KISH, WILLIAM. Synthesis and Binding Studies of HWRGWV Affinity Resins.
(Under the direction of Dr. Ruben G. Carbonell.)

Previous work has reported on the identification and characterization of hexapeptide ligand HWRGWV for the affinity capture of IgG through specific binding to its Fc-fragment. HWRGWV affinity resins were synthesized by two different approaches. The first methodology utilized two synthetic steps, implemented Mal-dPEG-NHS ester (with d = 0, 4, 8 and 12) as a spacer arm and HWRGWV-miniPEG-C as ligand. All syntheses from this approach were conducted onto Toyopearl AF-Amino-650M resin. The observed peptide coupling efficiencies for these syntheses were in the range of 23-98% with a decreasing spacer arm coupling efficiency upon increasing the number of PEG units. The resulting resins from this synthetic methodology gave static binding capacities (SBC’s) with hIgG lower than 9 mg/mL. A fellow group member has recently improved upon these results and has synthesized PEG₀ based resins onto Gigacap with peptide densities of 75-89 µequivalents/gram that give SBC’s of 25-30 mg/mL.

The second synthetic approach involved the HATU-mediated coupling of three peptides onto solid phases Toyopearl AF-Amino-650M and Gigacap. The three studied peptides were HWRGWV, N-terminus acetylated HWRGWV (Ac-HWRGWV) and HWRGWVA. Peptide HWRGVWVA gave the best result for all Toyopearl based resins with an SBC of 23 mg/mL. Peptides Ac-HWRGWV and HWRGWVA proved to be the most effective ligands to couple to Gigacap, as these resulting resins gave SBC’s of 33.5 and 31.0 mg/mL, respectively. These values offer significant improvements over resins previously studied by our group, which gave SBC’s of 23 mg/mL under similar conditions (Yang et al. 2008).
The two resins that gave the highest SBC’s (0.5 and 0.75 Ac-HWRGWV-Gigacap) were characterized in dynamic binding studies. These resins gave dynamic binding capacities (DBC’s) of 23-25 mg/mL at a flow rate of 0.05 mL/min (87 cm/hr), which are higher than the 18.4 mg/mL value Naik et al. reported, but under different conditions. Additionally, the resins displayed the ability to isolate Mab1 out of Chinese Hamster Ovary (CHO) cell culture supernatant with yields and purity of 72 and 98%, respectively. These values represent a slight increase in purity, but a significant decrease in yield compared to results reported by Naik et al., who obtained yield and purity of 85 and 95%, respectively. The packing of the 0.5 and 0.75 Ac-HWRGWV-Gigacap resins was not optimized in these studies and it is likely that once this parameter is studied, that these resins will be able to isolate mAb1 from CHO with yields similar to Naik et al.’s findings. Finally, 0.5 Ac-HWRGWV-Gigacap was shown to be the most bio specific of the studied resins, as it bound more Fc fragment of hIgG and less Fab fragment of hIgG than 0.75 Ac-HWRGWV-Gigacap.
Synthesis and Binding Studies of HWRGWV Affinity Resins

by
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DEDICATION

I dedicate this thesis to the other engineers in my family: my father, William John Kish, my grandfather William Eugene Kish, my aunt Diane Kish Ritz and my uncle Robert Ritz. Their dedication to and passion for engineering inspired me to follow in their footsteps.
BIOGRAPHY

William Stanley Kish was born on October 12, 1986, in the small town of Corning, NY, USA, to William John Kish, an electrical engineer and Linda Lukasiak Kish, a businesswoman. His family moved to Wilmington, NC, in 1990, where he lived for 19 years. While in Wilmington, he developed a deep love for athletics as he competed in roller hockey, baseball and basketball. He attended E.A. Laney High School, where he found a strong curiosity in mathematics, physics, chemistry and biology before earning his diploma in 2005.

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Chapter 1: Introduction

1.1 The Monoclonal Antibody Industry

Sales of monoclonal antibody (mAb) based therapeutics exceeded $ 40 billion in 2010 and are expected to reach $70 billion by 2015. Most of the approved antibodies are targeting cancer and autoimmune diseases with the top 5 grossing antibodies populating these two areas. In addition, over 100 monoclonal antibodies are in Phase II and III of clinical development and numerous others are in various pre-clinical and safety studies [1].

Antibodies constitute an integral part of the immune system, as they identify and neutralize foreign objects such as bacteria and viruses [2]. The antibody binds a unique part of the foreign target, called an antigen through its variable region [2]. In this way, an antibody acts to label a microbe or an infected cell for attack by other parts of the immune system [2]. Also, the antibody can neutralize its target directly, for example, by blocking a part of a microbe that is essential for its invasion and survival [3]. Therefore, antibodies offer great therapeutic potential and have transformed the treatment of many cancers, infectious diseases and auto-immune diseases [4].

Antibodies (also called immunoglobulins, Igs) are 150 kDa glycoproteins that have a Y-shape with a constant fragment crystallizable (Fc) region and a variable region, as shown in Figure 1.1[2, 5]. Immunoglobulins are composed of two heavy chains and two light chains held together with disulfide bonds [6]. All immunoglobulins have very similar Fc fragments, making this part of the molecule an excellent target for bio separation ligands that could be used as universal binders. Affinity chromatography has been the most commonly used mAb
purification technique since it was introduced by Cuatrecasas and co-workers in 1968 and works through highly specific interactions between resin ligands and a target protein [6].

**Figure 1.1:** Structural diagram of human IgG with constant (left) and variable regions (right), separated by a blue line. Green lines denote the molecules light chains, while brown lines denote the molecules heavy chain. Each “C” structure denotes one of the IgG domains. The enzymatic fragments Fab, Fc, F(ab’)_2, Fc’ and pFc are also indicated; adopted from Yang et al. [7].

In affinity chromatography, a protein is loaded onto a column in one environment, binding of the target protein to the resin occurs, and unwanted impurities are removed with a washing step [8]. Then, the protein of interest is eluted from the column when the column environment is changed. Typically, the elution and load buffers have a different pH and/or a different ionic strength (e.g. salt concentration) which causes the protein binding and eluting events to occur. [8]. Therefore, the pH of a solution, among other factors, determines the three dimensional shape of a protein and extreme pH environments can cause denaturation of proteins and subsequent loss of activity [8]. The flow rate at which an affinity chromatography process is conducted is also an important parameter. It is particularly important in the load step of an affinity separation, as too fast a flow can keep a target protein from binding a ligand [8].
Protein A chromatography resins are the most commonly used affinity resins in the antibody industry and are excellent for general capture as they bind specifically to the Fc portion that all antibodies have. However, the Protein A ligand has many shortcomings, as it is extremely expensive, is sensitive to product residence time, and can leach from resin particles into elution fractions [1]. This requires even further purification downstream to reduce any leached Protein A to less than 10 ppm, as the ligand is immunogenic [1]. A large portion of antibody production costs is spent on downstream processing (50-80%), and one of the largest contributors to these costs involves the use of Protein A chromatography resin [6].

Protein A chromatography resins are excellent for general mAb capture, as they bind specifically to the constant Fc region that all mAbs and Fc fusion proteins have. However, in addition to its high costs, Protein A also requires elution at highly acidic conditions (pH 3) that can result in antibody aggregation [6]. In addition, repeated cycles of binding, eluting and sanitization with 0.1-0.5 M NaOH causes loss of activity of Protein A and may induce leaching from the resin into elution fractions [6, 7].

1.2 Protein A Alternatives and Peptide Ligand HWRGWV

There have been many attempts from academia and industry to replace Protein A. Roque et al. has summarized several synthetic ligands, including peptidic and non-peptidic ligands, which have been discovered for antibody purification [9]. Some of them have been extensively studied and are already commercialized, such as the hydrophobic charge induced ligand MEP (4-mercaptoethylpyridine) marketed as BioSepra MEP HyperCel [10,11], the
Protein A mimetic peptide Kaptiv-GY based on the sequence (RTY)\(_4\)K\(_2\)KG (TG19318) [12,13], the mixed-mode chromatographic ligand FastMabs A [14,15], and MAbSorbant A2P derived from a triazine derivative ligand 22/8 [16,17]. Smaller peptidic ligands exhibit higher specificity than small organic ligands, but lower binding avidity than Protein A and Protein G. They have the advantage of being able to be synthesized chemically under cGMP conditions, offering the potential for lower production costs [20].

For the past several years, the Carbonell group at NC State University has worked on discovering and characterizing hexamer peptide sequences that bind specifically to the Fc region of hIgG and could act as a Protein A alternative. The research group used a linear hexameric peptide library synthesized via one-bead-one-peptide (OBOP) technique with 18 of the 20 naturally occurring amino acids (methionine and cysteine were excluded) [18]. Four percent of the library was screened against \(^{14}\)C-hIgG and/or the \(^{14}\)C-Fc fragment of hIgG and nineteen of the strongest binders were sequenced via Edman degradation. Surprisingly, all 19 of the identified resin sequences had histidine in the first position (N-terminus) of the hexapeptide, showing a great deal of homology. The probability of randomly selecting 19 library resin beads with histidine at the N-terminus was \((1/18)^{19}\) or \(1.4 \times 10^{-24}\) [18].

Furthermore, the selected binders had a great deal of homology in the second position of the hexamer sequence, as most of the identified amino acids were hydrophobic and/or aromatic. The strongest binders could be grouped into two general families. Family I consisted of the identified sequences HYFKFD, HFRRHL and HWRGWV, or more generally for the first three amino acids: \{histidine, aromatic amino acid, positively charged amino acid\}. Family II included the sequences HVHYYW, HHLYYW and HHLWYY,
consisting of a family homology for the first three positions! The histidine, nonaromatic amino acid, hydrophobic amino acid. Family ligands displayed the ability to recognize IgG directly through its Fc portion in a way comparable to that of Protein A [18].

In a competitive environment, HWRGWV performed the best out of all of the identified Family I sequences, as it bound 59.7% of all $^{14}$C-hIgG out of complete minimal essential medium (cMEM) [18].

Experiments were conducted to determine if it was required that sequences were hexameric in length in order to maintain their IgG capture abilities (and not tetramers or pentamers). For instance, peptides HWRGW, HWG and HWRGWV were synthesized and bound less hIgG out of cMEM than the original sequence HWRGWV. This proved that hexameric sequences were the best choices for hIgG ligands and that the protein-peptide interaction occurred throughout the entire hexameric sequence [18].

HWRGWV has exhibited the ability to bind IgGs from bovine, mouse, goat and rabbit and was also able to isolate hIgG from complete minimum essential medium (cMEM) with purity and yields of 68 and 65%, respectively, while Protein A was able to do so with respective purity and yields of 96 and 61% [18]. After direct synthesis on Toyopearl AF Amino 650M resins, HWRGWV was able to purify hIgG from cMEM containing 10% fetal calf serum and 5% tryptose phosphate broth with both purity and recovery as high as 95% under optimized conditions [19].

