SYNTHESIS AND SCREENING OF A COMBINATORIAL PEPTIDE LIBRARY FOR LIGANDS TO TARGET TRANSFERRIN: MINIATURIZING THE LIBRARY

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Combinatorial libraries are used in the search for ligands that bind to target proteins. Fmoc solid-phase peptide synthesis is routinely used to generate such libraries. Microwave-assisted peptide synthesis was employed here to decrease reaction times by 80-90%. Two One-Bead-One-Compound combinatorial libraries were synthesized on 130μm beads (one containing 750 members and the other 16, 807). The use of smaller solid supports would have many important practical advantages including; increased library diversity per unit mass, smaller quantities of library needed to generate hits, and screening could be conducted by using a standard flow cytometer. To this end, a miniaturized peptide library was synthesized on 20 μm beads to demonstrate proof of principle.

A small sample from the 16,807-member library was screened against transferrin-AlexaFluro 647, a protein responsible for iron transport in vivo. A number of hits were identified and sequenced using techniques coupling nanomanipulation with nanoelectrospray mass spectrometry.
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CHAPTER I

INTRODUCTION

Combinatorial libraries are routinely employed in the discovery of new lead compounds for drugs [20] and in the search for high-affinity ligands to target proteins. [15; 21] Peptide-based libraries have proven invaluable in cancer research where efforts focus primarily on finding ligands for proteins that facilitate targeted binding to diseased cells. [21] Such libraries allow for the generation of broad chemical diversity through relatively straightforward techniques and procedures. [13] Their incarnations range from chemically-inspired one-bead, one-compound (OBOC) libraries, [11] also as known as split-mix libraries,[11; 12; 13; 14] to biologically-inspired ones such as phage display. [22; 23; 24]

Another widely used method for identifying targeting ligands that warrants mention is the use of aptamer libraries. This technique works in the same fashion as a peptide combinatorial library but with each member of the library being a nucleic acid instead of a peptide. Each member of the pool has a different sequence and, thus, a different set of chemical groups that will fold into different structures that have differing properties or are capable of different functions. Individual members of a pool are separated from one another on the basis of their ability to perform an arbitrary task. For example, nucleic acids that interact with protein targets have been trapped on nitrocellulose filters or separated from uncomplexed species on native polyacrylamide gels. Aptamers that bind small molecules such as cofactors or vitamins can be isolated by affinity chromatography. Unbound nucleic acids are washed away from bound, and
the bound species are then eluted by adding an excess of soluble ligand, changing solvent conditions, or cleaving from the solid support. [25]

In the case of this OBOC library, a method for cleaving the peptide from the solid support once a hit had been identified were needed as well as ones that facilitate the identification of the released peptides. In practice, a variety of approaches are available for releasing the peptide from an individual library bead for analysis. These schemes utilize a carefully chosen cleavage site that is installed between the peptide and resin bead, such as certain types of ester linkages that can be cleaved with ammonia gas,[3; 4; 5; 6] a CNBr-cleavable methionine residue, as well as a variety of other chemically and photolytically sensitive ones. Several techniques have been employed for sequencing the cleaved peptides including Edman degradation (ED), partial ED followed by matrix-assisted laser desorption ionization mass spectrometry [19] (PED MALDI MS), MALDI tandem mass spectrometry [3] (MALDI MS/MS), and electrospray ionization tandem mass spectrometry [15] (ESI MS/MS). In order to avoid sample loss and to reduce analysis time, the number of processing steps required specifically for hit identification should be kept to a minimum and, for those that are absolutely necessary, ones that are solvent-free are preferable. An inherent aspect of utilizing bead libraries is the need for manipulating relatively small amounts of both library compound and the solvents used for cleavage/extraction. Nanomanipulation, with spatial resolution as fine as 3.4 nm using current technology, offers one partial remedy to this dilemma. Nanomanipulation has already proven to be a useful technique for the analysis of trace analyte particles found on fibers [26] and for lipid analysis [27] with liquid-phase microextractions. Due to <200 μL volumes of solvent and picomolar sensitivity,
nanomanipulation is coupled to nanoelectrospray ionization mass spectrometry (NSI MS) for sensitive analysis.

Using the techniques described here, analysis of combinatorial library constituents on sub-micron-sized solid supports could theoretically be performed, which would allow for miniaturization of library synthesis, screening, and identification. Current methods for automated screening of combinatorial libraries utilizing traditional solid supports (90-300 μm) based on fluorescence measurements require the use of a non-standard flow cytometer specifically engineered to allow sorting of large particles.[28] This need could be obviated through the use of smaller solid supports, 30 μm and 20 μm resin beads. Another advantage of synthesizing libraries on smaller supports is the significant gains that can be made in library diversity on a per gram basis. For instance, there are 6.55x10^4 beads per gram of 300 μm TentaGel resin; if the bead size were reduced to 800 nm, there would be 3.45x10^{12} beads per gram (assuming similar compositions). This would enable screening of highly diverse libraries using only microgram quantities of beads.

Targeting cancer cells can be achieved through the attachment of ligands to the surface of the nanoparticle known to bind to receptors over-expressed on the surface of rapidly dividing cancer cells. Because of the high metabolic demands engendered by rapid proliferation, many cancer cells over-express transferrin, albumin, and folate receptor, which makes conjugation of transferrin, albumin, folate, or antibodies to these receptors a successful targeting approach. Some of the ligands developed here to target serum proteins for particle stealth will have the added benefit of targeting the nanoparticle (NP) to cancer cells, i.e., NPs coated with a ligand to transferrin or albumin
should show preferential uptake in cancerous cells. Anti-TfR-coated particles have been shown [29] to be efficiently internalized by seven different cancer cell lines, Table 1, including: B-cell lymphoma (Ramos, Sup-B8), cervical (HeLa), ovarian (SKOV3), brain (MGR3), prostate (LNCaP), and lung (H125). Conversely, nanoparticles coated with the isotype control, IgG1, were not. Furthermore, neither particle composition was taken up by the negative control cell line, mouse embryonic fibroblasts (MEF).

