivalent haptens as indicated in Figure 2C.[23-28] The hydrodynamic diameter of the antibody complexes extended to 90 nm indicating the formation of some polymers of bivalent hapten with trastuzumab. Combined, the binding and DLS results indicate the formation of antibody complexes in the presence of the bivalent hapten thus validating its use in the ABP^{BPH} method.

Effect of Bivalent Hapten and Ammonium Sulfate on Antibody Precipitation

The ABP^{BPH} method uses AMS to selectively precipitate antibody complexes from contaminants. AMS is commonly used in protein purification because it is available in high purity, with low cost, is soluble up to 4 M in water, and is non-denaturing.[15, 19] It was important to optimize the concentration of AMS to ensure high mAb recovery. If the concentration of AMS is too high, monomeric antibody will precipitate in the first step simultaneously with high molecular weight impurities. If the concentration of AMS is too low, not all of the antibody complexes will precipitate in the second step, hence lowering the recovery. To determine the optimal concentration of AMS, we prepared solutions of trastuzumab (6.7 µM, 1 mg/mL) alone or with an equal concentration of the bivalent hapten with increasing concentrations of AMS (Figure 3A). The samples were incubated at 4˚C for 4 hours to ensure equilibrium was reached then centrifuged to separate the precipitated antibody from the solution. The amount of antibody remaining in solution was determined using RP-HPLC by monitoring absorbance at 280 nm as described in the

methods section. At a concentration of 1.3 M AMS, all of the antibody precipitated with the bivalent hapten while ~93% of the antibody remained in solution in the absence of the bivalent hapten (Figure 3A). Based on the stoichiometry of the antibody, an equal concentration of the bivalent hapten was the minimum amount required to form complexes with all of the antibody. To determine if this minimum amount of bivalent hapten was optimal, the process was repeated with 20 µM and 33 µM bivalent hapten representing 3 and 5 times the antibody concentration respectively (Figure 3A). With the increased concentration of bivalent hapten, an AMS concentration of 1.2 M was sufficient to cause complete precipitation of antibody complexes (100%) without precipitating monomeric antibody. The increased bivalent hapten concentration increased the maximum potential recovery from 93% to 100% since it allowed the use of a lower concentration of AMS that did not result in the precipitation of monomeric antibody. Next, we determined the effect of increasing trastuzumab concentration on AMS induced antibody precipitation. This was accomplished by repeating the optimization of the AMS and bivalent hapten concentration at an antibody concentration of 20 µM (3 mg/mL, Figure 3B). Similar results were observed with the increased antibody concentration; an AMS concentration of 1.2 M did not precipitate monomeric trastuzumab and was able to cause complete precipitation of antibody complexes with a bivalent hapten concentration equal to 3 times the antibody concentration. Therefore, the experimental conditions of 1.2 M AMS and a bivalent hapten concentration equal to 3 times the antibody concentration were selected to ensure maximum recovery.

Antibody Purification from CHO Cell Conditioned Media and Ascites Fluid

To demonstrate the high antibody purity obtained with the ABP^{BPH} method, trastuzumab was purified from CHO cell conditioned media and ascites fluid, two typical sources for antibody production. For these experiments, known concentrations of trastuzumab were spiked into the contaminant source with the initial antibody concentration ranging from 3.3 to 33 µM (0.5 - 5 mg/mL). The AMS concentration was increased to 1.2 M to precipitate any larger protein contaminants which were separated and removed via centrifugation. Next, the bivalent hapten was added to the solution at a concentration equal to 3 times the antibody concentration causing the formation of antibody complexes which immediately precipitated from the solution. The antibody complexes were separated from the solution via centrifugation and the pellet was solubilized in PBS. The bivalent hapten was then removed using membrane filtration resulting in pure, bivalently active antibody. A more detailed description of each step is listed below.

Step 1: Removal of High Molecular Weight Impurities. Pure trastuzumab, CHO cell conditioned media, and ascities fluid were analyzed with reverse phase HPLC (RP-HPLC) as described in the Experimental Procedures and these chromatograms were used as references (Figure 4). Next, trastuzumab was spiked into CHO cell conditioned media and ascites fluid. The resulting antibody solutions were analyzed with RP-HPLC and the antibody peak intensity in the chromatograms is representative of the concentration of antibody relative to the contaminants (Figure 4). Next, AMS was added to the antibody solution to a final concentration of 1.2 M to precipitate high molecular weight proteins. The samples were centrifuged and the precipitate was discarded. This step ensured complete removal of protein contaminants that would otherwise precipitate with the antibody complexes and contaminant the product.