In addition, HWRGWV demonstrated the ability to purify monoclonal antibodies from two different commercial Chinese Hamster Ovary (CHO) cell culture media formulations with purities and recoveries higher than 85% and 94%, respectively, matching
very well results found with Protein G using the same cell culture fluids [20]. Additionally, HWRGWV was able to bind all subclasses of human IgG, whereas Protein A does not interact with the Fc portion of hIgG[18].

Mass spectrometry and docking simulations were used to determine that HWRGWV binds to the pFc portion of hIgG located in the CH₃ domain and interacts with the amino acids in the loop Ser383-Asn389 (SNGQPEN). The binding of this loop to HWRGWV is specific and involves hydrogen bonding, hydrophobic, and electrostatic interactions [7]. Therefore, due to its broad specificity and selectivity towards the Fc region that all antibodies have, it was proven that HWRGWV has excellent potential for IgG detection and/or purification of monoclonal or polyclonal antibodies, making it a potential alternative to Protein A or Protein G [7].

In addition, work has begun in the Carbonell group to determine HWRGWV’s utility as a biosensor, as it has been attached to gold plates to study the surface chemistry of the ligand and various spacer arms. Also, the Carbonell group is attempting to conjugate HWRGWV to non-woven fibers with the intent to make membranes for bioseparations. These membranes offer great potential in bioseparations, as their main mode of mass transfer is convection which is much faster than diffusion. Traditional chromatography resins rely on diffusion as their main mode of mass transfer.

1.3 Project Objectives

The main objective of this work was to find new synthetic strategies to make HWRGWV resins with increased dynamic binding capacities (DBC) for antibodies. Protein
A has the ability to bind mAb1 at a DBC > 35 mg/ml, setting a difficult goal for HWRGWV ligand resins [7]. Dynamic binding capacities are typically expressed in units of mg protein captured per mL resin. The DBC of a resin for its target is influenced by a variety of factors, including pH and buffer environment, flow rate, choice of support matrix, film mass transfer, pore diffusion, spatial arrangement of ligands and adsorption kinetics [20, 21]. Buffer pH has a great impact in bio separations, as it determines the charge of various amino acids functional groups on peptides and proteins. Therefore, the pH of a solution, among other factors, determines the three dimensional shape of a protein and extreme pH environments can cause denaturation of proteins and subsequent loss of activity [22].

Yang et al. investigated the peptide resins HWRGWV, HYFKFD and HFRRHL in equilibrium binding experiments and fitted data to the Langmuir isotherm model, giving maximum capacities, $q_M$ of 28.4, 27.0 and 33.6 mg/mL respectively [19]. In previous work, HWRGWV- Toyopearl resin has displayed the ability to bind mAb1 with excellent selectivity at a DBC of 18.4 mg/mL [20]. The mAb1 is a humanized mAb of IgG4 subclass and is a widely used commercial therapeutic product [20]. Increasing the DBC of HWRGWV affinity resins would make them much more attractive to pharmaceutical companies who are accustomed to the high DBC’s of commercial resins that are typically used in industry. This project was focused on finding ways to make HWRGWV resins with maximal DBC’s for hIgG, while keeping the resin specificity for the Fc fragment of hIgG.

Presented in this thesis are studies from two different synthetic approaches to make high capacity HWRGWV affinity resins. Chapter 2 details findings from a two-step synthetic approach that implemented a polyethylene glycol spacer arm that attached the ligand to the
resin surface. This spacer arm was intended to give the ligand more flexibility and make it more bioavailable to the Fc portion of antibodies. The presented findings from this approach are not very encouraging, but have since been improved upon by other members of our group and these updated results can be found in the Chapter 2 conclusions section. In Chapter 3, an HATU-mediated synthetic approach was investigated. The resulting resins from these HATU syntheses offer significant improvement over all previously studied HWRGWV resins both in terms of binding capacity and monoclonal antibody separation capabilities out of CHO cell culture supernatant, a commercially relevant monoclonal antibody fluid.

**References**


Chapter 2: Synthesis and Binding Studies of HWRGWV-miniPEG-C-nPEG-Toyopearl Resins

2.1 Introduction

Of particular concern to making an efficient affinity resin is the spatial arrangement of ligands, which consists of the length from the resin surface, $L_1$ and the distance between ligands, $L_2$. This idea is illustrated in Figure 2.1, below, which also gives the spatial dimensions of human Immunoglobulin G, hIgG (see Chapter 1 for more information about hIgG, the ligand HWRGWV and Protein A) [1,2].

![Figure 2.1: Illustration of the spatial geometry of an affinity resin with $L_1$ and $L_2$ parameters (left) and spatial properties of human immunoglobulin G (right) [1, 2].](image)

Our group has conducted several studies on HWRGWV-Toyopearl resins that use a spacer arm that is ~12 atoms long, which might limit the binding capacity of the resins [3-6]. GE Healthcare uses a 12 atom spacer arm for Protein A, which is appropriate for small proteins, as they can reach out away from the resin to bind mAb’s. Various other literature findings indicate that longer spacer arms are required for affinity ligands that have molecular weights less than 1000 Daltons [1]. HWRGWV has a molecular weight of 840 Daltons; therefore, it is likely that increasing the spacer arm length, $L_1$, will increase the binding
capacity of HWRGWV-Toyopearl for hIgG [1].

Increasing $L_1$ gives the peptide more mobility and flexibility which should increase the bioavailability of the ligand. However, using a spacer arm that is too long could cause affinity ligands to shield themselves from interacting with hIgG [1]. Ideally, $L_1$ will be just long enough so that the HWRGWV ligand can readily access the Fc portion of a hIgG molecule which has diffused close to the surface of the resin. The presented study investigated polyethylene glycol (PEG) spacer arms that are 0, 4, 8 and 12 PEG units long, which correspond to arm lengths of 10.4, 24.8, 39.2 and 53.3 Å, respectively (Figure 2.2) [7]. The studied Toyopearl base resin has a small amine linker that is ~10-15 Å long (approximate because this information is confidential). Additionally, the studied peptide, HWRGWV-miniPEG-C, has a built-in two PEG unit spacer that adds an additional 7 Å to the $L_1$ length. Therefore, the study investigated total $L_1$ lengths in the range of 27-70 Å.

![Figure 2.2: Mal-nPEG-NHS is a heterobifunctional crosslinker that will act as a spacer arm. The study will investigated n =0, 4, 8 and 12 [7].](image)

Generally, the distance between respective peptides, $L_2$ is dictated by the average density of the peptides that are linked to a resins surface. It was expected that the optimal $L_2$ value lies in the range of 50 – 70 Å, as an hIgG molecule is roughly 40 Å wide [1,2].
Figure 2.3: The reaction scheme to form the HWRGVW-miniPEG-C-PEGx-Toyopearl resin will occur in two steps. First, Mal-PEGx-NHS will react with the primary amines on the resin to form an amide bond. Second, the thiol on HWRGVW-miniPEG-C will attack the spacer’s maleimide group to form the resin of interest [1].

Synthesis of the affinity resin occurred via a two-step methodology, as given in Figure 2.3. First, the spacer arm was conjugated to the resin by formation of an amide bond in which the NHS ester acts as a leaving group. The second reaction conjugated HWRGVW-miniPEG-C to the maleimide group of the spacer arm. The added cysteine introduces a thiol functional group to the ligand which allows the reaction to take place in a pH range of 6.5-7.5, similar to analogous cross linking reactions [8]. At a pH of 7, the reaction of the maleimide group with thiols proceeds at a rate 1,000 times greater than its reaction with amines [8].

2.2 Materials and Methods

2.2.1 Materials

Phosphate buffered saline, (PBS, NaCl - 0.138 M; KCl - 0.0027 M, PO₄ - 0.01 M) pH 7.4, monobasic phosphate, dibasic phosphate, polypropylene solid phase extraction tubes
14

(SPE, 3 mL) with polyethylene frits (20 μm pore size) were obtained from Sigma-Aldrich (St. Louis, MO). Ethylenediaminetetraacetic acid (EDTA, Ultrapure Bioreagent grade) was obtained from J. T. Baker (Phillipsburg, NJ). EMCS, MAL-PEG₄-NHS ester, MAL-PEG₈-NHS ester and MAL-dPEG₁₂-NHS ester cross linking reagents were obtained from Quanta Biodesign Limited (Powell, OH). HWRGWV-miniPEG-C was obtained from Genscript (Piscataway, NJ). Anhydrous dimethylformamide (DMF) was obtained from Acros (New Jersey). Technical DMF and centrifuge tubes (3 mL and 15 mL) were obtained from VWR International (West Chester, PA). Toyopearl AF Amino-650 M resin was obtained from Tosoh Biosciences (King of Prussia, PA). An 8453 model UV-Vis spectrophotometer was purchased from Agilent (Cary, NC). Human Immunoglobulin G (hIgG, powder form) was obtained from Equitech-Bio (Kerrville, TX). A Mini LabRoller rotator with accessories to accommodate twenty-eight 3 mL SPE tubes was obtained from LabNet International (Edison, NJ).

2.2.2 Synthesis of MAL-nPEG-Toyopearl AF-Amino-650 M Resins

A solution of 20% DMF (v/v) in 10 mM PBS, pH 7.4 was prepared in a graduated cylinder and is referred to below as ‘buffer A’ below. A buffer containing 50 mM PO₄ and 10 mM EDTA, pH 6.8, was prepared via standard methods. DMF was added to the buffer to bring it to total concentration of 20% DMF (v/v) and is referred to below as ‘buffer B’. 100 μL of swollen Toyopearl AF Amino 650-M resin (originally in 20% methanol) was added to a 3 mL SPE tube, rinsed with 10 mL of PBS, pH 7.4 and equilibrated in 1 mL of the buffer
overnight at 4°C. All PBS, pH 7.4 was flushed from resin, 3 mL of buffer A was added and allowed to equilibrate for 30 minutes.

N-epsilon-Malemidocaproyl-oxysuccinimide ester (EMCS, 23 mg) was dissolved in 1.5 mL of technical DMF and 6 mL of PBS, pH 7.4 by mixing via vortex. 1.5 mL of the EMCS solution was added to the resin for a theoretical spacer coverage of 600 µequivalents of spacer/ gram of dry resin (here, a µequivalent is equal to a µmol; 25 mg of dry Toyopearl Amino- 650 M swells to approximately 100 µL in 20% methanol). The reaction was briefly mixed via vortex, placed on a rotisserie rotator and allowed to react for 45 minutes. The reactions were removed from the rotisserie rotator and 15 mL buffer A was rinsed through the resin.