Table 1 - Internalization of nanoparticles as a function of surface ligand for several cancer cell lines. Particles were conjugated with either anti-TfR or IgG1, an isotype control.
CHAPTER II
EXPERIMENTAL
Peptide Synthesis

A four-glycine peptide chain was synthesized on 130 µm TentaGel S NH₂ (0.29 mmol amine/g) resin beads. The Kaiser test was used to ensure the presence or absence of the free primary amine group. The solutions used for this test were ninhydrin, 5 g ninhydrin in 100 ml ethanol, phenol, 80 g phenol in 20 ml ethanol, and pyridine/KCN, 2 ml of 0.001 M KCN in 98 ml pyridine. This test consisted of adding three drops of each of the solutions to a small sample of the resin beads, 1-2 mg, in a glass reaction vial. The sample was then heated to 120° C for two minutes in the CEM Discover SPS microwave. A positive result shows a dark blue colored solution indicating the presence of a free primary amine. A negative result shows a yellow colored solution indicating no presence of any free primary amines.

Initially the resin beads alone were tested to show the presence of a free primary amine. A small sample of beads was swollen in dimethylformamide (DMF) for 30 minutes. The beads were then washed three times with dichloromethane (DCM) to remove all DMF. They were then put in the microwave and a positive result was obtained.

Several methods for the synthesis of the peptide chain were tested. One 100 mg sample, Sample A, of resin beads (1 equiv) was swollen for 30 minutes in DMF. During this time a linker, 4-hydroxymethylbenzoic acid (HMBA, 3 equiv) and the reagent hydroxybenzotriazole (HOBt, 3 equiv) were dissolved in separate 1 ml solutions of DMF. These were then added to the resin beads along with 1,3-diisopropylcarbodiimide (DIC,
6 equiv). The solution was left to stir overnight and then washed with DMF and DCM 3 times each. The Kaiser test was performed and gave a negative result.

The chain was continued on Sample A by adding glycine (Fmoc-Gly-OH, 5 equiv) to the beads and sufficient amount of DMF to make the beads mobile. DIC (6 equiv) and 10% 4-dimethylaminopyridine (DMAP) dissolved in DMF (3 equiv) were also added to the resin beads. The solution was left to stir overnight and was followed with 3 washes of DMF. A 30% piperidine solution in DMF was used for the fmoc deprotection. The amount of deprotection reagent used was just enough to cover the beads, 1-2 ml. The solution was left to stir for five hours and then washed with DMF and DCM. The Kaiser test was performed and a positive result was obtained. In a separate vial the next amino acid, Fmoc-Gly-OH, (5 equiv), DIC (6 equiv), HOBt (3 equiv) are mixed in 1 ml 1-methyl-2-pyrrolidone (NMP). The mixture is added to the resin after 2 minutes. The solution was placed in a microwave vessel and then put in the microwave for 10 minutes at 75° C (SPS mode, max power 10W ΔT = 3° C). The beads were washed with DMF and the subjected to the Kaiser test, which gave a negative result. The deprotection step was repeated but the Kaiser test gave another negative result. This sample was discarded.

The linker, H MBA, was added the same way using the same equivalents to Sample B as to sample A, but was only left to stir for one hour rather than overnight. The Kaiser test gave a negative result. Fmoc-Gly-OH (5 equiv), 10% DMAP solution in DMF (3 equiv), and DIC (6 equiv) were mixed in a separate vial then added to the resin beads. The sample was stored overnight and then washed with DMF. Piperidine (30% in DMF) was added and the beads were stirred for 15 minutes, the results of a Kaiser
test were positive. Fmoc-Gly-OH (5 equiv), HOBt (3 equiv), DIC (6 equiv) and NMP were mixed in a separate vial and then added to the beads. The sample was placed in the microwave for 10 minutes at 75° C (SPS mode, max power 10W, ΔT=3° C). The beads were washed five times with DMF and 30% piperidine (1-2 ml per washing). The sample was put in the microwave reactor for 2.5 minutes at 75° C, but gave a negative result when the Kaiser test was performed. The sample was de-protected again with a 30% piperidine solution, 1-2 ml, and allowed to stir for 15 minutes. The Kaiser test again gave a negative result and Sample B was discarded.

One hundred milligrams of beads (1 equiv), Sample C, was swelled in DMF for 30 minutes. H MBA (3 equiv) and HOBt was dissolved in DMF in a separate vial and then added to the beads. DIC (6 equiv) was then added and the vessel was placed in the microwave for 10 minutes at 75° C with the stir rate set to medium and power at 10W. The beads were then washed with DMF and DCM 5 times each. The Kaiser test gave a negative result, as expected. Ten percent DMAP in DMF (3 equiv), Fmoc-Gly-OH (5 equiv), DIC (6 equiv) and DMF were mixed in a separate vial and then added to the resin. It was then placed in the microwave for 10 minutes at 75° C. The beads were washed five times with DMF and then de-protected using a 30% piperidine solution in DMF, 1-2 ml. After two minutes of stirring, a Kaiser test was performed and a positive result was obtained as expected. Fmoc-Gly-OH (5 equiv), HOBt (3 equiv), DIC (6 equiv) and NMP were mixed in a separate vial and then added to the beads. The sample was then placed in the microwave for 10 minutes at 75° C (SPS mode, max power 10W, ΔT=3° C). The Kaiser test gave a negative result. Thirty percent piperidine solution in DMF, 1-2 ml, was added to the beads followed by stirring for two minutes.
The Kaiser test gave a positive result indicating the peptide chain now contains two glycines. The third and fourth glycine units were added to the chain using the same procedure for the second one.

