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Research Paper

Cross-Interaction Chromatography: A Rapid Method to Identify Highly Soluble Monoclonal Antibody Candidates

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Purpose. To develop a high-throughput cross-interaction chromatography screening method to rapidly identify antibody candidates with poor solubility using microgram quantities of purified material.
Methods. A specific recombinant antibody or bulk polyclonal IgG purified from human serum was chemically coupled to an NHS-activated chromatography resin. The retention times of numerous monoclonal antibodies were determined on this resin using an HPLC and compared to the solubility of each antibody estimated by ultrafiltration.
Results. Retention times of the antibodies tested were found to be inversely related to solubility, with antibodies prone to precipitate at low concentrations in PBS being retained longer on the columns with broader peaks. The technique was successfully used to screen microgram quantities of a panel of therapeutic antibodies to identify candidates with low solubility in PBS.
Conclusions. The cross-interaction chromatography methods described can be used to screen large panels of recombinant antibodies in order to discover those with low solubility. Addition of this tool to the array of tools available for characterization of affinity and activity of antibody therapeutic candidates will improve selection of candidates with biophysical properties favorable to development of high concentration antibody formulations.

KEY WORDS: aggregation; cross-interaction chromatography; monoclonal antibody; protein solubility.

INTRODUCTION

Therapeutic monoclonal antibodies (mAb) represent one of the fastest growing classes of therapeutic drugs, with at least 22 mAbs currently approved and more than a hundred in various stages of clinical trials for indications including autoimmune diseases, oncology, cardiovascular diseases, allergies, and transplant rejections (1). Technology advances over the last decade have enabled the discovery and optimization of monoclonal antibody therapeutic candidates using a variety of strategies ranging from transgenic mice to *in vitro* evolution. Libraries of antibodies derived by these new methods can be rapidly screened for activity, specificity and affinity so that it is frequently possible to obtain multiple high affinity antibody candidates (2,3). However, despite admirable *in vitro* activity and potency, successful development of candidate therapeutic antibodies can be limited by their solubility and aggregation properties. Protein solubility properties are governed by a variety of forces that contribute to self-interaction propensity and selection of mAb candidates with the optimal balance of activity, affinity, stability and solubility can be challenging at the discovery stage.

As monoclonal antibody technology has advanced, the use of mAbs for chronic therapy has become more common, and the need for convenient delivery strategies has become increasingly important. Thus, although the earliest mAb therapies were delivered by intravenous injection, many newer mAb therapies rely primarily on subcutaneous injection. Subcutaneous administration places high demands on the biophysical properties of antibodies due to the constraints of dosage volume (preferred at <1.5 mL) and high doses (>100 mg) (4). Subcutaneously delivered mAbs are often formulated at very high concentrations under conditions that maintain low viscosity, chemical integrity, and a monomeric state. Thus, solubility is one biophysical property that is important to the success of formulation development. In contrast to the high-throughput screening methods available for purification, activity, and affinity characterization of antibodies (5–11), there are few methods available for characterizing the solubility properties of proteins at small scale. Indeed, characterization of the solubility properties of proteins has traditionally relied on larger quantities of protein that are not routinely available at the screening stage of antibody discovery.

Many variations exist around methods used to measure or estimate protein solubility, with some of the most common

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ABBREVIATIONS: CIC, cross-interaction chromatography; Fab, fragment antigen binding; mAb, monoclonal antibody; PBS, phosphate buffered saline; pIgG, polyclonal immunoglobulin.

techniques including filtration and precipitation methods (12). Ultrafiltration is perhaps the most simple method used to estimate the solubility of a given protein in given solution. In this method, a protein solution is filtered through a membrane with an appropriate molecular weight cut-off until an amorphous precipitate is observed. After precipitation, the solution can be filtered and the protein concentration determined by measuring the absorbance at 280 nm. One advantage of this technique is that solubility can be determined in a pharmaceutically relevant solution. However, this method suffers from the reality that for some antibodies, it is difficult to reach the concentration at which the protein precipitates without consuming large quantities of material due to solubility over 100 mg/mL. Unfortunately, ultrafiltration requires the generation of milligram quantities of the purified material in order to estimate solubility, thus making the screening of larger sets of molecules with this method difficult. An alternative measure of solubility is to determine the concentration of protein in solution in the presence of increasing concentrations of precipitant, usually ammonium sulfate or polyethylene glycol. The data generated from such experiments is then extrapolated to zero precipitant concentration to determine the solubility (13–16). Finally, there are numerous additional techniques that have been described to estimate or directly measure protein solubility using dialysis, evaporation, and chromatographic methods (12).