Step 2: Precipitation of Bivalently Active Antibody. With the larger protein contaminants removed, the supernatant contained the active antibody, damaged antibody and contaminants smaller than the antibody. The bivalent hapten was added at 3 equivalents to the antibody concentration causing the antibody to precipitate through the formation of insoluble complexes. The samples were incubated for 4 hours at 4˚C to ensure maximum antibody recovery followed by centrifugation to separate the precipitated antibody complexes from the supernatant. At this point, the supernatant contained only contaminants and was discarded (Figure 4). The antibody pellet was washed with a 1.2 M AMS solution in PBS to ensure complete removal of contaminants. After the wash, the antibody pellet was redissolved in PBS and analyzed with the RP-HPLC and the only contaminant detected was the bivalent hapten (Figure 4).

Step 3: Separation of the Antibody from the Bivalent Hapten. The solubilized antibody pellet from step 2 contained the bivalent hapten and trastuzumab. The bivalent hapten was separated from the antibody using a 10 kDa molecular weight cut off spin concentrator (Millipore). The concentrated antibody was then diluted in PBS and the spin concentrator process was repeated once more to ensure complete separation of the antibody from the bivalent hapten. The resulting antibody product was >97% pure and the overall antibody recovery was >85%. We also evaluated the use of a dialysis cassette in place of the spin concentrator and found that the dialysis cassette also effectively separated the antibody from the bivalent hapten, however, the antibody recovery dropped to 75-80%. These results demonstrated that both dialysis cassettes and protein spin concentrators are capable of separating the antibody from the bivalent hapten with the spin concentrator resulting in slightly higher antibody recovery. Additionally,

the high recovery of the antibody indicates that both the cyclic and polymeric complexes of the antibody easily dissociate during membrane filtration. The antibody purity was determined using RP-HPLC (Figure 4) and SDS-PAGE (Fig. S3†).

Host Cell Proteins and Host Cell DNA are Efficiently Removed

Pharmaceutical antibodies are most commonly produced in transfected CHO cells. As a consequence, the CHO cells are the largest source of contaminants which include host cell proteins (HCP) and DNA. We therefore evaluated the effectiveness of the ABP^{BPH} method at removing both HCP and DNA. Using a third-generation CHO HCP ELISA kit from Cygnus Technologies, (see Experimental Procedures) the concentration of HCP present in the initial trastuzumab/CHO solution was compared to the purified product. The concentration of HCP was normalized to the antibody concentration. Prior to purification, the HCP concentration was 9850 ± 250 ng/mg mAb and after purification the HCP concentration was reduced to 185 ± 15 ng/mg mAb. The log reduction value (LRV) was 1.72 which is comparable to the HCP removal using protein A chromatography.[11, 29] Next, the concentration of DNA in the initial antibody/CHO and antibody/ascites solutions were compared to the purified products using the high-sensitivity Quant-iT PicoGreen dsDNA kit from Invitrogen (see Experimental Procedures). The concentration of DNA in each sample was normalized to the antibody concentration (Table 1). The LRV values were 2.14 and 2.08 when trastuzumab was purified from CHO cell conditioned media and ascites fluid respectively. These LRV values are similar to those obtained with protein A chromatography.[11, 30] Combined, these results further support the high level of purity (>97%) obtained with the ABP^{BPH} method.

Purified Antibodies Retain Binding Activity

Next we evaluated the activity of purified trastuzumab by evaluating the binding of the antibody to cell lines that express the target protein. Trastuzumab targets the human protein HER2 and was evaluated using the breast cancer cell lines BT-474 and SK-BR-3 which both overexpress human HER2.[31] Prior to evaluating the purified antibody samples, it was necessary to determine the appropriate antibody concentration. The activity of the antibody must be evaluated at a sub-saturating concentration in order to determine any adverse effects in the antibody activity. Consequently, native trastuzumab was incubated with both BT-474 and SK-BR-3 cell lines at increasing concentrations for 60 minutes on ice. Binding was detected using a secondary Fc specific fluorescein labeled antibody (Fig. S4†). Under these conditions, 5 nM trastuzumab demonstrated near saturating levels of binding and no significant increase