Through many trials a method was obtained and used in peptide synthesis from this point on. This method consisted of allowing the resin beads to swell in DMF for 30 minutes and then washing with 3 resin bead volumes of DMF prior to use. The resin beads (1 equiv, 0.29 mmol NH₂) were added to a 25 mL microwave reaction vessel (CEM Corporation) and swollen as described above. H MBA (3 equiv) and HOBT (6 equiv) were dissolved in DMF and then transferred to the vessel containing the beads. The vessel was heated to 75°C for 10 minutes (SPS mode, max power 10W,ΔT=3°C) using microwave irradiation. A small sample of resin beads, 2-5 mg, was removed, washed with DMF then DCM three times each and then subjected to the Kaiser test to confirm that the reaction had gone to completion. The resin was washed with DMF (10 x 2 min each) and then stirred with aqueous sodium hydroxide (1M NaOH:DMF, 1:1 v/v) for 15 min. The resin was washed with DMF/H₂O (1:1 v/v, 5 x 2 min each) followed by DMF (10 x 2 min each).

Fmoc-Gly-OH (3 equiv) was dissolved in DMF, which was then added to HMBA beads. DIC (4 equiv) was added followed by the drop-wise addition of DMAP dissolved in DMF (0.1 equiv, 0.05 M stock solution). The reaction vessel was heated to 75°C for 10 minutes (SPS mode, max power 10WΔT=3°C) using microwave irradiation. The resin was washed with DMF (2 x 2 m in each). Glycine coupling was repeated using identical amounts of each reagent to ensure complete coupling of the first amino acid. After the second reaction, the resin was washed with DMF (5 x 2 m in each) and any
remaining hydroxyl groups were acetylated by the addition of DMF, Ac₂O (6 equiv), and DMAP (0.1 equiv, 0.05 M stock solution). After 1 h, the resin was washed with DMF (10 x 2 min each).

Fmoc deprotection was achieved by the addition of 30% piperidine in DMF (v/v) followed by gentle agitation for two minutes. The solution was removed and the deprotection steps were repeated two more times. The resin was washed with DMF (5 x 2 min each). A small sample of resin beads (2-5 mg) was removed, and then subjected to the Kaiser test to confirm deprotection. Fmoc-amino acid-OH (5 equiv), HOBt (5 equiv), and DIC (5 equiv) were dissolved in DMF and stirred for 10-15 min. This solution was added to the resin and the reaction vessel was heated to 75°C for 10 minutes using microwave irradiation. A small sample of resin beads (2-5 mg) was removed, washed with DMF and DCM three times and then subjected to the Kaiser test to confirm coupling. The resin was washed with DMF (10 x 2 min each). This procedure was repeated for each additional amino acid.

Side chain de-protection was conducted as follows. All the amino acids used except glycine have a side chain protection group. In order to use and analyze the peptides the protecting groups need to be removed. The first step in this process is to remove the Fmoc protecting group from the last amino acid on the chain. This was achieved by allowing the sample to stir for two minutes with 30% piperidine solution in DMF. The solution was removed and the deprotection steps were repeated two more times. The sample was then washed several times with DCM. A cleavage cocktail consisting of trifluoroacetic acid (TFA), triisopropylsilane (TIS), and water (95:2.5:2.5) was used for the side chain deprotection. The solution was added to the beads and
allowed to stir for 90 minutes. The sample was then washed with TFA, DCM, and ether (3x 2min each).

One Bead One Compound Library Synthesis

A bulk sample of H-GGGG-HMBA-TentaGel (750 mg) was prepared using the procedures outlined above. The resin was then divided into six aliquots (125 mg each) and one of six different amino acids ($X_1 = G, S, H, R, Y, or W$) coupled to each aliquot using the coupling procedures outlined above. The six aliquots were mixed after rinsing, dried under vacuum for 1 h, and then weighed (533 mg, 71% recovery). The beads were again split into six aliquots (89 mg each) and one of six different amino acids ($X_2 = G, S, H, R, Y, or W$) coupled to each aliquot. During rinsing, one of the samples was lost due to a malfunctioning frit. The remaining five aliquots containing $X_2 = G, S, H, R, or W$ were pooled after rinsing, dried under vacuum and then weighed (383 mg, 89% recovery). For $X_3$, the resin was split into five aliquots (76 mg each) instead of six, due to the aforementioned material loss, and each coupled to a different amino acid, $X_3 = G, S, H, R, or W$. The five aliquots were pooled after rinsing, dried under vacuum and then weighed (322 mg, 84% recovery). For $X_4$, the resin was again split into five aliquots (64 mg each), and each coupled to a different amino acid, $X_4 = G, S, H, R, or W$. The five aliquots were pooled after rinsing, dried under vacuum and then weighed (295 mg, 92% recovery). After accounting for the loss of one of the samples, the resulting library consisted of 750 unique peptides of the general formula $H - X_4X_3X_2X_1GGGG-HMBA-TentaGel$, $X_1 = G, S, H, R, Y, or W$; and $X_2 = X_3 = X_4 = G, S, H, R, or W$ and a total yield of 39%.