A major limitation of the methods described above is the requirement of relatively large amounts of protein to complete such studies, often on the order of 10 mg or more. This report describes an antibody solubility screening method based on cross-interaction chromatography (CIC) that requires only microgram amounts of protein. By limiting the amount of material used to microgram quantities, this process becomes amenable to the screening of samples produced by small-scale, transient expression in mammalian cells, therefore minimizing cost and time. The method can be used to screen multiple antibody candidates identified during activity and affinity characterization in order to eliminate those molecules with low solubility. We demonstrate that the solubility screening results from this method compare well with estimates of solubility generated by ultrafiltration and that it is highly effective in ranking the relative solubilities of antibodies in a specific buffer. For the purpose of this study, solubility is defined as the maximum protein concentration that can be achieved before the observance of amorphous precipitate. Thus, the term *solubility* is used here to describe amorphous solubility rather than crystalline or thermodynamic solubility (17). This definition does not exclude the possibility that such molecules may form oligomers in solution that are not observed by visual inspection. The results presented here demonstrate that the CIC method can be successfully used as a rapid screening tool to determine the relative solubility of a large number of antibodies.

MATERIALS AND METHODS

Reagents

Phosphate buffered saline (PBS, 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.4) was purchased from Invitrogen. All mAbs were produced by transient transfection in HEK293 or CHO cells followed by purification using Protein A resin.

Coupling of Antibodies to Chromatography Resins

mAb1 was coupled to Toyopearl AF-tresyl-650 M chromatography resin (Tosoh Biosciences, Montgomeryville, PA) by adding 10 mL of a 1 mg/mL solution of the antibody in coupling buffer (0.1 M $NaHCO_3$ pH 8.1, 0.5 M NaCl) to 1 mL of 0.25 g of resin swollen in 1 mL of coupling buffer. The reaction was allowed to proceed for 5 h at room temperature before washing twice with 10 mL of coupling buffer. Coupling efficiency was estimated by measuring the protein concentration left in solution by absorbance at 280 nm. The remaining unreacted tresyl groups were blocked by incubating the resin in 0.1 M Tris-HCl pH 8.1, 0.5 M NaCl overnight at 4°C. The coupled resin was packed in a 6.6×50 mm borosilicate chromatography column (Omnifit, Boonton, NJ) in PBS at a flow rate of 1 mL/min until the bed height stabilized. Final volume of the column was ~0.8 mL. The integrity of the packing procedure was verified by injecting a 10 μL sample of 10% acetone. A control column was generated by blocking 1 mL of swollen resin in 0.1 M Tris-HCl pH 8.1, 0.5 M NaCl overnight at 4°C without coupling any protein to the surface. This resin was then packed into an identical column as described above.

Purified IgG from human serum was obtained from Sigma (catalog # 14506-100MG) and resuspended in 0.1 M NaHCO3 pH 8.1, 0.5 M NaCl at a concentration of 8.2 mg/mL. This solution was passed back and forth over a 1 mL HiTrap NHS-activated column (GE Healthcare # 17-0716-01) every 30 min for 5 h at room temperature. Coupling efficiency was estimated by measuring the protein concentration left in solution by absorbance at 280 nm and assuming that any protein not remaining in solution was bound to the resin. Any remaining reactive groups were blocked overnight at 4°C in 0.1 M Tris-HCl pH 8.1, 0.5 M NaCl.