This procedure was repeated 4 additional times for a total of five samples for the spacer arm EMCS. The procedure was also repeated for spacer arms: MAL-PEG₄-NHS ester, MAL-PEG₈-NHS ester and MAL-PEG₁₂-NHS ester for a total of 20 samples, all using the same molar addition technique of 600 µequivalents of spacer coverage / gram of dry resin.

2.2.3 Synthesis of HWRGWV-miniPEG-C-nPEG-Toyopearl Resins

A slow stream of nitrogen gas was bubbled through buffer B to purge it of oxygen and prevent oxidation of cysteine terminated peptide. Residual buffer A was discarded from each resin with a syringe plunger. 3 mL of buffer B was added to the resin, allowed to equilibrate for 10 minutes and then discarded from the SPE tubes. HWRGWV-miniPEG-C was massed out into a 15 mL centrifuge tube and dissolved in buffer B to make a stock 1.17 mg miniPEG-peptide/ mL solution. Peptide solution was added to each resin sample
according to Table 2.1, below. The peptide was added in 1/5th increments every minute so that the whole amount was added over the course of 5 minutes.

**Table 2.1:** Amount of peptide added to each respective resin sample. This protocol was followed for four groups of spacer arms (PEG0, PEG4, PEG8 and PEG12) for a total of 20 samples.

<table>
<thead>
<tr>
<th>Sample #</th>
<th>Amount of HWRGWV-miniPEG-C Added</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0 µequivalents of peptide/ gram of dry resin</td>
</tr>
<tr>
<td>2</td>
<td>15 µequivalents of peptide/ gram of dry resin</td>
</tr>
<tr>
<td>3</td>
<td>30 µequivalents of peptide/ gram of dry resin</td>
</tr>
<tr>
<td>4</td>
<td>60 µequivalents of peptide/ gram of dry resin</td>
</tr>
<tr>
<td>5</td>
<td>90 µequivalents of peptide/ gram of dry resin</td>
</tr>
</tbody>
</table>

To ensure adequate mixing among reaction solution and resin beads, buffer B was added to some reactions so that all reaction volumes were at least 1.5 mL. All reactions were placed onto a tumbling rotator and allowed to react for 9 hours at room temperature. This procedure was repeated three additional times to cover all four studied spacer arms, giving rise to four different peptide load solutions and twenty resin samples total.

### 2.2.4 Quantification of Peptide Coupling Efficiency

Resins were removed from the tumbling rotator and unreacted miniPEG-peptide was rinsed from them with 5 mL of buffer B. All flow through were collected in 15 mL centrifuge tubes for a total rinse volume of 7.5 mL and analyzed via $A_{280}$ spectrophotometry.
One Hundred, 50, 25, 12.5 and 8.3 fold dilutions of HWRGWV-miniPEG-C solution (originally 1.17 mg/mL) were made and analyzed via $A_{280}$ spectrophotometry for a standard curve. The obtained standard curve equation was used to quantify the amount of unreacted peptide in all rinse fractions and the amount of peptide coupled to each resin.

### 2.2.5 Static Binding Studies

All resins were equilibrated in 1.0 mL of PBS, pH 7.4 for 30 minutes. A 5.0 mg/mL human immunoglobulin G (hIgG) solution was prepared in PBS, pH 7.4. Any residual PBS, pH 7.4, was flushed from the resins and 1 mL of hIgG solution was added to the resins, placed onto a tumbling rotator and incubated for 30 minutes. The resins were removed from the rotator, rinsed with an additional 9 mL of 10 mM PBS, pH 7.4, and a flow-through fraction was collected in a 15 mL centrifuge tube. This procedure was repeated for all 20 samples and each obtained flow through fraction was analyzed for hIgG content via $A_{280}$ spectrophotometry. Static binding capacities were calculated by determining the amount of target protein that the resins did not bind.

The hIgG load solution was diluted with PBS, pH 7.4, to make standard curve samples that were 0.05, 0.10, 0.20, 0.40 and 0.60 mg/mL. The samples were analyzed for protein content via $A_{280}$ spectrophotometry and the resulting standard curve equation was used to quantify protein content in flow through fractions and static binding capacities. All syntheses and binding studies were repeated an additional time to prove reproducibility, except for the PEG$_0$ spacer, which was only synthesized and characterized once.
2.3 Results and Discussion

2.3.1 Peptide Coupling Efficiencies

The diluted HWRGWV-miniPEG-C samples gave rise to a standard curve with the equation $A_{280} = 6.9055[\text{peptide concentration (mg/mL)}] - 0.0099$ with an $R^2$ value of 0.9965. The slope (or extinction coefficient), 6.90, is lower than the slope obtained for HWRGWV, 8.09. This makes sense, as the miniPEG peptide has a higher molecular weight (1088 g/mol) than HWRGWV (840 g/mol) but both have two tryptophan residues per molecule, which is the absorbing moiety at 280 nm. HWRGWV has a higher Tryptophan density (in terms of amount of absorbing moiety per unit mass) than the miniPEG peptide and therefore gives the higher extinction coefficient. The $R^2$ (linear regression) value of 0.9965 indicates that there is very little scatter in the data, which is expected in a standard curve.

![HWRGWV-miniPEG-C Standard Curve](image)

**Figure 2.4:** The standard curve for HWRGWV-miniPEG-C show very little scatter with an $R^2$ value of 0.9965. The obtained equation was used to calculate various coupling efficiencies, which appear below.
Figure 2.5: Coupling efficiency graph for the HWRGWV-miniPEG-C + PEG\(_0\) + Toyopearl Amino- AF 650-M matrix. At loads of 15, 30, 60 and 90 µequivalents/gram dry resin, peptide coupling efficiencies of 98 %, 94 %, 91 %, and 88% were obtained, respectively.

The obtained standard curve equation was used to quantify the coupling efficiencies of all reactions, which appear in Figures 2.5-2.8. These coupling efficiencies were obtained by directly quantifying the peptide reaction yields without directly following the spacer arm reaction yields. A commonly observed general trend was that peptide coupling efficiency decreased upon increasing length of the spacer arm used. Because the yield of the first reaction was not directly measured, it is difficult to determine if this observed phenomena occurs because of differences in spacer arm reaction yields (reaction 1) or because of differences in reactivity of coupled spacer arms with peptide (reaction 2).

The PEG\(_0\) spacer proved to be the most efficient of all the studied systems in terms of peptide coupling efficiency. HWRGWV-miniPEG-C coupled to the spacer arm at yields ranging from 88-98%, with the more efficient coupling occurring at lower peptide loads. Because all resins are initially reacted with the same 600 µequivalents of spacer, it is likely
that each resin has roughly the same number of maleimide groups available for reaction with the peptide. Therefore, the decrease in coupling efficiency upon increasing peptide load occurs simply because the fraction \( \frac{\text{Surface Maleimide Groups}}{\text{Unreacted Peptide Molecules}} \) has decreased which decrease’s the reaction velocity.

The PEG\(_4\) spacer gave peptide coupling efficiencies in the range of 78 to 87\%, with decreasing coupling efficiencies upon increasing the peptide loading. There was one outlier in the data, as one of the 15 µequivalent additions only coupled ~8 µequivalents of the peptide. It is important to notice the decrease in coupling efficacy from 88-98\% to 78-87\% upon increasing \( L_1 \) from a PEG\(_0\) to a PEG\(_4\) spacer. The phenomenon of decreasing peptide coupling efficiency upon increasing spacer arm length was observed throughout the study, especially at peptide additions of 60 and 90 µequivalents/gram.

**Figure 2.6:** Coupling efficiency graph for the HWRGWV-miniPEG-C + PEG\(_4\) + Toyopearl Amino- AF 650-M reaction. At loads of 15, 30, 60 and 90 µequivalents of peptide / gram dry resin, average peptide coupling efficiencies of 70 % (86% if you exclude the repetition 1 outlier), 87 %, 85 %, and 78% were obtained, respectively.
The PEG$_8$ system showed surprisingly high coupling efficiencies at the 15 and 30 µequivalent peptide loading regimes (each 85%) but then saw a rapid decrease to 65 and 43% at peptide loads of 60 and 90 µequivalents, respectively. The best explanation for this data is that less of the 600 µequivalent of the PEG$_8$ coupled to the Toyopearl AF-Amino 650-M than for respective PEG$_4$ and PEG$_0$ reactions. However, the reactivity of the maleimide group on the coupled PEG$_8$ spacer is likely more available to peptides (than in coupled PEG$_0$ and PEG$_4$ resins) because of the added length from the resin surface. This means that the fraction of \( \frac{\text{Surface Maleimide Groups}}{\text{Unreacted Peptide Molecules}} \) can be slightly lower for longer spacer arms, while still yielding coupling efficiencies comparable to the shorter spacer arms (continued on pp 32).

**Figure 2.7:** Coupling efficiency graph for the HWRGWV-miniPEG-C + PEG$_8$ + Toyopearl Amino- AF 650-M matrix. At loads of 15, 30, 60 and 90 µequivalents/ gram dry resin, average peptide coupling efficiencies of 85 %, 85 %, 65 %, and 43% were obtained, respectively.
Figure 2.8: Coupling efficiency graph for the HWRGWV-miniPEG-C + PEG<sub>8</sub> + Toyopearl Amino- AF 650-M matrix. At loads of 15, 30, 60 and 90 µequivalents/ gram dry resin, average peptide coupling efficiencies of 68 %, 53 %, 32 %, and 27% were obtained, respectively.

However, because there are so few PEG<sub>8</sub> spacers coupled to the surface of the resin, there is a point at which addition of more peptide will not yield a significant increase in the number of coupled peptide. For the PEG<sub>8</sub> system, this point seemed to be a point around the 60 µequivalent peptide addition mark, where coupling efficiencies plummeted to 65 % and eventually 43 % for the 60 and 90 µequivalent additions, respectively. This likely means that there is not much more than 80 µequivalents/g of PEG<sub>8</sub> spacer coupling to the resin which is very inefficient considering a 600 µequivalent/g addition was made.

Finally, the PEG<sub>12</sub> system displayed the lowest coupling efficiencies of all spacer arms studied, with efficiencies of 68, 53, 32 and 27% at respective peptide loads of 15, 30, 60
and 90 µequivalents. The best explanation for this data is that PEG\(_{12}\) couples to the resin very inefficiently in relation to PEG\(_0\), PEG\(_4\) and PEG\(_8\).

In summary, the most important findings from peptide coupling experiments was that increasing spacer arm length led to decreasing peptide coupling efficiencies during the second reaction. There was likely much more PEG\(_0\) and PEG\(_4\) spacer coupled to the resin than in the PEG\(_8\) and PEG\(_{12}\) situations. However, this did not keep the PEG\(_8\) and PEG\(_{12}\) coupled resins from giving reasonable high peptide coupling efficiencies at the lower peptide additions of 15 and 30 µequivalents/gram dry resin.