A second library was synthesized in the same fashion as mentioned above.
starting with 3 g of resin beads. The sample was split into seven aliquots (485 mg) and one of the seven amino acids \((X_1 = G, R, S, W, H, Y, \text{ or } K)\) was coupled to each sample. All samples were mixed (2.5 g) and split into seven aliquots (350 mg). The same amino acids were added to each of the seven samples. This process was repeated until five random amino acids were added to the chain giving a total of nine amino acids in the peptide. The general formula of the peptides is \(H-X_5X_4X_3X_2X_1GGG-HMBA-\text{TentaGel}\). This gives 16,807 different peptide combinations.

**Gas-Phase Cleavage**

Peptide was released from the resin (after deprotecting the final amino acid and side chains as outlined above) by exposure to ammonia/tetrahydrofuran (THF) vapor on a Schlenk line. Briefly, one manifold of the Schlenk line (app. 335 mL volume) was purged with ammonia gas. The gas contained in the manifold was condensed into a round bottom flask containing degassed THF (30 mL) cooled in liquid \(\text{N}_2\). Separately, a flask containing resin beads was evacuated. The two flasks were isolated from the rest of the Schlenk line, followed by opening stopcocks between the two flasks. The ammonia/THF solution was then allowed to warm to room temperature and the apparatus was left undisturbed for 20 hours. After the reaction, ammonia gas was condensed back into the flask containing THF by cooling in liquid \(\text{N}_2\), and the flask containing resin beads was carefully removed from the Schlenk line.

Peptide was also released from the resin (after deprotecting the final amino acid and side chains as outlined above) by exposure to high-pressure \(\text{NH}_3\) gas in a general-purpose pressure vessel (Parr Instrument Company). The sample to be cleaved was transferred to a 35 mm glass bottom dish that was then placed inside the pressure
vessel. The top was secured and the vessel was pressurized with NH₃ gas (anhydrous ammonia, 99.99%) and then left undisturbed for the desired reaction time. Several different times at a pressure of 100 psi were tested and compared to cleavage on the Schlenk line. At the end of the reaction, the vessel was vented, the top disengaged, and the sample removed.

Sequencing the Peptide

Bulk samples and single beads can be analyzed and sequenced with a DECA Ion Trap MS. For bulk samples, peptide-coated beads that have been cleaved were placed in a 1 mL solution consisting of 50:50 methanol/water and 1% acetic acid. The sample was vortexed and the beads were left to settle to the bottom of the eppendorf tube. The solution was extracted and then injected into the electrospray ionization source. The parent peak was identified and tandem MS was performed to identify each amino acid in the peptide chain.

Single beads were isolated on a glass bottom dish and located with a TE 2000 inverted microscope (Nikon). Following identification of a single bead, the L200 nanomanipulator (Zyvex) was utilized to land a 1 μm nanoelectrospray tip (New Objective) in close proximity to the bead. The solvent was injected onto the bead with a PE2000b four-channel pressure injector (MicroData Instruments). The solvent was allowed to extract on the bead for approximately 30 seconds before being filled back into the tip. Once the peptide-containing solution was retrieved, the tip was transferred to the nanoel ectrospray ionization source (Proxeon Biosystems) for analysis. These single bead extractions were completed with an injection pressure of 30 psi and a fill pressure of 40 psi.
During the analysis of a peptide, the parent peak is identified after about one to two minutes into the run time. This peak is the most dominant in the scan and is often the singly protonated form of the peptide. The doubly protonated peak is also identified and the tandem mass spectra is conducted on this species to provide more charged fragments that can be detected. The analysis is allowed to run for an additional two or so minutes or until the doubly protonated peak is gone. From the data, the b and y ions are identified. From these ions each individual amino acid and its place in the peptide chain can be determined. [30] This method can be used for a bulk sample or an individual bead.

Screening the Peptide Library for Ligands that Bind Transferrin

Initial attempts to screen for ligands that bind to transferrin (Tf) were performed by using both Tf-AlexaFluor 647 (red fluorescent marker) and Tf-AlexaFluor 488 (green fluorescent marker) in different combinations. A small sample of beads from the library, 25 mg, 8.87x10^5 beads/gram with a 280-330 pmol capacity/bead, was first swelled in DCM for 30 minutes. It was then washed with DCM, DMF, DMF/H_2O (7:3, 5:5, 3:7) and H_2O (5 x 1 minute each). A blocking buffer was prepared which consisted of 10% skim milk, 2% chicken egg albumin, and 0.05% Tween 80 in phosphate buffered saline (PBS). Blocking solution (0.5 mL) was added to the sample and left to incubate for 30 minutes. The beads were then washed 5 x 1 minute with 0.05% Tween 80 in PBS (PBS-Tween). A stock solution of the Tf marker was made by adding 1.2 mg of the protein to 1 ml ultra pure water. From this an 83 nM concentration of the Tf marker in blocker solution was made by adding 2.8 µL of the desired Tf marker stock solution to 500 µL of blocker solution. This was added to the sample and allowed to incubate
overnight in the refrigerator. The beads were then washed with PBS-Tween (10 x 1 min), PBS (5 x 1 min), and water (3 x 1 min). The beads were taken up into an aqueous solution (0.5 mL), transferred to a 6-well cell culture plate, the excess water removed by pipette, and the beads were left to dry.

This process was performed on five different 25 mg samples from the peptide library. Each sample was incubated overnight in the refrigerator as follows: Sample D – red fluorescent marker in the blocker solution; Sample E – red fluorescent marker in PBS without the blocker solution; Sample F – red and green fluorescent marker in the blocker solution; Sample G – green fluorescent marker in the blocker solution; Sample H – no marker, only the blocker solution.