Chromatography

CIC retention times were determined by diluting each antibody in PBS to a concentration of 0.1 mg/mL and injecting 10 or 20 μL onto the column at a flow rate of 0.1, 0.2 or 0.5 mL/min using a mobile phase of PBS pH 7.4. The retention time of each sample was also determined on a control (no antibody coupled) column in the same way. All chromatography measurements were made using a Beckman Coulter System Gold HPLC or an Ettan-LC HPLC system equipped with an autosampler at room temperature. All columns were equilibrated with at least 3 column volumes of PBS between runs. Peaks were visualized by monitoring absorbance at 214 nm or 215 nm and 280 nm using a UV detector. Retention times were determined using Karat32 software. k′ values were calculated using equations described previously.

$$k' = \frac{Vr - Vo}{Vo} = \frac{Tr - Tm}{Tm} \tag{18}$$

where V_r represents the elution volume of the sample on the protein coupled column, V_o the elution volume from a

control column, T_r the retention time on the protein coupled column and T_m the retention time on the control column. The retention time is defined as the elution time of the peak maximum.

Solubility Determination by Ultrafiltration

Samples were concentrated using VIVAspin6 concentrator with 10 K MWCO (vs0602) membrane and centrifuged at speeds of 4 K to10 K RPM at 23°C. Prior to concentration, each sample was dialyzed into the same PBS buffer that was used as a running buffer in CIC experiments. Each sample was intermittently checked by visual inspection for any signs of precipitation until the desired volume was obtained. After concentration, all samples were transferred to clean glass tubes and stored overnight at 4°C. The next day, the concentration of the solution was determined by diluting the sample 50 fold in PBS and measuring the absorbance at 280 nm using a Nanodrop Spectrophotometer.

RESULTS

Cross-Interaction Chromatography Based on a Monoclonal Antibody

We tested the utility of cross-interaction chromatography (CIC) in screening solubility using mAb1. mAb1 was originally isolated as a Fab fragment by phage display and converted to a full-length antibody by standard methods. The full length mAb was found to have limited solubility near neutral pH as judged by the formation of an amorphous precipitate at a concentration of 13 mg/mL in PBS at pH 7.4 (Table I). The pI of mAb1 was measured to be 7.2 by isoelectric focusing (data not shown), which may explain the limited solubility observed at neutral pH. Ten mg of this antibody were coupled to 1 mL of Toyopearl AF-tresyl-650 M resin and packed into a column for development of the CIC method. The retention times of eight antibodies (mAbs1-6, mAbB, and pavilizumab (19)) and the Fc-fusion protein etanercept (20) were tested on this mAb1 coupled column. Antibodies mAb1-6 are closely related, with mAbs2-6 each exhibiting greater than 98% sequence identity to mAb1. In spite of this sequence similarity, mAbs1-6 have different solubilities as estimated by ultrafiltration in PBS pH 7.4 (Table I). Pavilizumab, mAbB, etanercept, and mAb6 were found to remain in solution in PBS at concentrations greater than 87 mg/mL after ultrafiltration. mAb1, mAb2, mAb3, mAb4, and mAb5, however, all precipitated at concentrations below 25 mg/mL during ultrafiltration (Table I). The CIC data for these proteins are shown in Fig. 1. Although all proteins exhibited a similar profile on the control column where no protein is coupled to the resin (data not shown), mAbs1-5 produced broad chromatographic profiles with delayed retention times on the mAb1 coupled column (Fig. 1), indicative of an interaction with mAb1. mAb6 together with mAbB, pavilizumab, and etanercept eluted as sharper peaks and with reduced retention time in comparison to the others, indicating diminished interactions with mAb1. These results are in agreement with the solubility estimates from ultrafiltration, suggesting that the CIC method is an efficient technique to identify antibodies and Fc-fusion proteins with low solubility.

Table I. Solubility of mAbs in PBS pH 7.4

Sample	Maximum Observed Concentration (mg/mL)
mAb1	13
mAb2	25
mAb3	13
mAb4	17
mAb5	12
mAb6	>163
pavilizumab	>140
mAbB	>126
etanercept	>87

The maximum observed concentration represents the highest concentration that each protein reached by ultrafiltration.