in binding was observed beyond 10 nM. Based on these results, the activity of the purified antibody samples were evaluated at 5 nM to ensure that if any of the antibody was inactive, differences in binding could be observed. Using the described method, the binding activity of trastuzumab purified from CHO cell conditioned media and ascites were compared to native trastuzumab. Our results demonstrated that trastuzumab purified via the ABP^{BPH} method had native levels of binding activity to both BT-474 and SK-BR-3 cells (Figure 5A). These results confirmed that the ABP^{BPH} method did not have any adverse effects on the antibody activity, including both antigen detection and Fc recognition.

Purification of Trastuzumab from Antibody Mixtures

To demonstrate the specificity of the ABP^{BPH} method for a single antibody, trastuzumab was purified from a mixture containing an equimolar solution of both trastuzumab and rituximab, a pharmaceutical mAb targeting CD-20. Both rituximab and trastuzumab are IgG1κ pharmaceutical antibodies. Due to the similarities between these antibodies, they cannot be separated with protein A chromatography. However, using the ABP^{BPH} method, trastuzumab was purified from rituximab with >95% purity and >85% recovery (Figure 5B). Traditional methods for determining antibody purity cannot distinguish between antibodies of the same isotype. Consequently, the recovery and yield were based on the amount of antibody recovered compared to the amount of trastuzumab in the initial antibody solution and by comparing the binding activity of the purified antibody and the initial antibody solution to native trastuzumab using $HER2^{+}/CD20^{-}$ cell lines BT-474 and SK-BR-3 (Figure 5B).[31] These results demonstrate the selectivity of the ABP^{BPH} method for a single antibody in a complex solution containing multiple antibodies of different specificities.

Purification of Bivalently Active Trastuzumab from a Solution of Active and Denatured Antibody

Finally, to demonstrate that the ABP^{BPH} method is specific for active antibody, trastuzumab was purified from a solution of active and chemically denatured antibody. Trastuzumab was denatured using 5 M guanidine hydrochloride and by storing the antibody at room temperature for 3 weeks. We confirmed that the antibody was denatured by complete loss of binding to HER2 expressing cell lines BT-474 and SK-BR-3 cells (Fig. S4†). Next, 4 solutions of trastuzumab at 6.7 μM were prepared: (i) 100% native (ii) 75% native, 25% denatured, (iii) 50% native, 50% denatured, and (iv) 25% native, 75% denatured. Typically the amount of denatured antibody in a sample is unknown; consequently, the same concentration of the bivalent hapten (20 μM) was used to purify all 4 samples. The binding activities of

the antibody samples before and after purification were evaluated at a 5 nM total antibody concentration using the HER2 expressing cell lines BT-474 and SK-BR-3. Prior to purification, the samples containing 75, 50, and 25% native antibody had 78, 61, and 30% binding activity respectively (Figure 5C). After purification, all 4 antibody samples demonstrated native biding activity demonstrating the selectivity of the ABP^{BPH} method for active antibody (Figure 5C).

Discussion

The ABP^{BPH} technique is an effective and straightforward method for the facile purification of mAbs from complex biological solutions. The ABP^{BPH} method was demonstrated by purifying trastuzumab from CHO cell conditioned media, ascites fluid, other antibodies, and denatured antibodies. The purified product consistently had >97% purity with yields in excess of 85% and displayed native binding activity. The utility of the ABP^{BPH} affinity-based precipitation method was demonstrated by varying the trastuzumab concentration from 0.5 to 5 mg/mL representing the range of antibody concentrations in lab scale and industrial scale antibody production.[3]