Hit beads were identified by manual screening of the sample for increased fluorescence. It was noted that the beads have an auto-fluorescence. Because of this, using both the red and green marker together would not give a true positive hit. To solve this problem only the red fluorescent marker was used for all future screenings. Using this method, hit beads were identified by manually looking for an increased fluorescence emission in the far red (ex. 650 nm, em. 665 nm) with no similar increase in the green channel (ex. 495, em. 519). Observing enhanced fluorescence only in the far red makes it less likely that the increased fluorescence is a result of the inherent auto-fluorescence of the beads themselves.

Positive hits were removed manually with a syringe needle and placed into a different well on the 6-well cell culture plate. To denature the transferrin 2 µL of 8 M guanidine hydrochloride was added to each bead. The stock solution was made by adding 3.8 g guanidine hydrochloride to 5 ml DI water. The denaturing solution was left
on the bead for 20 minutes and then extracted with a pipette. 3 µL of water was then added to each bead and extracted after 1-2 minutes of standing. This step was repeated once more and the beads were left to dry. They were then transferred to a glass bottom dish using a syringe needle. The dish with the individual beads separated out was then placed in the pressure-vessel to cleave the peptide from the bead. The cleavage process and sequencing of the individual beads was performed as mentioned above.

Synthesis and Comparison of Positive and Negative Hit Beads

From the positive hits identified one was chosen for further analysis. This bead sequence was WKRVRGGGG. This peptide was synthesized using the same procedure outlined above. The Kaiser tests showed that the HMBA linker and each amino acid were added successfully. A negative bead with the sequence HWHWHGGGG was also synthesized in the same fashion.

A 5 mg bead sample from both the positive and negative hits was washed with DCM, DMF, DMF/H₂O (7:3, 5:5, 3:7) and H₂O (3 x 1 minute each). A blocking buffer was made which consisted of 10% skim milk, 2% chicken egg albumin, and 0.05% Tween 80 in phosphate buffered saline (PBS). Blocking solution (0.2 mL) was added to the sample and left to incubate for 30 minutes. The beads were then washed 3 x 1 minute with 0.05% Tween 80 in PBS (PBS-Tween). A 500 nM solution containing the Tf-AlexaFluor 647 was made by adding 6.7 µL of the stock solution to 0.2 ml blocker solution. This was then added to each sample and left to incubate overnight at 4°C. The beads were then washed with PBS-Tween (5 x 1 min), PBS (3 x 1 min), and water (3 x 1 min). The beads were taken up into an aqueous solution (0.5 mL), transferred to
a 6-well cell culture plate, the excess water removed by pipette, and the beads were left to dry. Both samples were placed in the same well to compare the fluorescence.

Peptide Synthesis on Smaller Beads

The original synthesis of peptides mentioned above was performed using 130 µm beads. There are many benefits to miniaturizing the library as outlined above. The first step in the synthesis of the miniature library was to add a four-glycine peptide chain to 20 µm and 30 µm resin beads. The same procedure described in the section “Peptide Synthesis” was used for the smaller beads. The same equivalents were used and the starting amount was 100 mg for each. The major difference in the procedure used here compared to above was that since the beads are much smaller a different reaction vessel had to be used. The reaction vessel that had been used up to this point had a frit with 30 µm pore size, which would not retain these smaller beads. The manufacturer was contacted in an attempt to have reaction vessels made with smaller pore size frits; however, they were unable to accommodate. The new reaction was a 2 ml centrifugal filter device, Ultrafree-CL, from Millipore Corporation. These vessels could not be placed alone into the microwave and the reagents would leak out during the reaction time. To solve this problem parafilm was used to seal the bottom of the vessel. It was then placed in the original microwave vessel. The Kaiser tests were as expected for the addition of the HMBA linker and four glycines to both the 30 µm and 20 µm beads.

The analysis of these peptides was attempted using the same procedure employed for the bigger beads. Data was obtained for a bulk sample of the 30 µm beads but analyzing a single bead is still in the process. Both bulk and single bead analysis is also still under investigation for the 20 µm beads.
Synthesis of a Peptide Library on 20 µm Beads

Five hundred milligrams of 20 µm beads were used to synthesize the peptide library. It was initially split into two 250 mg samples because this was the maximum amount of sample that could be conveniently manipulated in the smaller reaction vessels. The same methods described above were used in the synthesis of the miniature library. The linker, HMBA, was successfully added to both 250 mg samples as verified by a negative Kaiser test. The next four glycines were also successfully added to both samples with coupling verified via the Kaiser test. Both samples were dried under vacuum for 15 minutes, mixed together, and weighed. The total weight came to 313 mg. This was then split into six aliquots with approximately 45 mg of beads in each. One of six different amino acids ($X_1 = S, H, R, Y, K, $ or $W$) was coupled to each aliquot using the coupling procedures outlined above. The Kaiser test for all six was negative indicating the successful addition of the fifth amino acid. All six samples were mixed and deprotected with 30% piperidine in DMF, and gave positive Kaiser test. The sample was dried under vacuum and weighed (440.6 mg). It was then split again into six aliquots weighing approximately 65 mg each. One of six different amino acids ($X_2 = S, H, R, Y, K, $ or $W$) was coupled to each aliquot as before giving a negative Kaiser test for each indicating the successful addition of the sixth amino acid. The samples were mixed and deprotected giving a positive Kaiser test. The sample was dried under vacuum and had a total weight of 284 mg. The beads were split into six aliquots weighing approximately 42 mg each.