Cross-Interaction Chromatography Based on Polyclonal Antibodies

We further tested this method using an NHS-activated sepharose column coupled to polyclonal IgG (pIgG) isolated from human sera to find whether the relationship between the CIC profiles and solubility can be generalized to other mAbs and to rule out any specific effect due to the coupling of mAb1 to the column. Polyclonal IgG has an advantage over any single monoclonal antibody to be used as the coupling protein due to the diversity of antibody sequences within, as well as the ease of obtaining large quantities from commercial sources. mAb1, mAbB, pavilizumab, etanercept, mAbC, and mAbD (Table II) were injected onto the polyclonal IgG column and the retention times determined. Fig. 2 shows that the proteins tested exhibited a range of retention times. The retention times of these proteins on a control column with no protein coupled vary slightly (Fig. 2A). However, the range of retention times is considerably smaller on the control column than that observed on the pIgG coupled column (Fig. 2B). This indicates that protein-protein interactions between the soluble and bound antibodies are responsible for the differences in retention times between the control column and the pIgG coupled column. mAb1 again showed the broadest peak and the longest retention time, similar to that measured on the mAb1 coupled column. The retention time and profiles of the other molecules also rank in a similar manner to those measured on the mAb1 coupled column, demonstrating that the CIC method using pIgG can also be used to identify antibodies and Fc-fusion proteins with low solubility.

Optimization of the Cross-Interaction Chromatography Method

We next sought to extend the CIC approach to a screening method with maximum resolution and sensitivity by optimizing several parameters using pIgG. The parameters investigated are resin type, flow rate, injection volume, and amount of antibody coupled to the column. We considered two types of resin, Toyopearl AF-Tresyl-650 M and NHS activated Sepharose, using coupling amounts of 30 mg pIgG per mL of resin. Three antibodies, mAb15, mAb16, and

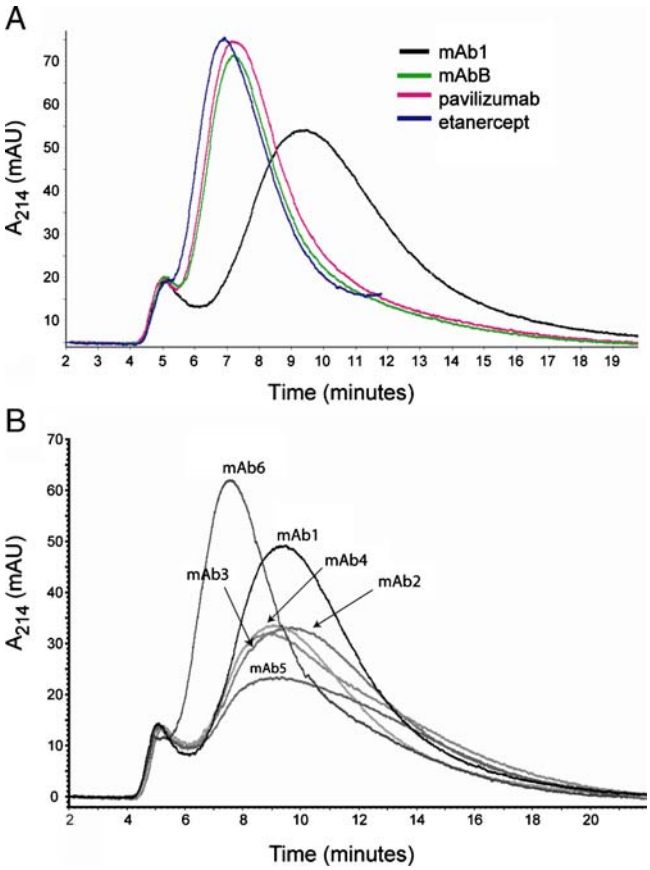

Fig. 1. CIC experiments with mAb1 coupled column in PBS. **A** Retention of soluble molecules mAbB, etanercept and pavitizumab. **B** Retention of mAb1 variants mAb2-6. Retention was monitored by absorbance at 214 nm.

mAb453, were used for this study because they represent a wide solubility range as estimated by ultrafiltration. Fig. 3 demonstrates that the NHS resin yielded better resolution. This resin has the additional advantage that it is available as a pre-packed column, whereas the Toyopearl AF-Tresyl resin must be manually packed.