In all likelihood, once spacer arms have coupled to the resin, introduced peptide molecules will react with them equally well. This means that the spacer arm reaction is likely the limiting reaction in terms of obtaining high peptide coupling efficiencies. This also means, in effect, that high ligand coverage or density cannot be economically obtained with PEG\(_8\), PEG\(_{12}\) and possibly PEG\(_4\) spacers. Past work from our group has shown that a range of 21-600 HWRGWV ligand molecules are required to bind a single hIgG molecule for peptide densities ranging from 22-550 µequivalents/gram, with an increasing number of ligands required to bind a molecule of hIgG as ligand density increases [3]. Also, the 22 and 550 µequivalents/gram resins gave static binding capacities of 17 and 27.2 mg/mL after a 5.0 mg/mL load, which were very similar to the experimental conditions give here [3].

In order to make a high capacity HWRGWV affinity resin, it makes sense to make a resin with just enough coupled ligand to obtain a maximal capacity, but is still bio specific. This philosophy, combined with the obtained peptide coupling efficiencies suggests that making an HWRGWV resin with a spacer arm can only be justified if PEG\(_0\) is used, as the
PEG₄, PEG₈ and PEG₁₂ spacer arms proved to be too inefficient in terms of coupling efficiencies and also were likely to give low binding capacities.

### 2.3.2 Static Binding Capacities

From a practical standpoint, the observed static binding capacities of the synthesized resins are the most important data in the whole study, as it gives insight into the usefulness of the end product. Unfortunately, none of the studied resins performed very well, as they all bound hIgG at static binding capacities less than 9 mg/mL, which is obviously much lower than the goal of this study, which was to improve upon Yang et al.’s static binding capacity of ~27 mg/mL at the same hIgG load of 5 mg/mL [3].

The PEG₀ based resins were unique in that they were the only resins in the study that gave an observable trend in terms of the obtained static binding capacities in light of the amount of peptide coupled to the resin (Figure 2.9). For PEG₀ based resins, increasing the amount of coupled ligand led to an increase in SBC, except for the resin that had 78 µequivalent/g of ligand coupled to it, which bound hIgG the same (with-in experimental error) as the resin that had 53 µequivalent/g of coupled ligand.
Figure 2.9: Apparent static binding capacities for HWRGWV-miniPEG-C-PEG$_0$-Toyopearl resins were obtained by 30 minute incubation with 1.0 mL of 5.0 mg/mL hIgG solution. Values for the amount of coupled peptide were determined as given in the methods and materials section.

Figure 2.10: Static binding capacities for HWRGWV-miniPEG-C-PEG$_4$-Toyopearl resins were obtained by 30 minute incubation with 1.0 mL of 5.0 mg/mL hIgG solution.
For PEG₄ resins, (Figure 2.10) there was a surprisingly high amount of binding for resins that were never even introduced to peptide, as they each bound hIgG at approximately 5.8 mg/mL, which is higher than the ~4.3 mg/mL capacity of Toyopearl AF-Amino-650M (found in previous studies). This binding was likely due to nonspecific interactions that the PEG₄ spacer has with hIgG. This amount of nonspecific binding was not observed for any other PEG₀, PEG₈ or PEG₁₂ resins that were not introduced to peptide. Therefore, it is likely that PEG spacers that are 4 units long bind a part of hIgG, while longer length PEG spacers fail to do so. The rest of the PEG₄ resins, which has varying amounts of coupled ligand bound hIgG at a capacity less than 3.0 mg/mL. This data indicates that adding peptide to PEG₄ resins kept the spacer arm from interacting with hIgG and that the main mode of binding for the PEG₄ resins were nonspecific, which is the exact opposite of what is desired for affinity resins.

All PEG₈ based resins displayed extremely low capacity for hIgG except for two, which had peptide coverages of 24.7 and 26.6 µequivalents/g and gave identical SBC’s of 8.4 mg/mL. As previously mentioned in the introduction of this chapter, it was expected that there would be optimal L₂ (or peptide density) values for different L₁ (spacer arm lengths). It is likely that the 24.7 and 26.6 µequivalents/g peptide densities for the PEG₈ spacer offer the desired optimal geometry to efficiently bind hIgG. However, the amount of coupled ligand seems to limit the resins overall capacity. Finally, all studied PEG₁₂ resins gave SBC’s lower than 2.0 mg/mL and such low values can be explained by the length of the spacer and the low amount of ligand coupled to the resin. In all likelihood, the 12-unit PEG spacer is so long that it overlaps with neighboring spacer arms, shielding the ligand from interacting with hIgG.
**Figure 2.11**: Static binding capacities for HWRGWV-miniPEG-C-PEG$_8$-Toyopearl resins were obtained by 30 minute incubation with 1.0 mL of 5.0 mg/mL hIgG solution.

**Figure 2.12**: Static binding capacities for HWRGWV-miniPEG-C-PEG$_{12}$-Toyopearl resins were obtained by 30 minute incubation with 1.0 mL of 5.0 mg/mL hIgG solution.
2.4 Conclusions

Unfortunately, none of the studied resins performed very well, as they all bound hIgG at static binding capacities less than 9 mg/mL, which is much lower than the goal of this study, which was to improve upon Yang et al.’s static binding capacity of ~27 mg/mL at the same hIgG load of 5 mg/mL [3]. The biggest issues with the PEG₄, PEG₈ and PEG₁₂ based affinity resins seems to be extremely low spacer arm coupling efficiencies, which lead to very low ligand densities. However, the PEG₀ spacer arm gave decently high coupling efficiencies, showing promise as effective affinity resin component.

More resin syntheses which implement a PEG₀ spacer coupled to a Gigacap support matrix have been conducted by Dr. Alex G. Tkachenko, a fellow member of the Carbonell bio separations group. Gigacap is a polyamine functionalized polymethacrylate resin that has approximately twice the ligand density of Toyopearl AF-Amino-650M. These HWRGWV-PEG₀-Gigacap resins are a significant improvement over the resins presented here, as they have displayed SBC’s in the range of 25-30 mg/mL after obtaining peptide densities ranging from 75-89 μequivalents/gram.

One alternative to this two-step approach is to use HATU, a coupling reagent, to couple the C-terminus of HWRGWV directly to a resin surface. This chemistry affords the desired affinity resin in one step, instead of the two steps that spacer arm syntheses require. Also, HATU-mediated synthesis promises to make the resin slightly cheaper, as HATU is much cheaper than PEG₀ spacer arms. Chapter 3, which is the bulk of this thesis, outlines the findings from an extensive study using HATU-mediated synthesis to make HWRGWV
affinity resins that were able to improve upon Yang et al.’s and Naik et al.’s findings, both in
terms of binding capacity and separation abilities out of industrially relevant fluids [3].

References


Chapter 3: HATU-mediated Synthesis of HWRGWV Affinity Resins and Binding Studies

3.1 Introduction

O-(7-azabenzotriazol-1-)-N,N,N0,N0-tetramethyluronium hexafluorophosphate (HATU) has shown great utility in the efficient coupling of many biomolecules. It was first studied by Carpino and collaborators in the early 1990s and proven to be an excellent peptide coupling additive [1]. Carpino was able to show that HATU was more enantioselective than HBTU, which was believed to be the best peptide coupling additive at that time [2]. Since then, it has been mainly been used to facilitate solid phase peptide synthesis [3,4]. Lately, HATU has found utility in many other difficult amide bond forming reactions such as in the solution phase conjugation to synthesize siRNA [5].

In the presented work, HATU is used to enable the coupling of the carboxyl terminus of derivatives of a hexapeptide, HWRGWV to the amino groups of a solid phase support to make an affinity chromatography resin. HATU-based chemistry is conducted in an organic solvent (typically DMSO or DMF) and employs a catalytic base (typically diisopropylethylamine, DIPEA). The mechanism by which HWRGWV forms an activated ester with HATU, followed by nucleophilic attack from an amine or amino functionalized based resin is given in Figure 3.1.

As in most methodologies, the use of HATU chemistry to make an HWRGWV resin has advantages and disadvantages. One advantage to using this technique is that it fairly simple to conduct the synthesis and purification. Unfortunately, the chemistry lacks true synthetic specificity, as the HWRGWV peptide contains multiple functional groups which can act as a nucleophile to attack the HWRGWV-HATU activated ester, yielding side
products that may decrease the performance of the affinity resin (Figure 3.2). For instance, the guanidino group, (pKa = 12.5) of HWRGWV’s arginine residue can act as a nucleophile to form cross-mers (Figure 3.2, reaction 4). Additionally, the N-terminus of an HWRGWV molecule (pKa = 9.2) can nucleophilically attack the C-terminus of another HATU-activated peptide molecule to form oligomers (Figure 3.2, reaction 1). This side reaction can be avoided by using HWRGWV that has an acetylated N-terminus (Ac-HWRGWV). Yang et al. showed that acetylating the N-terminus of the peptide does not have a noticeable impact on hIgG binding capacity [7].

Figure 3.1: Mechanism by which HATU reacts with the C-terminus of HWRGWV to form an activated ester. This is followed by the nucleophilic attack of the resins amine or amino group to form the desired affinity resin [4, 6].

Using HATU chemistry to make an affinity HWRGWV resin is an alternative to another methodology, which has been used by our group in the past. This technique involves making the resin by synthesizing the peptide, by building it- amino acid by amino acid,
directly onto Toyopearl AF-Amino-650 M, a polymethacrylate based resin [7-11]. The biggest issue with this methodology is that it is prone to give truncated peptide sequences which may cause the resin to lose specificity. When the resin is made via HATU based chemistry, the peptide is separately synthesized via solid-phase techniques onto polystyrene or polyethylene glycol based resins. The peptide is then cleaved from the support matrix with trifluoroacetic acid and purified via HPLC to yield an HATU chemistry starting reagent that is at least 93% pure (typically > 98.5% pure).

Therefore, the presented methodology acts to circumvent the issue of peptide truncation, which is an issue only when the solid-phase synthesis technique is used, as shown by Figure 3.2 (on following page). Making the affinity resin with ligands that are only whole hexapeptide sequences is also likely to increase its dynamic binding capacity, while keeping the resin as bio specific as possible, which was the main goal of this project.