The seventh amino acid, ($X_3 = S, H, R, Y, K, $ or $W$), on the peptide chain was added successfully to all six samples according to the negative Kaiser tests. The
samples were mixed and deprotected giving a positive Kaiser test. The beads were dried under vacuum and weighed 271.2 mg. The sample was split into six aliquots weighing about 40 mg each. The eighth amino acid, \( X_4 = S, H, R, Y, K, \) or \( W \), was added and each sample gave a negative Kaiser test. All six samples were mixed and deprotected giving a positive Kaiser test. The beads were dried under vacuum and weighed 184 mg. The beads were split into another six aliquots weighing about 30 mg each. The ninth and last amino acid, \( X_5 = S, H, R, Y, K, \) or \( W \), was added giving a negative Kaiser test for each of the six. The samples were mixed and dried under vacuum. The final weight came to 195.4 mg giving a 39% yield. The general formula of the peptides is \( H-X_5X_4X_3X_2X_1GGGG-HMBA-TentaGel \) where \( X = R, S, W, H, Y, \) or \( K \). This gives 7,776 different peptide combinations in the library.
CHAPTER III
RESULTS AND DISCUSSION

Introduction

Various biological screenings can be utilized using a combinatorial library. A library of the general formula \( H-X_6X_5X_4X_3X_2X_1-GGGG-HMBA-TentaGel \), where \( X = \) randomized amino acid is ideal for this project. Four to six randomized amino acids were chosen because these lengths have been reported to be sufficient for the discovery of high-affinity ligands to a variety of proteins.[2] Furthermore, the addition of four glycine units before each chain was used to give some flexibility to the binding portion of the peptide to allow for productive interactions with the target of interest; an approach that has also been reported by others.[3] An HMBA resin linker was selected because the cleavage reaction can be achieved under solvent-free conditions via exposure to \( NH_3 \). Most other cleavage strategies require long reaction times or have other significant detractors such as the requirement of a multistep synthesis to prepare the linker, or extensive processing steps pre- or post-cleavage. The use of HMBA has been reported in the literature;[3; 4; 5; 6; 7; 8; 9] however, the experimental procedures that have been employed to date involving \( NH_3 \) are unnecessarily long and cumbersome as will be highlighted below. The goal was to demonstrate the ability to (1) synthesize an O BOC peptide library using microwave-assisted, solid-phase peptide synthesis, (2) rapidly and efficiently
cleave the peptide from the solid support, (3) sequence the peptide from individual members of the library with the aid of mass spectrometry, (4) identify a ligand to target transferrin, (5) measure the avidity of positive hits ligands, (6) generalize peptide synthesis for smaller supports such as 30 µm and 20 µm resin beads, (7) synthesize a miniaturized version of the library on 20 µm beads, and (8) sequence peptide on the 30 µm and 20 µm individual beads.

Peptide Synthesis

HMBA was coupled to TentaGel S NH₂ resin using a slightly modified version of published procedures described by Mellor, et al. [7] The procedure was modified to reduce reaction time by conducting the coupling reaction at 75°C for 10 minutes under microwave heating. This is the first report of microwave-assisted HMBA coupling, which reduced reaction time from 2-24 hours to 10 minutes. Amino acid coupling was conducted using an Fmoc protection scheme and either 4-(N,N-dimethylamino)pyridine (DMAP)/1,3-diisopropylcarbodiimide (DIC) or 1-hydroxybenzotriazole (HOBT)/DIC in DMF for activation of the C-terminal or all other amino acids, respectively. Coupling of the C-terminal amino acid was repeated once to ensure that the reaction went to completion. Coupling reactions for both the C-terminal and all remaining amino acids were conducted at 75°C for 10 minutes under microwave heating to reduce reaction times.[10] A Kaiser test was also performed after the linker coupling, each amino acid coupling, and deprotection to ensure the reaction took place. The Kaiser test is used to trace the presence of free primary amines. After each coupling, no primary amines should be present which gives the solution a yellow color, a negative result. After removal of the Fmoc protecting group, a free primary amine is present which gives a
One Bead One Compound Combinatorial Library

The first OBOC library was synthesized using the split-mix method. A bulk sample of H-GGGG-HMBA-TentaGel was prepared and then divided into six equal aliquots. A different amino acid ($X_1 = G, S, H, R, Y, \text{ or } W$) was coupled to each aliquot, and after repeated washings the samples were combined. The beads were again split into six aliquots; however, during coupling of the second randomized amino acid, ($X_2$), a sample was lost due to equipment malfunction. The remaining five aliquots containing $X_2 = G, S, H, R, \text{ or } W$ were pooled. For $X_3$, the resin was split into five aliquots instead of six, due to the aforementioned material loss, and each was coupled to a different amino acid, $X_3 = G, S, H, R, \text{ or } W$. The split mix procedure was repeated one final time where $X_4 = G, S, H, R, \text{ or } W$. After accounting for the loss of one of the samples, the resulting library consisted of 750 unique peptides of the general formula H-$X_4X_3X_2X_1$GGGG-HMBA-TentaGel, $X_1 = G, S, H, R, Y, \text{ or } W$; and $X_2 = X_3 = X_4 = G, S, H, R, \text{ or } W$. Although the microwave-assisted synthesis of $\beta$-peptide and peptoid OBOC libraries have been reported, this is the first account of the microwave-assisted synthesis of an OBOC peptide library composed entirely of standard amino acids.

The second OBOC peptide library was synthesized using the same method as mentioned above. To expand the number of possible peptide combinations, the peptide chain was lengthened and two more random amino acids were used. The random amino acids chosen were G, R, S, W, H, Y, and K. These were chosen because of their high rate of occurrence in other peptide-based targeting ligands. The general
formula of the peptide library is H-X₅X₄X₃X₂X₁GGGG-HMBA-TentaGel, X₁-₅ = G, R, S, W, H, Y, and K. This gives a total of 16,807 possible peptide combinations.