Additionally, the ABP^{BPH} method has significant advantages over traditional affinity chromatography methods including low cost, rapid purification, easily scalable, concentrates the antibody of interest, does not require the use of specialized equipment, is selective for bivalently active antibody, and can be utilized to purify any bivalent antibody including IgG, IgE, and IgD. The ABP^{BPH} method also eliminates the possible contaminants inherent to protein A chromatography including leached Protein A and damaged, denatured or aggregated antibody caused by the acidic conditions required for elution. Finally, the ABP^{BPH} method brings a significant improvement over the recently described affinity-based precipitation method using trivalent haptens.[20] Despite the success of our previous work, there were some major limitations regarding the synthesis and use of the trivalent hapten. The trivalent hapten was synthesized in 3 stages. First, the peptidic hapten portion was synthesized, second a monodispersed ethylene glycol ligand was conjugated to the hapten as a linker, and finally three copies of the hapten-ethylene glycol ligand was conjugated to the core molecule. These non-standard peptide synthesis techniques result in an overall low yield and the required use of specialized equipment limits the broad applicability of the affinity-based method for antibody purification. In addition, during the separation of the trivalent hapten via membrane filtration in the last step of antibody purification, the trivalent hapten formed highly stable bicyclic trimers with the target antibody that necessitated the addition of guanidinium chloride, a protein denaturant, to dissociate the complexes.[20] To overcome the limitations

introduced by the use of the trivalent hapten, in this study we designed a linear bivalent hapten where the two haptens are linked by a semi-rigid hydrophilic amino acid linker. By eliminating branching and by replacing the ethylene glycol linker with amino acids, the entire bivalent peptidic hapten can be synthesized using standard peptide synthesis techniques thereby enabling automated synthesis via peptide synthesizers. Importantly, the simplified bivalent peptidic hapten can be recombinantly expressed in cells thereby enabling the production of the potentially large quantities required for the industrial scale.[32] In addition, the bivalent hapten forms cyclic dimers with the target antibody that have reduced stability compared to the bicyclic trimers as indicated by the 6 fold reduction in affinity of the bivalent hapten for trastuzumab compared to the trivalent hapten.[20] The reduced stability of the cyclic dimers eliminates the need for guanidinium chloride to dissociate the antibody complexes in the final step of the ABP^{BPH} method. Overall, the bivalent hapten design utilized in the ABP^{BPH} method for the purification of active antibodies brings a significant advancement to the field of antibody purification. A remaining potential challenge in the application of the ABP^{BPH} method is the identification of a hapten for the antibody of interest. However, with the advancements in high throughput screening techniques such as micro arrays and phage display it is now feasible to identify short peptide sequences specific for a vast number of antibodies for use as haptens, making the ABP^{BPH} method broadly applicable. Altogether, the ABP^{BPH} technique provides a rapid and efficient method for the purification of antibodies from lab scale to industrial scale with the potential to decrease production costs while increasing product quality.

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Tables

Table 1. DNA Content in Initial Trastuzumab Solutions and Purified Products

Sample	Initial DNA (ng/mg mAb)	Final DNA (ng/mg mAb)	LRV
Trastuzumab + CHO	22000 ± 1500	160 ± 100	2.14
Trastuzumab + Ascites	15000 ± 1000	125 ± 100	2.08

Figures with captions

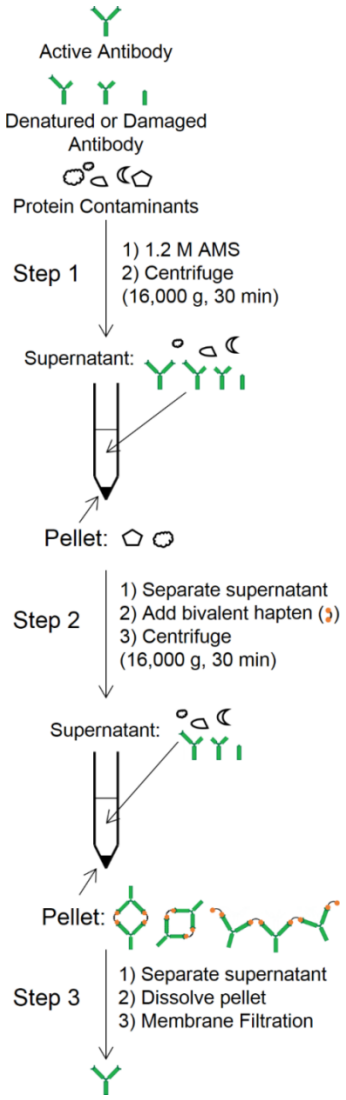

Figure 1. Graphical representation of the ABP^{BPH} method for the purification of bivalently active monoclonal antibody from complex biological solutions. (1) The concentration of AMS in the crude antibody solution was increased to 1.2 M to precipitate high molecule weight contaminants which were separated via centrifugation and discarded. The supernatant from step 1 contained the antibody and low molecular weight contaminants. (2) The addition of the bivalent hapten caused the formation of antibody complexes which immediately precipitated from the solution. The supernatant now contained only impurities and was separated and discarded via centrifugation. (3) The antibody pellet was dissolved in PBS and separated from the bivalent hapten using membrane filtration yielding pure, bivalently active monoclonal antibody.