HWRGWV is a selective binder of the Fc portion of human Immunoglobulin G (hIgG) and is in the process of becoming a more viable Protein A alternative (see Chapter 1 for more information regarding HWRGWV, hIgG and Protein A). In order to become a more viable Protein A alternative, the dynamic binding capacity (DBC) of HWRGWV-based resins must be increased. Protein A has the ability to bind mAbs at a DBC > 30 mg/mL, leaving much to be desired for HWRGWV, which, until this work, displayed DBC’s of 18.4 mg/mL and equilibrium maximum capacities of 28 mg/mL [7, 8]. This project was focused on discovering new synthetic strategies to make HWRGWV resins with high binding capacities, while keeping the resins as bio specific for the Fc fragment of hIgG as possible.
Figure 3.2: Synthesis of an HWRGWV affinity resin via HATU based chemistry lacks true synthetic selectivity, as the peptide can undergo two side reactions multiple times. The N-terminus of one peptide molecule can react with the activated C-terminus of another peptide molecule to form oligomers (reaction 1). This side reaction can be circumvented by acetylating the N-terminus of the peptide prior to coupling. The R-group of arginine can also act as a nucleophile to attack the activated ester C-terminus of the peptide to form ‘cross-mers’ (reaction 4). Despite these side reactions, HATU based chemistry is probably superior to the solid phase synthesis technique to make the affinity resin. The biggest issue with this methodology is that it is prone to give peptide ligands with truncated sequences that are likely weaker binders of the target protein and undergo more non-specific binding to unwanted proteins.
3.2 Materials and Methods

3.2.1 Materials

Gigacap-HC resin (ligand: polyamine; base resin: HW-65 M; particle size: 40-90 µm) and Toyopearl-AF Amino 650-M resin (base resin: HW-65 M, mean particle size: 65 µm) was obtained from Tosoh Biosciences (King of Prussia, PA) and micro centrifuges were obtained from Heathrow Scientific LLC (Illinois, IN). Anhydrous DMF was obtained from Acros (NJ), technical DMF was obtained from VWR International (Westchester, PA) and ethanol (200 proof) was obtained from Pharmco Products (Brookfield, CT). Peptides HWRGWV (98.8% pure), acetylated HWRGWV (97.3% pure) and HWRGWVA (92.7% pure) were all obtained from Genscript (Piscataway, NJ). HATU was obtained from Matrix Innovation (Quebec City, Quebec, Canada) and N, N-diisopropylethylamine solution (DIPEA, 0.749 g/mL) was obtained from Sigma-Aldrich (St. Louis, MO).

3 mL fritted solid phase extraction (SPE) tubes (I.D. = 9 mm) were obtained from Supelco (Bellefonte, PA), 5 cc disposable Luer lock syringes were obtained from Becton Dickinson & Co. (Franklin Lakes, NJ), and 0.22 µm syringe driven, low protein binding PVDF filters were obtained from Millipore (Tullagreen, Ireland). Human immunoglobulin G (hIgG, lyophilized powder, 99.8% pure by Bradford Assay) was obtained by Equitech-Bio Inc. (Kerrville, TX). Glycine was obtained from Sigma Aldrich (St. Louis, MO), hydrochloric acid (HCl, 12.1 N) was obtained from Fischer Scientific (Fair Lawn, NJ) and used to make 0.1 M Glycine-HCl buffer, pH 2.5. 10mM phosphate buffered saline, pH 7.4 (PBS, containing 10 mM phosphate, 0.138 M NaCl and 0.0027 M KCl) was obtained from Sigma-Aldrich (St. Louis, MO).
Dichloromethane (DCM), was obtained from Sigma-Aldrich (St. Louis, MO) and a Roto-Tourque heavy duty rotator was obtained Cole Parmer Instruments (Vernon Hills, IL). All static binding experiment solutions were analyzed using an Agilent 8453 UV-Visible spectrophotometer from Agilent Technologies (Santa Clara, CA). All chromatographic experiments were carried out on a Waters 626LC system. Absorbance of protein samples was measured at a wavelength of 280 nm using a built-in 2487 dual wavelength UV detector from Waters (Milford, MA, USA). NuPAGE Novex 4–12% Bis-Tris gels, NuPAGE reducing agent, NuPAGE MOPS SDS running buffer, See-Blue Plus2 pre-stained molecular weight marker and SimplyBlue Safestain were all from Invitrogen (Carlsbad, CA, USA).

Amicon Ultra centrifugal filters (MWCO = 3,000) were obtained from Millipore (Tullagreen, Ireland). NuPAGE® Novex Gels (4-12% Bis-Tris), NuPAGE® MOPS running buffer, NuPAGE® LDS sample buffer, NuPAGE® reducing agent, SeeBlue plus 2® pre-stained molecular weight marker, SimpleBlue™ Safestain and a Novex® mini cell gel box were all obtained from Invitrogen (CA, USA).

3.2.2 Resin Synthesis

Gigacap resin slurry was added to a 0.3 mL centrifuge tube and centrifuged at 2000 × g for 30 seconds to pellet the resin. Gigacap slurry was then added or removed until the pelleted resin volume level was exactly 50 µL. The slurry was transferred to a 0.5 mL centrifuge tube and centrifuged at 2000 × g for 30 seconds. The supernatant was discarded, 450 µL of ethanol was added to the resin, and mixed via vortex for 3 seconds. The slurry was centrifuged, the supernatant was discarded and fresh ethanol was added to the slurry. This
process was repeated once more with ethanol, twice with technical DMF and finally once with anhydrous DMF. After pelleting the resin the last time, all of the anhydrous DMF supernatant was removed while avoiding disturbance of the pelleted resin.

Peptide HWRGWV (38.8 mg) and 35.0 mg of HATU were added to a 1.3 mL centrifuge tube. 610.6 µL of anhydrous DMF was added to the solids and mixed via vortex, totally dissolving them to form a 100 mM peptide solution (which additionally contained 200 mM HATU). 10.0 µL of the peptide solution was mixed by vortex with 0.69 µL of diisopropylethylamine (DIPEA) and the color of the solution changed from clear to yellow, indicating that the peptide had been activated by HATU. The activated peptide solution was immediately added to the drained resin and mixed via vortex for 5 seconds. The reaction was placed onto a vortex mixer set to an agitation rate of 70 % of the maximum and allowed to react for 9 hours. This exact process was repeated five more times, using the peptide, HATU and DIPEA amounts listed in Table 3.1 (on the following page).

The reaction tubes were removed from the vibrator and the resins were transferred into 3 mL SPE tubes. The resins were rinsed with 6 mL of technical DMF, 6 mL of ethanol and 6 mL of 10 mM PBS, pH 7.4. An additional 1.5 mL of 10 mM PBS, pH 7.4 was added to the resins and they were allowed to equilibrate for 45 minutes tumbling on a rotator.
Table 3.1: Synthesis parameters used to make HWRGWV-Gigacap resins. Peptide load refers to the moles of peptide reacted/moles of –NH$_2$ groups available on the resin. All reactions were conducted at a scale of 50 µL of drained resin and allowed to run for 9 hours at room temperature while undergoing agitation on a vortex mixer set to 70% of the maximum.

<table>
<thead>
<tr>
<th>Peptide Load</th>
<th>HWRGWV</th>
<th>HATU</th>
<th>DIPEA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>1.0 µmol</td>
<td>2 µmol</td>
<td>4 µmol</td>
</tr>
<tr>
<td>0.25</td>
<td>2.5 µmol</td>
<td>5 µmol</td>
<td>10 µmol</td>
</tr>
<tr>
<td>0.5</td>
<td>5.0 µmol</td>
<td>10 µmol</td>
<td>20 µmol</td>
</tr>
<tr>
<td>0.75</td>
<td>7.5 µmol</td>
<td>15 µmol</td>
<td>30 µmol</td>
</tr>
<tr>
<td>1.0</td>
<td>10.0 µmol</td>
<td>20 µmol</td>
<td>40 µmol</td>
</tr>
<tr>
<td>2.0</td>
<td>20.0 µmol</td>
<td>40 µmol</td>
<td>80 µmol</td>
</tr>
</tbody>
</table>

In addition to the batch of reactions given above, various other syntheses were conducted using peptide concentrations ranging from 10 to 200 mM, however, most were conducted at 100 mM. All reactions were conducted at a scale of 50 µ of drained resin and maintained a 1:2:4 molar ratio of peptide: HATU: DIPEA. This technique of using a 1:2:4 molar ratio proved to be the most effective methodology from previous studies, which are not presented here. Syntheses were also conducted using peptides acetylated-N terminus HWRGWV and HWRWVA. Additionally, reactions using all three peptides were conducted at various peptide concentrations onto Toyopearl AF-Amino 650-M, in place of Gigacap.

3.2.3 Static Binding Studies

Human immunoglobulin G (hIgG, 52.5 mg) was dissolved in 7 mL of 10 mM PBS, pH 7.4 and mixed via vortex to make a 7.5 mg/mL hIgG stock solution. The stock solution was passed through a 0.22 µm low protein binding PVDF filter with a disposable luer lok
syringe into a fresh 15 mL centrifuge tube. All 10 mM PBS, pH 7.4 (from equilibration step in A) was discarded from the resins with a 10 mL syringe. 1.0 mL of the filtered stock hIgG solution was added to each drained resin, mixed via vortex and placed on a rotator, tumbling for 30 minutes. This process was repeated for all the resins that appear in Table 3.1, ensuring they were in contact with the stock hIgG solution for 30-35 minutes.

Resins were periodically removed from the rotator, their SPE tubes were placed into 15 mL centrifuge tubes and a flow-through fraction was collected after rinsing with 9 mL of 10 mM PBS, pH 7.4. The first 5 mL of the flow-through step occurred at flow rate of ~ 1 mL/min and the last 5 mL of PBS, pH 7.4 was forced through the resin with a syringe plunger at a flow rate of ~4 mL/min. All binding buffer was fully discarded from the resin using a syringe plunger, 2.0 mL of 0.1 M glycine-HCl, pH 2.5 was added to the resin and placed on a rotator tumbling for 15 minutes. This procedure was repeated for all resins.

Resins were periodically removed from the rotator, their SPE tubes were placed into 15 mL centrifuge tubes and an elution fraction was collected at ~0.5 mL/min. An additional 2.0 mL of 0.1 M glycine-HCl was rinsed through the resins at a flow rate of ~0.5 mL/min. All elution buffer was forced through the resins with a syringe plunger and collected for a total elution fraction volume of 4 mL. Finally, all resins were stored in PBS, pH 7.4 at 4 °C.

A hIgG standard curve was constructed using stock hIgG solution that was unused during the binding study and appropriate dilutions with 10 mM PBS, pH 7.4. Samples of 0.075, 0.15, 0.30, 0.60 and 0.90 mg/mL hIgG were made and analyzed via A$_{280}$ spectrophotometry. Finally, all elution and flow-through fractions were mixed via vortex and analyzed for protein concentration via A$_{280}$ spectrophotometry using an Agilent 8453 UV-
Visible spectrophotometer. The entire static binding procedure (hIgG solvation to A\textsubscript{280} spectrophotometry) was conducted in 2.5 hours or less to ensure that hIgG integrity was maintained in all buffers.