Flow rate is another parameter that is expected to affect the resolution significantly. Three flow rates of 0.1, 0.2, and 0.5 mL/min were tested using two mAbs: mAb15 and mAb16. The flow rate that provided the best resolution between the antibodies was found to be 0.1 mL/min (Fig. 4A). This is not surprising, since slow flow rate allowed better separation at the expense of longer experimental time in previous cross-interaction chromatography studies (21). Using the flow rate of 0.1 mL/min, therefore, represents a compromise between resolution and throughput.

In order to find the smallest amount of protein injected that still gives a reliable profile, injection volume of 10 uL and 20 uL of 0.1 mg/mL antibodies were tested. Three antibodies—mAb15, mAb16, and mAb453— were used in this study. It was found that 20 μL injections yielded more consistent data, although a 10 μL injection provided interpretable data (comparing Figs. 3A and 4B).

Finally, we investigated the effect of the amount of pIgG coupled to NHS Columns. Three columns were packed at 30, 20, and 10 mg of pIgG per mL of resin, respectively. Twenty microliters of mAb1, mAb15, mAb16, or mAb453 solutions at 0.1 mg/ml were used in this study. Figure 4D shows the representative data on mAb453 when flowed over columns with different concentrations of pIgG coupled to the resin. Coupling at 30 mg of pIgG per mL of resin was found to give the best resolution of soluble and insoluble antibodies.

Relationship Between Cross-Interaction Chromatography and Solubility for a Larger Number of Antibodies

Using the optimized experimental parameters described above, the k′ values (see "Methods") of a larger set of antibodies against a variety of targets were determined in PBS buffer. The data in Table II compares the k′ values observed with the highest measured concentration of each antibody in PBS following ultrafiltration. Unfortunately, limiting solubility of many of these antibodies was not determined due to solubility greater than 100 mg/mL and limiting amounts of recombinant material. The limiting solubility of only antibodies mAb1 and mAb453 were determined by ultrafiltration as these antibodies visibly precipitated from solution at the concentration shown in Table II. The results presented in Table II support the conclusion that antibodies prone to precipitate in PBS are retained longer on the pIgG column.

Applying Cross-Interaction Chromatography Screening to a Pool of Therapeutic Antibody Candidates

Having established a relationship between the k′ value of antibodies on the polyclonal IgG column with solubility estimated by ultrafiltration, we used this method to screen a novel set of therapeutic antibody candidates with unknown solubility that target the same antigen in order to rank the relative solubility of these candidates. These antibodies were selected by phage display panning experiments in a Fab

Table II. Antibody Retention on Polyclonal hIgG Coupled NHS-Activated Sepharose Column Represented by k′ Values

mAb	k′-CIC	Maximum Observed Concentration (mg/mL)
mAbC	0.02±0.01	>180
mAbL	0.07±0.10	>143
mAbM	0.07±0.01	>140
mAbB	0.25±0.11	>126
mAbO	0.07±0.02	>120
mAbK	0.12±0.03	>119
mAbN	0.07±0.03	>116
mAbQ	0.02±0.04	>114
mAbF	0.08±0.11	>110
mAbP	0.06±0.08	>106
mAbD	0.08±0.02	>105
mAb453	0.66±0.08	70
mAb1	1.37±0.12	13

The maximum observed concentration represents the highest concentration that each protein reached by ultrafiltration. All samples, except mAb453 and mAb1, remained in solution at the listed concentration. Error values for k′ represent the standard deviations from three independent measurements.