High Pressure Ammonia Gas Cleavage

Exposure of a resin-bound, HMBA-coupled peptide to NH₃ leads to cleavage of the ester bond anchoring the peptide to the resin bead and formation of the corresponding peptide amide. The mechanism for this reaction is shown in Figure 1. The combination of HMBA/NH₃ as a cleavage strategy benefits tremendously from its conceptual simplicity. Because it can be performed under solvent-free conditions, it is inherently green, which means less waste generated, and no energy is needed for product separation. Reaction work-up is accomplished in one easy step by just venting excess NH₃. Another benefit is that the peptide is left in place on the resin bead for future retrieval and analysis without any unnecessary dilution that could jeopardize detection and/or sequencing (no attempt was made to determine if peptide was transferred to the walls of the Parr reactor; however, in all cases sufficient peptide remained on the bead to facilitate MS analysis).

Figure 1 – Mechanism of ammonia gas cleavage of the peptide.
While the technique is conceptually very simple, the initial cleavage experiments conducted used a complicated procedure whereby a Schlenk line was used to manipulate NH$_3$ gas. NH$_3$ gas was first condensed into a flask containing degassed THF cooled in liquid N$_2$. Beads containing peptide to be cleaved were then placed in another vessel and put onto a separate port on the Schlenk line. The flask containing beads was evacuated followed by exposure to the THF/NH$_3$ solution overnight. All reports detailing the use of HMBA/NH$_3$ involve similar steps that use dry ice or liquid N$_2$ to manipulate NH$_3$. We hypothesized that the same results could be obtained if the beads were exposed to NH$_3$ gas in a high-pressure reactor. While such a reactor has been described,[7] NH$_3$ was condensed into the reactor in much the same manner as described above, and that the reaction was run overnight. Several tests were done to observe whether or not peptide cleavage could be accomplished in shorter reaction times by simply putting the beads inside an appropriate reactor and then pressurizing with NH$_3$ gas.

In order to examine the effect of time on the cleavage of HMBA-coupled peptides from TentaGel resin, the cleavage of two peptides was investigated: H-RHYWSGSG-HMBA-TentaGel (1a) and H-PWSG-HMBA-TentaGel (2a). Peptide precursor 1a was chosen because it represents the target length of constituents in the peptide library and 2a was to be used as an internal standard for quantifying the relative amounts of cleaved peptide, 1b, obtained under a variety of cleavage conditions. Samples to be cleaved were placed inside a general purpose pressure vessel and, without purge or evacuation, the vessel was brought to 117 psi using NH$_3$ gas. A series of 10 mg bulk bead samples were analyzed as a function of time Table 2.
Table 2 - Cleavage of H−RHYWSGSG−HMBA−TentaGel as a function of time using high pressure NH₃ gas

<table>
<thead>
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<th>time (min)</th>
<th>normalized peak area</th>
<th>Percent completion</th>
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<tr>
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<td>2.4</td>
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<td>1440</td>
<td>433.8</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>Schlenk line</td>
<td></td>
</tr>
<tr>
<td>1440</td>
<td>358.9</td>
<td>82.7</td>
</tr>
</tbody>
</table>

^a peak areas were normalized using an internal standard, ^b cleavage under high pressure for 24 h was designated as 100% completion.

There was a slight dependence of the amount of cleaved peptide on incubation time; however, the reaction was >70% complete in just 5 minutes and was almost 90% complete within 30 minutes (the data was normalized to 24 h of incubation at 117 psi). The amount of 1b obtained after 24 hours using the complicated Schlenk line method was only approximately 13% higher than that obtained in 5 minutes using the new, much simpler, high-pressure technique. Furthermore, the maximum amount of 1b obtained via this new technique was >20% higher than that obtained using the Schlenk line method. No significant decomposition was observed in samples cleaved at high NH₃ pressure. Cleavage and MS analysis of peptides from 1-8 amino acids in length were carried out to demonstrate the technique’s broad applicability, and though only one other example has been studied thus far, this technique is not limited to having glycine as the anchoring residue. This technique has also been expanded to single...
bead analysis, and unless otherwise noted, the sequencing data reported below was collected from bulk or single bead samples cleaved at 117 psi for 30 minutes.

Peptide Sequencing

A schematic representation of the nanomanipulation device is shown in Figure 2. The base, which has four stage controller units, sits atop an inverted microscope. Each stage controller can be fitted with one of the following: a nanoelectrospray tip, microgrippers, a tungsten end effector probe, or a microcapillary. Resolution of movement is roughly 3.4 nm in the x,y-plane and 100 nm in the z-direction. A single stage controller unit fitted with a nanoelectrospray tip was used in each of the studies outlined below.