### 3.2.4 Determination of 10% Breakthrough Point on HPLC Setup

The HPLC system was fitted with a zero dead volume union in place of a chromatography column. Stock solutions with hIgG concentrations of 0.25, 0.5, 0.75, 1.0 and 1.25 mg/mL in PBS, pH 7.4 were prepared by standard procedures. 100 µL of the 0.25 mg/mL stock solution was injected into the system and PBS, pH 7.4 was flowed at 0.02 mL/min for 20 minutes. The absorbance was monitored at 280 nm to determine the maximum absorbance that a 0.25 mg/mL solution would obtain under these conditions. This procedure was repeated for all of the prepared stock solutions to construct a standard curve which could be used to determine the exact absorbance at which 10% of breakthrough was obtained in dynamic binding capacity experiments.

100 µL of 0.1 M acetone in PBS, pH 7.4 was injected into the HPLC system and PBS, pH 7.4 was flowed at 0.05 mL/min for 20 minutes. The following equation was used to determine the dead volume of the HPLC system,

\[
DV [mL] = T [min] * FR \left[ \frac{mL}{min} \right]
\]

where DV is dead volume, T is the time at which the first deflection of the acetone peak occurred (defined as A\textsubscript{280} = 0.01) and FR is the flow rate at which the HPLC run was conducted. This dead volume was used to calculate dynamic binding capacities.
3.2.5 Dry Packing, Swelling and Equilibration of Resins

The two resins that gave the highest static binding capacities (0.5 and 0.75 Ac-HWRGWV-Gigacap) were chosen for further characterization in dynamic studies. The resins were placed into SPE tubes and washed with 3 mL of 0.85% phosphoric acid, 6 mL of PBS, pH 7.4, 3 mL of DI H₂O, 6 mL of ethanol, 6 mL of technical DMF and 6 mL of dichloromethane. A syringe plunger was used to force air through the resin beads until they were dry enough to be poured easily.

Resins were dry packed into a 30 mm X 2.1 mm I.D. microbore column (0.1 mL). The column was placed in-line with a Waters HPLC system with a 600s flow controller and a 2487 wavelength detection unit. 20% methanol was flowed through the column at a rate of 0.1 mL/min (174 cm/hr) for 2 hours to swell the resin beads. The resin was equilibrated by flowing PBS, pH 7.4 at a rate of 0.05 mL/min (87 cm/hr) for 2 hours.

3.2.6 Determination of Dynamic Binding Capacity Using Breakthrough Curves

Human Immunoglobulin G (hIgG, 15.1 mg) was massed out and dissolved with 1.51 mL of PBS, pH 7.4 to make a 10.0 mg/mL stock hIgG solution. This solution was injected onto the column using a 1 mL sample loop and PBS, pH 7.4 was flowed at 0.05 mL/min (87 cm/h) for at least 15 minutes (some runs utilized a longer flow-through period, but this is insignificant as all runs flowed PBS, pH 7.4 for a long enough time that 10 % of breakthrough was achieved). Then, PBS, pH 7.4 was flowed at 0.2 mL/min (348 cm/hr) for 5 to 10 minutes.
Column regeneration and elution of bound hIgG occurred in a single step by flowing 0.85% phosphoric acid at 0.3 mL/min (522 cm/hr) for 10 minutes. Finally, the column was re-equilibrated by flowing PBS, pH 7.4 at 0.40 mL/min (696 cm/hr) for 15-20 minutes. This exact procedure was repeated with a loading flow-rate of 0.02 mL/min PBS, pH 7.4 for at least 40 minutes. Dynamic binding capacities were calculated using the following equation:

\[
DBC \left(\frac{mg}{mL}\right) = \frac{T_{10\%BT}[min] \times FR \left(\frac{mL}{min}\right) - DV \left[\frac{mL}{mL}\right]}{CV \left[\frac{mL}{mL}\right]} \times hIgG C_0 \left(\frac{mg}{mL}\right)
\]

where DBC is dynamic binding capacity, \(T_{10\%BT}\) is the time of the HPLC run after injection at which 10% of breakthrough occurs, FR is the flow rate at which the experiment is run, hIgG \(C_0\) is the concentration of hIgG in the feed and CV is the resin column volume.

3.2.7 Chromatographic Capture and Elution of hIgG Fc and Fab Fragments

Stock hIgG-Fab fragment solution (11.4 µL of 4.4 mg/mL) was mixed with 38.6 µL of PBS, pH 7.4 to make 50 µL of 1.0 mg/mL Fab solution. This solution was injected onto the column using a 50 µL sample loop and PBS, pH 7.4 was flowed at 0.05 mL/min (87 cm/hr) for 10 minutes and at 0.2 mL/min (348 cm/hr) for 5 minutes. Column regeneration and elution of bound Fab occurred in a single step by flowing 0.85% phosphoric acid at 0.3 mL/min (522 cm/hr) for 10 minutes. Finally, the column was re-equilibrated by flowing PBS, pH 7.4 at 0.40 mL/min (696 cm/hr) for 10 minutes. The flow-through and elution peaks were integrated to find the percentage of Fab binding. This exact same procedure was repeated with a 100 µL injection of 1.0 mg/mL hIgG-Fc fragment.
3.2.8 Chromatographic Capture and Elution of Human IgG-4 from Cell Culture Supernatant

CHO cell culture supernatant containing 1.5 mg/mL hIgG₄ was thawed from -20 °C to 4°C. 100 µL of the solution was injected onto the column and 10 mM PBS, pH 7.4 was flowed at 0.05 mL/min (87 cm/hr) for 10 minutes and increased to 0.20 mL/min (348 cm/hr) for 10 additional minutes. The first 0.5 mL of the flow-through peak was collected (beginning when A₂₈₀= 0.1). To elute IgG₄ from the column, 200 mM acetate buffer, pH 4.0 was flowed through the system at 0.30 mL/min (522 cm/hr) for 10 minutes and the elution peak was collected (beginning and ending when A₂₈₀ =0.1). Column regeneration occurred by flowing 0.85% phosphoric acid at 0.2 mL/min (348 cm/hr) for 10 minutes. Finally, the column was re-equilibrated by flowing PBS, pH 7.4 through the column at 0.4 mL/min (696 cm/hr) for 20 minutes.

To show the reproducibility of the separation, this exact procedure was repeated, except that the column was re-equilibrated at the end of the run in PBS, pH 7.4 that contained 0.5 M NaCl (in addition to the 0.14 M NaCl that is present in standard 10 mM PBS, pH 7.4). To determine the effect of salt concentration on the performance of the column, 0.5 M NaCl in PBS, pH 7.4 was used as the flow-through buffer for the first two flow steps in place of PBS, pH 7.4, using the same flow rates and times as given above. All other proceedings stayed the same as in previous experiments. The column was re-equilibrated in 0.5 M NaCl in PBS, pH 7.4, and this exact procedure was repeated once more to prove reproducibility. All collected fractions were stored at 4°C.
3.2.9 Concentration of Fractions from CHO Cell Culture Supernatant Separation and Analysis via SDS-PAGE

Fractions from the CHO cell culture supernatant separation were pipetted into Amicon Ultra centrifugal filters and centrifuged for 15 minutes at 20,817 × g and 15°C. The resulting filtrate was discarded; filters were turned around in their respective centrifuge tubes and centrifuged again for 5 minutes at 2,655 × g and 15°C to eject the concentrated retentate from the filters. 20 µL of the concentrated fractions were combined with 9 µL of NuPAGE LDS buffer and 6 µL of NuPAGE reducing agent in micro centrifuge tubes. The samples were mixed via vortex and placed into a boiling water heat bath for 10 minutes. 17 µL of the boiled samples and SeeBlue plus 2⃝ pre stained molecular weight marker were pipetted into the wells of a NuPAGE® Novex Gel (4-12% Bis-Tris).

The gel was run in a Novex® mini cell gel box using NuPAGE® MOPS running buffer and Coomassie-stained with SimpleBlue™ SafeStain. The IgG purity was determined by densitometric analysis of Coomassie-stained gels by means of ImageJ 1.32j software (National Institutes of Health, MD, USA). The purity of the product was calculated as the fraction of the total area equivalent to the IgG bands at 25 and 50 kDa.

3.3 Results and Discussion

3.3.1 Impact of Peptide Selection, Reaction Peptide Concentration, Peptide Load and Support Matrix Selection on Static Binding Capacities

Equilibrium (static) binding capacities were obtained for various peptide-solid phase resins that were synthesized using the technique above. By changing the synthetic variables
of peptide selection, peptide concentration, peptide load and solid-phase selection, the impact of these variables on apparent static binding values of the resultant resins was elucidated. In the data that follows, peptide load is defined as mol peptide reacted/ mol -NH$_2$ groups on the resin surface. Syntheses occurred employing multiple peptide concentrations, with the 100 mM technique usually giving the best results. To be certain that using a 100 mM technique is optimal, it will be necessary to complete similar curves (by varying peptide load from 0.1 to 2.0), for peptide concentrations of 50 and 150 mM. This work will be completed in the early part of 2012, prior to being submitted for publication.

By monitoring the equilibrium binding capacity as a function of peptide load, three distinct regimes existed for most of the studied resins. There was an under-loaded regime, in which resins simply had too little peptide ligand coupled to them to achieve a maximal equilibrium binding capacity. In many cases, there seemed to be an over-loaded regime, where there was too much peptide added to the resin, which either decreased or gave a slightly negative impact on equilibrium binding capacity. Finally, there was an optimally-loaded regime, where the right amount of ligand was added to the resin to yield a resin and gave the highest equilibrium binding capacity for a given peptide-solid phase combination.

### 3.3.2 Gigacap Based Resins

Figure 3.3 details the results from HWRGWV-Gigacap resins, which did not give a true maximum equilibrium binding capacity and can be considered to be only in the under-loaded regime. For the HWRGWV-Gigacap resins studied, using a peptide load of 3.0 gave the highest equilibrium binding capacity of 26.5 mg/mL. It is likely that making resins with
peptide loads higher than 3.0 might give even higher equilibrium binding capacities than the ones in Figure 3.1. However, this would be a very inefficient approach in terms of the amount of peptide used and in light of results from other experiments that are detailed later in this document. In addition, it is highly likely that using higher peptide loads would cause the resin to have a considerable amount of non-specific binding to unwanted proteins.

It is interesting to note the apparent differences in binding capacities obtained by making HWRGWV-Gigacap resins using different peptide reaction concentrations. Resins made using a 30 mM peptide concentration technique gave lower capacities than those synthesized using peptide concentrations of 50, 150 and 400 mM, which all gave capacities similar to resins made with the 100 mM technique at identical peptide loads. Evidently, a 30 mM concentration is too dilute to drive the desired reaction forward, while the other studied concentrations (50, 100, 150 and 400 mM) gave resulting affinity resins that bound hIgG at the same capacity with-in experimental error.
Figure 3.3: Equilibrium binding capacities of various HWRGWV-Gigacap resins after a 30 minute incubation with a 7.5 mg/mL hIgG solution. The resins were synthesized using the indicated peptide concentrations.