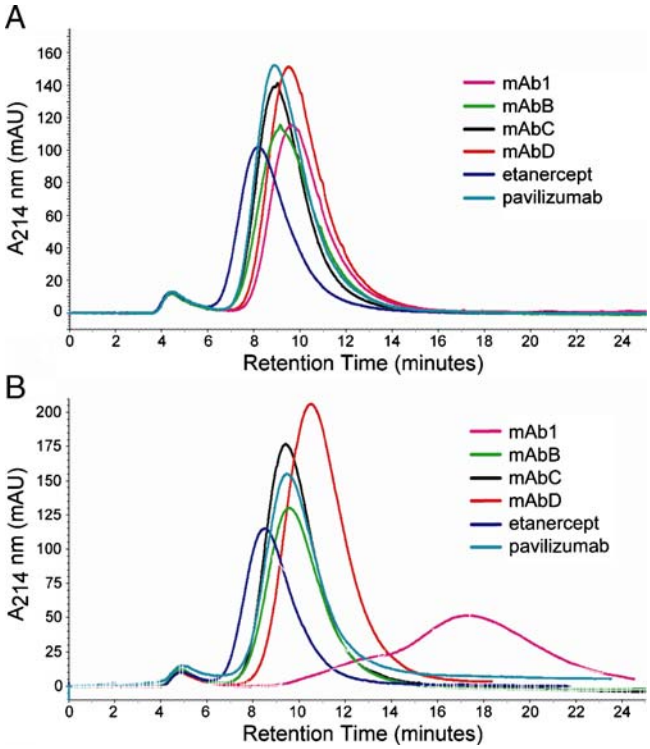

Fig. 2. Retention of test antibodies on pIgG CIC column in PBS. **A** Retention on uncoupled control column. **B** Retention on polyclonal IgG coupled column. IgG was coupled at 30 mg/mL. Elution was monitored by absorbance at 214 nm.

format and were converted to full IgG for analysis. Nineteen antibodies that showed potent activity in biological assays were subject to solubility screening using the CIC method outlined above. Fig. 5 shows the chromatographic profiles of a subset of these antibodies. Sixteen of the nineteen antibodies screened were eluted with a k′ value of <0.4. Two antibodies, mAb106 and mAb107, however, had much longer retention times, resulting in k′ values of 0.94 and 1.01 respectively. Antibodies mAb106, mAb107, mAb109, mAb112, mAb114, and mAb115 were subsequently scaled up and the solubility of each estimated by ultrafiltration. The results confirmed that mAb106 and mAb107 indeed have drastically reduced solubility in comparison to the other antibodies tested as these antibodies precipitated during concentration (Table III). Thus, the CIC screening results of this set of therapeutic antibody candidates successfully predicted the comparatively lower solubility of mAb106 and mAb107 using only 1 microgram of material.

DISCUSSION

This work describes a simple antibody solubility screening method based on the principle of cross-interaction chromatography. Most important for high throughput utility, this screen can be completed with as little as a few micrograms of recombinant protein compared to multi-milligram quantities of protein required for traditional methods of estimating solubility, and can be easily implemented without requirement for special instrumentation. CIC screening results were compared to solubility estimates made by ultrafiltration. Although the ultrafiltration method has several limitations (see "Introduction") we find it to be a simple and reliable way of inducing precipitation of relatively insoluble antibodies. One shortcoming of this method is that for many of the antibodies we tested, the upper solubility limit could not be determined as the antibody did not precipitate at the highest concentrations reached. Thus, a true correlation curve between solubility and CIC retention time could not be drawn. Hence, it is not possible from the data presented here to determine if an antibody with a k′ value of 0.02 is more soluble than an antibody with a k′ value of 0.12. However, the data obtained shows empirically that the most soluble antibodies tested have considerably lower k′ values than those that precipitated during ultrafiltration and that the antibodies whose k′ values are greater than 0.6 are generally significantly less soluble.

The results of this screening method can be used to predict the ability of an antibody to remain in solution at concentrations up to 100 mg/mL, which is beneficial for high concentration formulation. Although this property does not ensure the ability of the molecule to be successfully administered subcutaneously, as a number of other factors could also make a molecule inadequate for this mode of delivery, it increases the chance of a successful formulation development effort. Likewise, it remains possible that an antibody that precipitates at lower concentrations can be formulated in such a manner to improve the solubility enough for injection, by altering solution conditions, such as changing the pH and adding appropriate excipients (22,23).