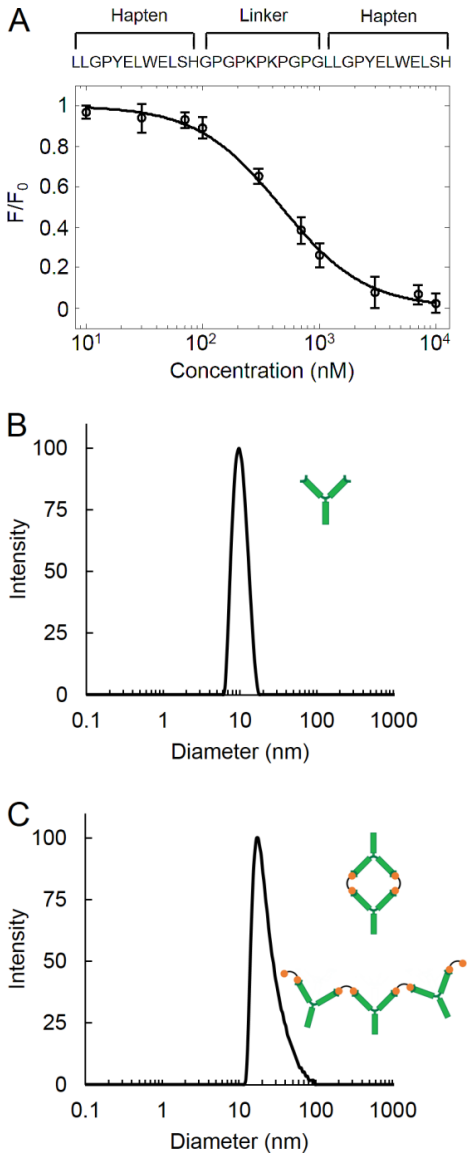

Figure 2. Characterization of bivalent hapten-trastuzumab interactions. (A) A DNP-labeled version of the bivalent hapten was used to determine the binding constant to the pharmaceutical antibody trastuzumab in a fluorescence quenching assay. The bivalent hapten had a K_d of 460 nM. Data represents the mean ± SD of triplicate experiments. (B) In the absence of the bivalent hapten trastuzumab had a hydrodynamic diameter of 9.7 nm as determined using DLS. (C) After the addition of the bivalent hapten, complexes formed with a hydrodynamic diameter of 17.4 nm.