Acetylated HWRGWV-Gigacap showed three distinct peptide loading regimes and a precise maximum binding capacity (Figure 3.4). For this combination, resins were in the under-loaded regime from 0 to ~0.25, in the optimally loaded regime from 0.5 to ~1.0 and in the over-loaded regime above 1.0. The 0.5, 0.75 and 1.0 peptide load Ac-HWRGWV-Gigacap resins produced equilibrium binding capacities of 32.1, 33.5 and 31.0 mg/mL, respectively. These values represent the highest obtained results from the presented study and are much higher than the HWRGWV-Gigacap matrix. This indicates that oligomerization of unacetylated peptide likely occurs at the studied conditions, acts to decrease the resins capacity and can be avoided by using acetylated peptide.
Figure 3.4: Equilibrium binding capacities of various N-terminus acetylated HWRGWV-Gigacap resins after a 30 minute incubation with a 7.5 mg/mL hIgG solution. The resins were synthesized using the indicated peptide concentrations.

Introducing peptide loads greater than 1.0 means that there are likely more HATU-activated peptide molecules in the system than there are resin –NH\textsubscript{2} groups. In this situation, it is likely that a large percentage of the Ac-HWRGWV is coupling to the resin as desired, followed by cross-mer formation (Figure 3.2, reaction 4), which likely decreases the binding capacity compared to the optimally loaded resins.

Study of HWRGWVA-Gigacap resins (Figure 3.5) showed that adding an alanine residue to the end of the original hexapeptide sequence increases the effectiveness of HATU mediated coupling. The R-group for alanine (−CH\textsubscript{3}), is less bulky than the R-group for valine (−CH-(CH\textsubscript{3})\textsubscript{2}) and the former is also more electron donating than the latter. Therefore,
HATU-activation and subsequent nucleophilic attack by resin –NH₂ groups occurs more easily with an alanine residue at the C-terminus of the peptide than with a valine.

Comparison of Figures 3.3 and 3.5 shows the obvious superiority of peptide with an additional alanine at the end of it. The HWRGWVA-Gigacap resins were apparently in the under-loaded peptide regime from 0 to ~0.25, in the optimally loaded regime from ~0.5 to ~1.0 and in the over-loaded regime in loads over ~1.0. At peptide loads of 0.5, 0.75 and 1.0, HWRGWVA-Gigacap gave equilibrium binding capacities of 30.7, 30.8 and 31.0 mg/mL, respectively.

![Equilibrium Binding Capacity of Gigacap-HWRGWVA Resins at 7.5 mg/mL hIgG Load](image)

**Figure 3.5:** Equilibrium binding capacities of various HWRGWVA resins after a 30 minute incubation with a 7.5 mg/mL hIgG solution. The resins were synthesized using the indicated peptide concentrations.

Capacities obtained from HWRGWVA-Gigacap resins were higher than those from HWRGWV-Gigacap resins but slightly lower than those obtained from Ac-HWRGWV-Gigacap resins. It is likely that HWRGWVA resins are superior to HWRGWV resins simply
because of increased reactivity at the C-terminus, which helps with the formation of the HATU-peptide intermediate. Conversely, Ac-HWRGWV-Gigcap resins show superiority over HWRGWV-Gigacap resins because the former avoids the side reaction of oligomerization, which the latter likely undergoes.

Combining these two ideas, gives a logical answer to the question: ‘which peptide should be studied next via HATU based methodologies?’ N-terminus acetylated HWRGWV-A will be studied in the near future, as it offers added reactivity of the C-terminus, combined with only one possible side reaction (cross-mer formation) and therefore, is likely to give static binding capacities greater than 33 mg/mL.

3.3.3 Toyopearl Based Resins

HWRGWV-Toyopearl resins showed three distinct peptide loading regimes as they were in the under-loaded regime from 0 to ~0.5, the optimally loaded regime at ~0.75 and in the over-loaded regime at peptide loads greater than ~0.75. Figure 3.6 reveals that HWRGWV-Toyopearl resins gave SBC’s that increased upon increasing the synthetic peptide concentration from 10 to 100 mM and from 25 to 100 mM. However, concentrations of 50, 67, 100 and 133 mM all gave SBC’s that were the same with-in experimental error at constant peptide loads.

One would think that using acetylated peptide (Ac-HWRGWV), in place of HWRGWV, would increase the binding capacity of Toyopearl based resins, as one of the possible side reactions (oligomerization) would be avoided. Surprisingly, this was not at all the case with Ac-HWRGWV-Toyopearl, as these resins gave extremely low results, as the
highest observed binding capacity was an abysmal 4.8 mg/mL using 70 mM peptide reaction concentration at a 1.0 peptide load. Because these capacities were so surprisingly low, multiple resins that appear in Figure 3.7 were re-synthesized and re-screened against hIgG but all gave the same observed results within 0.5 mg/mL experimental error. The optimal peptide reaction concentration for Ac-HWRGWV appears to be in the overly broad range of 50 to 142 mM. However, Ac-HWRGWV-Toyopearl SBC values are so low that the differences observed between data points might simply be due to experimental error.

Ultimately, the optimal peptide concentration for Ac-HWRGWV-Toyopearl resins need not be determined, as the observed SBCs are much lower than other studied resins to justify further investigation in light of this project’s main goal, which is to maximize the resin’s binding capacities.
Figure 3.6: Equilibrium binding capacities of various HWRGWV-Toyopearl resins after 30 minute incubation with a 7.5 mg/mL hIgG solution. The resins were synthesized using the indicated peptide concentrations.

Figure 3.7: Equilibrium binding capacities of various Ac-HWRGWV-Toyopearl resins after 30 minute incubation with a 7.5 mg/mL hIgG solution. The resins were synthesized using the indicated peptide concentrations.
Figure 3.8: Equilibrium binding capacities of HWRGWVA-Toyopearl resins after a 30 minute incubation with a 7.5 mg/mL hIgG solution. The resins were synthesized using the indicated peptide concentrations.

HWRGWVA proved to be the most effective peptide to couple to Toyopearl using HATU based chemistry. Figure 3.8 gives the results for these resins, which displays an under-loaded regime from 0 to ~0.75, an optimally loaded regime at ~1.0 and an overloaded regime when the peptide load is greater than 1.0. Resins utilizing a 1.0 load with peptide reaction concentrations of 100 and 50 mM gave resulting equilibrium binding capacities of 23.0 and 22.2 mg/mL, respectively.

Figure 3.8 does not have enough data points with peptide concentrations other than 100 mM to obtain a definitive relationship between this variable and resultant binding capacity. Ultimately, the most important finding from Toyopearl based studies is that HWRGWVA performed much better than HWRGWV, which performed much better than Ac-HWRGWV. The first of these relations can be explained by the added C-terminus.
reactivity that alanine offers over valine. However, explanation of the result that the acetylated peptide performed much worse than unacetylated peptide when coupling to Toyopearl is far more difficult to decipher. It is most likely that unacetylated peptide has increased hydrogen bonding capabilities when compared to acetylated peptide and this increased hydrogen bonding likely helps peptides undergoing HATU-mediated coupling to Toyopearl, but not necessarily to Gigacap.

3.3.4 Gigacap vs Toyopearl

Both Toyopearl AF-Amino-650 M and Gigacap are built onto an HW-65 M base matrix, each have similar chemical stability and pore sizes that are approximately 1000 Å in diameter. However, the two resins are different in the quantity and nature of the reactive groups present on each solid phase. Gigacap has roughly twice the functional ligand density that Toyopearl AF–Amino- 650 M has. Because Gigacap is not yet commercially available, it is not known exactly what kind of functional group is present on the surface of the resin, except that it is a ‘polyamine.’ It is the thought of members of the Carbonell group that poly(lysine) is the functional ‘polyamine’ ligand attached to Gigacap. Finally, it is highly likely that the functionalized groups on Toyopearl are oriented on the surface of the resin differently than Gigacap and therefore give different arrangements upon peptide coupling. In light of the apparent differences between the two resins, it is not surprising that they gave very different results when used in HATU coupling experiments and subsequent binding studies.
Table 3.2: Summary of the resins that gave the highest hIgG static (equilibrium) binding capacities, SBC’s, from each of the 6 peptide-solid phase combinations studied. Peptide load is defined (as mol peptide reacted/ mol –NH₂ groups on resin).

<table>
<thead>
<tr>
<th>Solid Phase</th>
<th>Gigacap (200 µmol –NH₂/mL)</th>
<th>Toyopearl (100 µmol –NH₂/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Peptide]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mM)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Peptide</td>
<td>3</td>
<td>0.75</td>
</tr>
<tr>
<td>Load</td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td>SBC</td>
<td>26.5</td>
<td>33.5</td>
</tr>
<tr>
<td>(mg/mL)</td>
<td></td>
<td>31.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>23.0</td>
</tr>
</tbody>
</table>

Finally, this study has shown that in terms of static (equilibrium) binding capacity, affinity resins made by coupling HWRGWV and its derivatives to Gigacap performed much better than those same ligands coupled to Toyopearl. Of all the studied resins, six different Gigacap based resins gave SBC’s over 30 mg/mL, while only two Toyopearl based resins broke the 20 mg/mL barrier. The two Gigacap resins that gave the highest SBCs, (0.5 and 0.75 Ac-HWRGWV) were chosen for further characterization via dynamic studies under flowing conditions.

3.3.5 Determination of 10% Breakthrough for DBC Studies

The four standard curve experiments resulted in the four chromatograms given in Figure 3.9, below. The maximum $A_{280}$ value obtained for each experiment was plotted against hIgG concentration to yield a standard curve equation of: $A_{280} = 0.8413 \times [\text{hIgG concentration}] - 0.0016$ with an $R^2$ value of 0.9998. Therefore, it was determined that 10% of
breakthrough occurred at $A_{280}$ values of 0.840 and 0.630 for 10 and 7.5 mg hIgG/mL injection dynamic binding experiments, respectively.

**Figure 3.9:** The four stacked chromatograms for protein concentrations of 0.25, 0.5, 0.75 and 1.0 mg/mL, respectively. The maximum $A_{280}$ values from each run was plotted against the concentration of the injection, yielding the above standard curve, which was used to determine exactly when 10% of breakthrough occurred during dynamic binding experiments.

### 3.3.6 Dynamic Binding Capacities

The two resins that gave the highest static binding capacities (0.5 and 0.75 Ac-HWRGWV-Gigacap) were chosen for further characterization in dynamic studies under flowing conditions. Breakthrough experiments were conducted at flow rates of 0.02 mL/min and 0.05 mL/min after a 1.0 mL injection 10 mg/mL hIgG solution (Figure 3.10). In addition, the resin 0.75 Ac-HWRGWV-Gigacap was studied at the same flow rates after a 7.5 mg/mL hIgG injection (Figure 3.11).
Figure 3.10: DBC Breakthrough curves at 0.02 and 0.05 mL/min of 0.5 and 0.75 Ac-HWRGWV-Gigacap resins after 10 mg/mL hIgG injections. Detailed protocols of these runs can be found in the methods section.