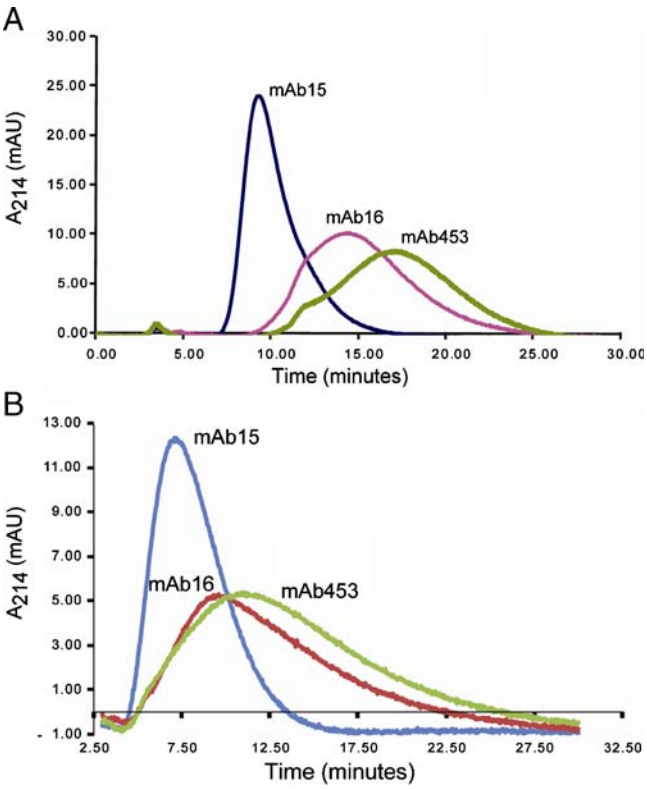

Fig. 3. Effect of chromatography media on pIgG CIC. pIgG was coupled to **A** NHS-activated Sepharose or **B** Toyopearl AF-tresyl-650 M resins. 20 μL of each antibody was injected at a flow rate of 0.1 mL/min.

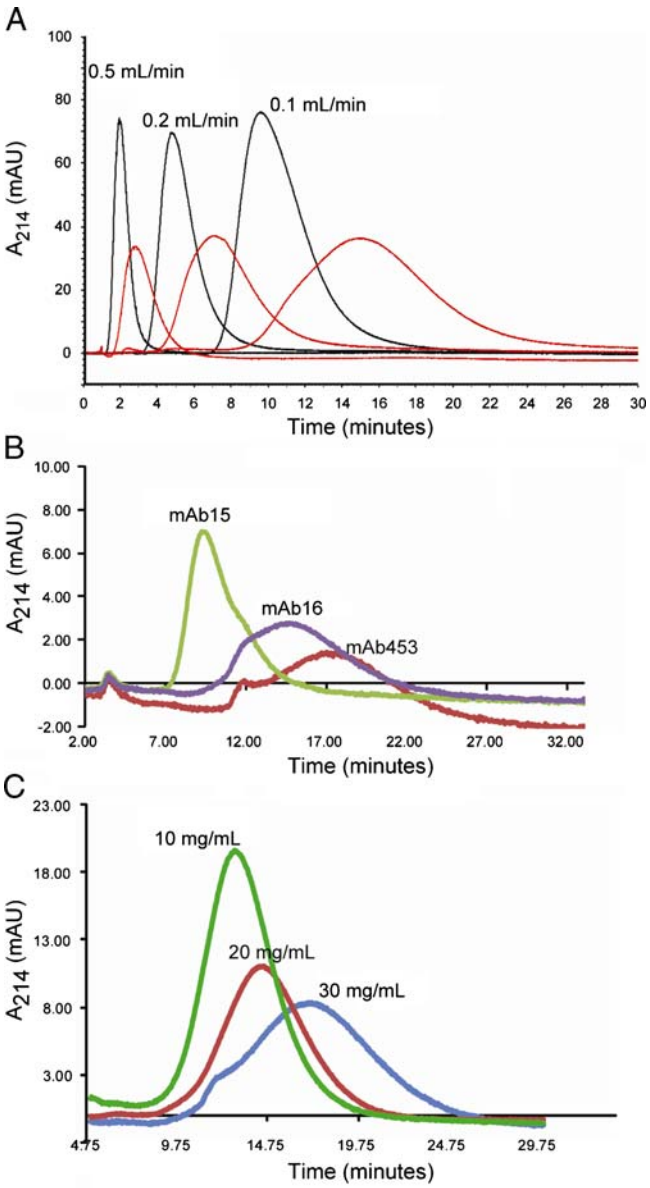

Fig. 4. Optimization of CIC methods. **A** CIC profiles obtained at flow rates of 0.1 mL/min, 0.2 mL/min, and 0.5 mL/min. Black lines represent traces from mAb15 and red lines from mAb16. **B** Effect of injection volume on CIC profiles. For each sample, 10 μL was injected. **C** CIC profiles with varying amounts of pIgG coupled to the resin.