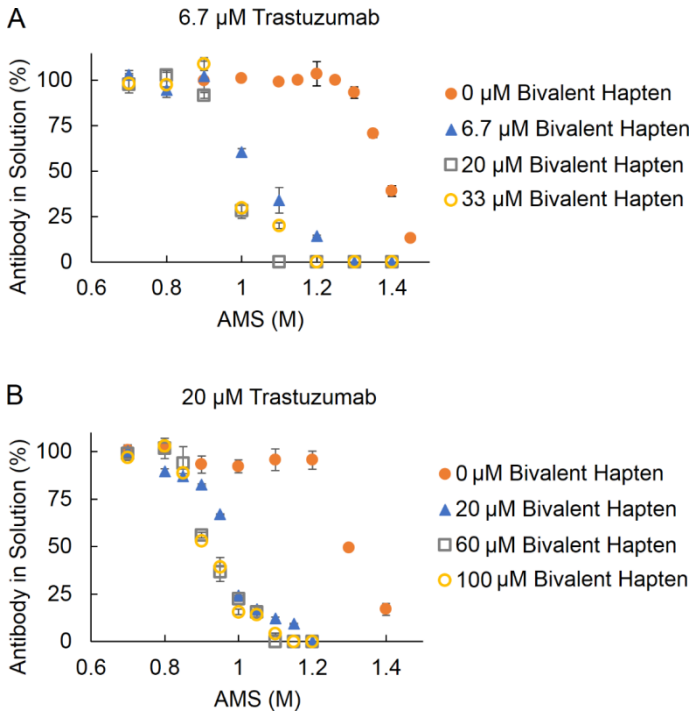

Figure 3. Optimization of the bivalent hapten and AMS concentration. (A) An AMS concentration of 1.2 M caused near complete precipitation of trastuzumab (6.7 μM) in the presence of 6.7 μM bivalent hapten and complete precipitation with bivalent hapten concentrations of 20 and 33 μM. In the absence of the bivalent hapten, trastuzumab did not begin to precipitate until a concentration of 1.3 M. (B) The optimization was repeated with increased trastuzumab (20 μM) and similar results were observed. In the absence of the bivalent hapten, trastuzumab did not being to precipitate until an AMS concentration greater than 1.2 M while this concentration of AMS caused complete precipitation of trastuzumab with 20, 60, and 100 μM bivalent hapten. Data represents the mean ± SD of triplicate experiments.

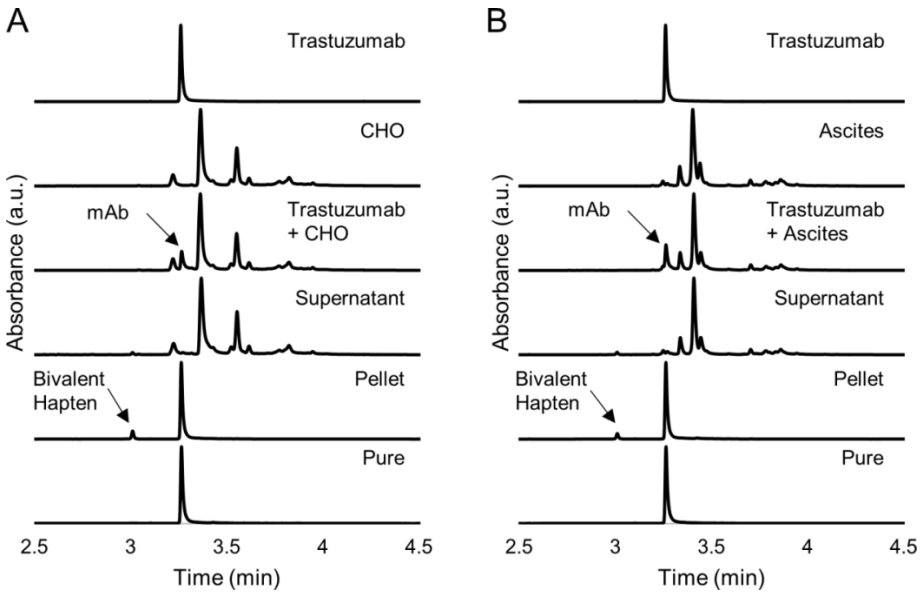

Figure 4. Analysis of samples from the ABP^{BPH} method using RP-HPLC. Trastuzumab was purified from (A) CHO cell conditioned media and (B) ascites fluid. Native trastuzumab, CHO cell conditioned media, and ascites fluid were used as references. The trastuzumab peak intensities in the samples containing the antibody with the contaminant are indicative of the concentration of antibody to contaminant proteins in the starting solution. The addition of the bivalent hapten to the initial antibody solution with 1.2 M AMS caused complete precipitation of trastuzumab as shown in the supernatant and pellet chromatograms. The bivalent hapten was then removed using membrane filtration. The purified trastuzumab contained a single peak demonstrating high purity.