Figure 3.11: DBC Breakthrough curves at 0.02 and 0.05 mL/min of 0.75 Ac-HWRGWV-Gigacap after 7.5 mg/mL hIgG injections. Detailed protocols of these runs can be found in the methods section.
With-in experimental error, both resins gave identical DBC values, which were expected, as they gave very similar static binding capacities. At a flow rate of 0.05 mL/min both resins gave DBCs of 23-25 mg/mL, which is a considerable improvement over the 18.4 mg/mL capacity that Naik et al. obtained while binding Mab1 out of CHO cell culture supernatant. However, these experiments aren’t truly comparable, as the culture fluid contained considerable impurities and the presented studies involved the use of pure hIgG in PBS, pH 7.4. All experimental DBC results are summarized in Table 3.3, below.

**Table 3.3:** Listed are apparent dynamic binding capacity values (mg/mL) calculated at 10% of breakthrough for 0.5 and 0.75 Ac-HWRGWV-Gigacap resins. SBC values are from experiments in which 50 µL of each resin was incubated with 1.0 mL of 7.5 mg/mL hIgG for 30 minutes.

<table>
<thead>
<tr>
<th>DBC’s (mg/mL)</th>
<th>10 mg/mL Injection</th>
<th>7.5 mg/mL Injection</th>
<th>SBC (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Flow-Rate (mL/min)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.02</td>
<td>0.05</td>
<td>0.02</td>
<td>0.05</td>
</tr>
<tr>
<td><strong>0.5 Ac-HWRGWV-Gigacap</strong></td>
<td>31.9</td>
<td>25.2</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>0.75 Ac-HWRGWV-Gigacap</strong></td>
<td>30.2</td>
<td>23.4</td>
<td>31.9</td>
</tr>
</tbody>
</table>

As expected, both resin’s DBCs approached their respective SBCs upon decreasing flow rates. Surprisingly, the 0.75 resin bound slightly more hIgG at a lower injection concentration of hIgG. In addition, the 0.5 Ac-HWRGWV resin bound slightly more hIgG than its 0.75 counterpart, which is the opposite of what would be predicted by comparing their respective SBC values. If indeed this truly is the case, then it is possible that the 0.5 Ac-HWRGWV-Gigacap resin has ligands coupled to its surface in a more efficient way the 0.75 Ac-HWRGWV-Gigacap resin. However, it is most likely that these small differences are simply due to experimental error. Also, the performance of the packing of these resins must
be studied and fully optimized (these are some of the first Gigacap-based resins studied by our group under flowing conditions). Once the optimal packing conditions for Gigacap resins are determined, more credibility can be given to obtained experimental DBC values analogous to the ones in Table 3.3.

### 3.3.7 Binding Specificity of Resins for Fab and Fc Fragments of hIgG

Even though the two resins gave similar DBC results, surprisingly, there was a significant difference between the two in terms of specificity, as 0.5 Ac-HWRGWV-Gigacap proved to be the superior resin. Figure 3.12 shows the chromatograms from Fab and Fc binding experiments and Table 3.4 details the results of integrating the peaks from these chromatograms.

![Figure 3.12](image.png)

**Figure 3.12:** Both Fab (left) and Fc (right) fragment experiments were conducted with a 50 µL load of 1 mg/mL solution of each respective fragment, followed by a flow-through of PBS, pH 7.4 at 0.05 mL/min (87 cm/hr). Elution and regeneration of the columns occurred in a single step by flowing 0.85% phosphoric acid at 0.3 mL/min (522 cm/hr) for 10 minutes.
Table 3.4: Summary of Fab and Fc binding studies that resulted from integration of the peaks that appear in Figure 3.12, above. Studies involved a 50 µL injection of 1.0 mg/mL fragment, flow-through with PBS, pH 7.4 at 0.05 mL/min and elution/regeneration with 0.85% phosphoric acid.

<table>
<thead>
<tr>
<th>Resin</th>
<th>Fab Binding</th>
<th>Fc Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 Ac-HWRGWV</td>
<td>3.4 %</td>
<td>87 %</td>
</tr>
<tr>
<td>0.75 Ac-HWRGWV</td>
<td>6.4 %</td>
<td>73 %</td>
</tr>
</tbody>
</table>

Resin 0.75 Ac-HWRGWV- Gigacap bound 6.4 % of the injected Fab, while 0.5 Ac-HWRGWV-Gigacap only bound 3.4 %. Therefore, when comparing the 0.75 and 0.5 Ac-HWRGWV-Gigacap resins, the former is made with ~50% more peptide, which is likely to form more cross-mers which are more likely to nonspecifically bind Fab.

Surprisingly, the 0.5 loaded resin bound ~14% more Fc than its 0.75 counterpart, helping make an even stronger case that the former is more specific and therefore better suited to be an affinity resin. The most likely explanation of these phenomena is again probably best explained by the formation of more cross-mers in the 0.75 resin. Cross-mers are formed on top of correctly oriented ligands in an ‘incorrect’ orientation that is unlikely to bind Fc-hIgG as well as the correctly oriented ligands would.

3.3.8 Analysis of Chromatographic Separation of IgG4 (mAb1) from CHO Cell Culture Supernatant

The concentration of Mab1 in the CHO cell culture supernatant was 1.5 mg/mL. Mab1 is a humanized MAb of IgG4 subclass and is a commonly used therapeutic product. In addition to the Mab1 target protein, the cell culture fluid also contained pluronic acid, yeast
hydrolysate, cholesterol, tropolone, amino acids, glucose, vitamins and various cellular components. Chromatograms from the separation of IgG4 using the 0.5 and 0.75 Ac-HWRGWV-Gigacap columns can be visualized in Figures 3.13 and 3.14, on the following pages. Inspection of these chromatograms shows single elution peaks with minimal regeneration peaks, showing that the target protein was successfully eluted using 200 mM acetate buffer, pH 4. This is an interesting result, as Naik et al.’s studies of the same fluid with HWRGWV resins synthesized via solid-phase synthesis gave split elution peaks using the same pH 4 buffer. This suggests that HWRGWV resins synthesized using HATU chemistry are slightly weaker binders of Mab1 and therefore require less acidic elution conditions than resins studied by Naik et al. [8]. Another possibility is that the resins studied by Naik et al. are less bio specific and bind more impurities than the presented 0.5 and 0.75 Ac-HWRGWV-Gigacap resins.
Figure 3.13: Chromatograms and resulting SDS-PAGE from chromatographic separation of IgG4 from CHO cell culture supernatant using 0.5 Ac-HWRGWV-Gigacap resin. Labels: MM- molecular weight marker; FT- flowthrough fraction; EL- elution fraction; CHO- cell culture supernatant that was loaded onto column; hlgG- pure hlgG in PBS, pH 7.4
**Figure 3.14:** Chromatograms and resulting SDS-PAGE from chromatographic separation of IgG4 from CHO cell culture supernatant using 0.75 Ac-HWRGWV-Gigacap resin. Labels: MM- molecular weight marker; FT- flowthrough fraction; EL- elution fraction; CHO- cell culture supernatant that was loaded onto column; hlgG- pure hlgG in PBS, pH 7.4.
Table 3.5: Listed are average yields and purities of IgG₄ in elution fractions after separation from CHO Cell Culture Supernatant. Yields were calculated using a standard curve conducted on the Waters HPLC system. Purity values were obtained by densitometry calculation of SDS-PAGE using Image J software. All runs were performed in duplicate to show reproducibility.

<table>
<thead>
<tr>
<th>Flow-Through Buffer</th>
<th>PBS, pH 7.4</th>
<th>0.5 M NaCl in PBS, pH 7.4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Resin</strong></td>
<td><strong>Yield</strong></td>
<td><strong>Purity</strong></td>
</tr>
<tr>
<td>0.5 Ac-HWRGWV-Gigacap</td>
<td>76% ± 4%</td>
<td>99% ± 0%</td>
</tr>
<tr>
<td>0.75 Ac-HWRGWV-Gigacap</td>
<td>69% ± 3%</td>
<td>99% ± 0%</td>
</tr>
</tbody>
</table>

The average yields from CHO separations using the 0.5 and 0.75 Ac-HWRGWV-Gigacap resins using two different flow through buffers is given in Table 3.5, above. The two SDS-PAGE runs of the fractions collected during separation of Mab1 from CHO show that 0.5 and 0.75 Ac-HWRGWV-Gigacap isolated Mab1 with a great deal of purity (Table 3.5). Seven out of eight elution lanes of the SDS PAGES were so pure that densitometry analysis found less than 0.2% impurities in them. The only elution peak fraction that showed any substantial impurities was still impressively 97% pure (from the first 0.5 Ac-HWRGWV-Gigacap run using 0.5 M NaCl in PBS, pH 7.4 as the flow through buffer). It is also important to note that while using 0.5 and 0.75 Ac-HWRGWV-Gigacap resins to purify Mab1 from CHO cell culture supernatant, adding 0.5 M NaCl to the flow-through buffer (standard PBS, pH 7.4) seems unnecessary, as it did not help separations in terms of yield or purity.
3.4 Conclusions

The SBC values of 31-33 mg/mL found in this study are a significant improvement over the 23 mg/mL value obtained by Yang et al. under similar conditions [7]. In addition, the obtained DBC values of 23-25 mg/mL are higher than the 18.4 mg/mL value that Naik et al. reported, but under different conditions [8]. However, the most impressive characteristics of the presented resins are their improved bio specificity over previously studied HWRGWV resins [8]. This increase in specificity arises from the fact that the presented HATU methodology uses peptide starting reagents that are at least 93% pure. Conversely, the previously studied resins were made by synthesizing the peptide, amino acid by amino acid directly onto Toyopearl AF-Amino 650-M, which resulted in some amount of peptide truncation and subsequent non-specific binding.

Both 0.5 and 0.75 Ac-HWRGWV-Gigacap resins displayed the ability to isolate Mab1 with yields and purity of 72 and 98%, respectively. These values represent a slight increase in purity, but a significant decrease in yield compared to results reported by Naik et al. (yield and purity of 85 and 95%, respectively) [8]. The packing of the 0.5 and 0.75 Ac-HWRGWV-Gigacap resins was not optimized in these studies and it is likely that once this parameter is studied, that these resins will be able to isolate mAb1 from CHO with yields similar to Naik et al.’s findings. Finally, 0.5 Ac-HWRGWV-Gigacap was shown to be the most bio specific of the studied resins, as it bound more Fc fragment of hIgG and less Fab fragment of hIgG than 0.75 Ac-HWRGWV-Gigacap.
References