Although we focused on solubility in PBS buffer at pH 7.4 for the preformulation studies described here, the method can be readily extended to other buffer systems. An array of CIC experiments is envisioned where buffers, pH, and excipient combinations can be interrogated, analogous to therapeutic protein formulation or crystallization screens. We have observed a limitation of the method when there is strong interaction between an antibody of interest and the resin matrix itself. In this case, the chromatographic profile on the control column will be poor, and antibody solubility must be assessed by an alternative method.

It is interesting to consider the mechanism of the protein-protein interaction observed by the CIC screening method at the molecular level. As polyclonal IgG coupled to the resin

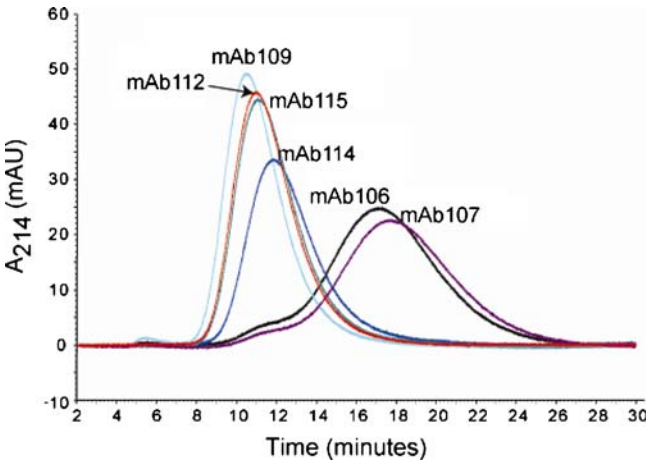

Fig. 5. Retention of selected therapeutic antibody candidates on polyclonal hIgG column.

can be used to successfully discriminate soluble and insoluble antibodies, it would appear that the majority of the interactions observed are nonspecific. However, given the high similarity in antibody sequences, we speculate that this method can also detect certain specific interactions between the variable region of the mobile phase antibodies, with the more conserved framework and constant regions of the antibodies coupled to the column. For example, we have evidence that one antibody determined to have a k′ value >1.0 using this method interacts with the hinge region of antibodies specifically through its CDRs (unpublished data). It is of course possible for an antibody to aggregate specifically only with itself. Such an interaction would most likely not be detected using this method. In that case, self-interaction chromatography methods might better predict the potential for aggregation or precipitation (21,24).

The method used to couple the stationary phase antibodies to the column matrix described here is through primary amines. Thus, certain antibody regions could be masked from the mobile phase due to this coupling, making them unavailable for protein-protein interactions. As such, we employ this method as a screening tool to eliminate lower solubility antibodies early on in the antibody discovery process. Potential lead molecules are then subjected to more rigorous solubility measurements using multiple methods after the number of candidates is narrowed down to only a few to enable larger scale expression and purification. We anticipate that as the technology to rapidly generate large

Table III. Polyclonal IgG Column Screening of Antibody Candidates

Sample	Tr (minutes)	k′	Maximum Observed Concentration (mg/mL)
mAb106	17.15	0.99±0.14	10
mAb107	17.74	1.02±0.19	9
mAb109	10.54	0.21±0.10	>108
mAb112	10.95	0.26±0.12	>124
mAb114	11.86	0.33±0.16	>109
mAb115	11.12	0.22±0.10	>119

Error values for k′ represent the standard deviations from three independent measurements.

panels of high affinity and activity antibodies advances, methods to screen for molecules with high solubility such as the one described here will become an integral part of a lead selection strategy.

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