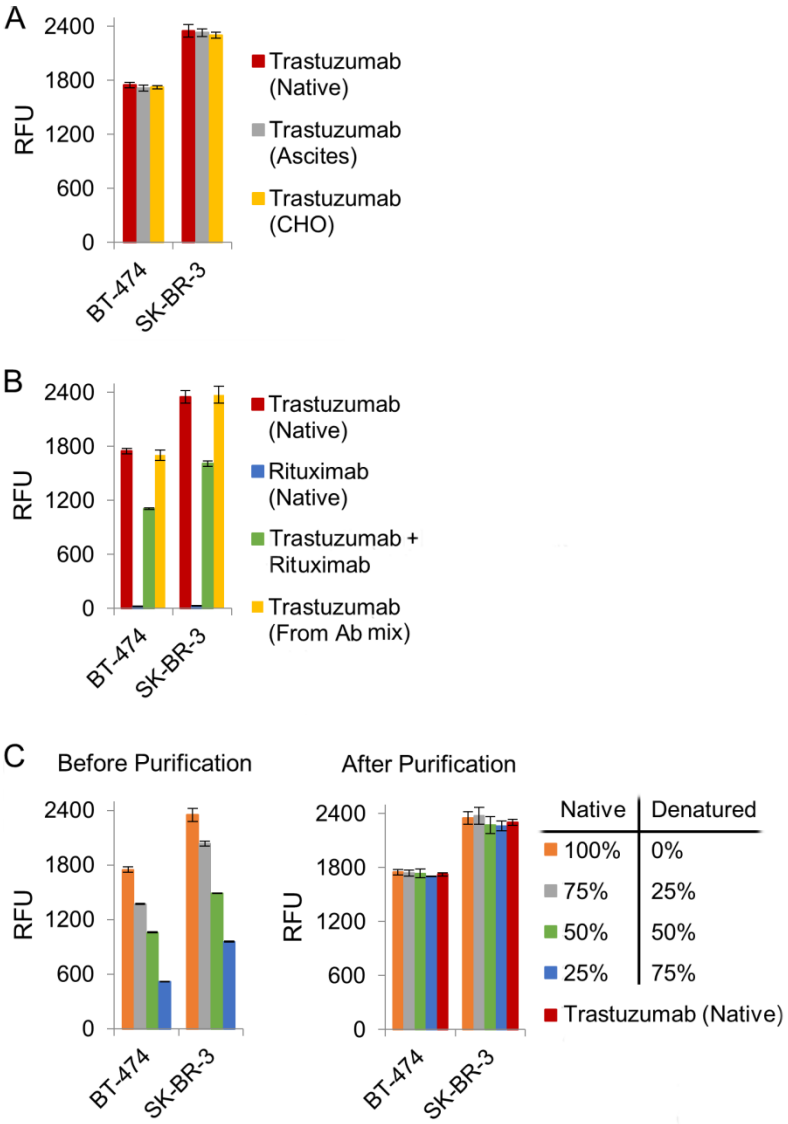

Figure 5. Binding activity of purified trastuzumab. (A) Trastuzumab purified from CHO cell conditioned media and ascites fluid had native binding activity. (B) Trastuzumab purified from a solution initially containing an equimolar solution of trastuzumab and rituximab had native binding activity. The initial antibody mixture had 65% binding activity and rituximab did not have any binding activity compared to native trastuzumab. (C) Trastuzumab purified from solutions of active and denatured antibody had native binding activity. The antibody solutions initially contained (i) 100% native, 0% denatured, (ii) 75% native, 25% denatured, (iii) 50% native, 50% denatured, and (iv) 25% native, 75% denatured. After purification, native binding activity was restored for all 4 samples. Antibody activity was demonstrated using the HER2 expressing cells lines BT-474 and SK-BR-3 at a total antibody concentration of 5 nM. Data represents the mean ± SD of triplicate experiments.

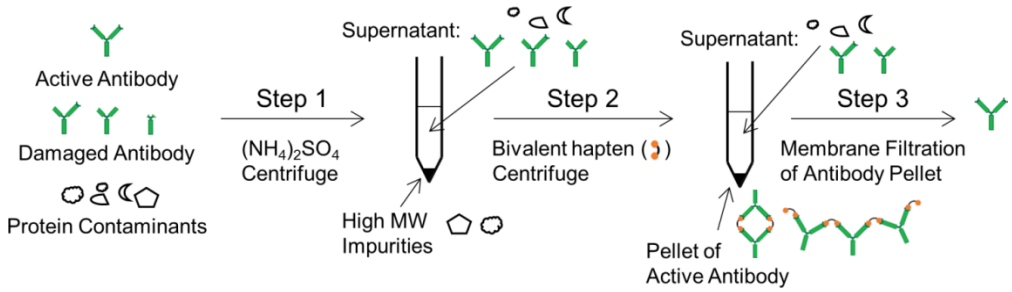

Table of contents graphic

An affinity-based precipitation method was developed for the rapid and facile purification of bivalently active antibodies from complex biological solutions.