![Figure 2 – Schematic of nanomanipulator.](image)

The spacer peptide (H-GGGG-NH2, 3b) was first synthesized to test the ability to extract peptide from individual resin beads. A bulk sample of 3b was collected from beads, post ammonia cleavage, by elution into a 5 0:50 MeOH:H2O 1% acetic acid solution and then analyzed by electrospray ionization mass spectrometry (ESI MS). Figure 3. Analysis of the MS data confirmed the presence of 3b in high abundance, but also indicated the presence of a small amount of a five-glycine peptide. From this data
it is believed that double coupling most likely occurred during the addition of the first amino acid where the hypernucleophile 4-\((N,N\text{-dimethylamino})\text{ pyridine (DMAP)}\) was used to catalyze ester bond formation. DMAP could also act as a base resulting in some fmoc deprotection in addition to its role as catalyst. DMAP's role in double coupling has been an area of concern,[6; 9] and is magnified here because the reaction conditions include microwave irradiation.
Figure 3 – A) ESI MS of 3b. B) NSI MS of 3b. [1]
Next, 3b was collected from individual resin beads by positioning the probe tip of the nanomanipulator within a few microns of the bead, dispensing a 50:50 MeOH:H₂O 1% acetic acid solution onto the bead, waiting 30 seconds for elution of 3b from the bead, and then retrieving the solution back into the probe tip, Figure 4. The probe tip was transferred directly to the mass spectrometer and the solution analyzed. Data obtained for 3b from the single bead extraction was virtually identical to that of the bulk sample.

![Figure 4](image)

Figure 4 – Injection of solvent onto a single bead (A). Extraction of solvent and peptide from bead (B).

Next, to investigate the possible origin of the double coupling product and to demonstrate the ability to sequence peptide on individual resin beads, H-PWSG-NH₂ (2b) was synthesized. Four different amino acids were chosen to allow unambiguous identification of any products due to double coupling reactions while keeping the C-terminal amino acid constant.

Again, a sample of 2b was analyzed by dissolving the cleaved peptide in a solution of 50:50 MeOH:H₂O 1% acetic acid. Samples of 2b were analyzed by both
conventional ESI MS (bulk peptide) and by NSI MS (individual beads). Both the protonated and sodiated ions of 2b were observed along with a small amount of a peptide resulting from double coupling during the addition of glycine. Peptides resulting from other possible double coupling reactions were not observed supporting the assertion that double coupling was likely due to molecule cleavage by DMAP during coupling of the first amino acid. Finally, MS/MS analysis of 2b was conducted on peptide taken from bulk samples and ones collected from individual beads via nanomanipulation, Figure 5. The MS/MS data of both samples of 2b were virtually identical with the expected fragmentation pattern being clearly observed. From the fragmentation data, a-, b- and y-ions were identifiable allowing the sequence of 2b on individual beads to be readily elucidated via standard methods of MS/MS analysis. Nanomanipulation/NSI MS analysis of 2b was repeated a total of eight times to ensure method reproducibility and each time the virtual identical MS/MS data was obtained.
Figure 5 - A) ESI MS/MS spectrum of 2b. H-PWSG-NH$_2$ + H+: 445.33 m/z, H$_2$O: 427.27 m/z, b3: 371.27 m/z, y 3: 348.27 m/z, y3*: 330.20 m/z, b2: 284.20 m/z, a2: 256.20 m/z; B) N SI MS/MS spectrum of 2b. H-PWSG-NH$_2$ + H+: 445.33 m/z, -H2O: 427.33 m/z, b3: 371.27 m/z, y 3: 348.27 m/z, y3*: 330.20 m/z, b2: 284.20 m/z, a2: 256.27 m/z. [1]
Hit screenings based on MALDI MS often necessitate the addition of extra non-random amino acid residues to library constituents to increase peptide molecular weight so that overlap of peptide-fragment peaks with those of the matrix material can be avoided. Therefore H-Y-NH₂, H-HY-NH₂, and H-WHY-NH₂ were synthesized and bulk samples were analyzed by ESI MS to establish if there were any restrictions on the minimum number of amino acid residues required for these analytical techniques. In all three instances, the expected peptide was present at high abundance Figure 6.
Figure 6 – ESI MS of H-Y-NH$_2$, H-HY-NH$_2$, and H-WHY-NH$_2$
Individual beads from the 750 member OBOC peptide library were analyzed to ascertain whether or not peptides 8-amino acids in length could be sequenced and what percentage of selected beads could be unambiguously sequenced from a given pool. Thirteen beads were chosen at random, the peptide cleaved, and analyzed as described above. The peptide sequences for all 13 beads analyzed were determined and listed in Table 3. The MS data is shown in Figures 7-8.

Table 3 - Sequences of 13 randomly selected beads from first OBOC peptide library

<table>
<thead>
<tr>
<th>Bead no.</th>
<th>Peptide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H–GGGSGGGG–NH₂</td>
</tr>
<tr>
<td>2</td>
<td>H-WGWRGGGG–NH₂</td>
</tr>
<tr>
<td>3</td>
<td>H-WRGHGGGG–NH₂</td>
</tr>
<tr>
<td>4</td>
<td>H-SGHYGGGG–NH₂</td>
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<td>5</td>
<td>H-WGSSGGG–NH₂</td>
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<td>6</td>
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<td>7</td>
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<td>8</td>
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<td>10</td>
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<td>12</td>
<td>H-GHWHGGGG–NH₂</td>
</tr>
<tr>
<td>13</td>
<td>H-SGWRGGGG–NH₂</td>
</tr>
</tbody>
</table>
Figure 7 - A) N SI MS spectrum of H-GWG-WGGGG-NH₂, bead number 11. H - GWGWGGGG-NH₂ + H⁺: 732.25 m/z; B) N SI MS/MS spectrum of H-GWGWGGGG-NH₂. H-GWGWGGGG-NH₂ + H⁺: 732.25 m/z, b7: 658.07 m/z, b6: 601.07 m/z, b5: 544.00 m/z, y6: 489.07 m/z, b4: 487.00 m/z, y5: 432.07 m/z, b3: 301.00 m/z, y4: 246.01 m/z, b2: 243.93 m/z. [1]
Figure 8  -  A) NI SI MS spectrum of H-SGWRGGGG-NH$_2$, bead number 13. H-SGWRGGGG-NH$_2$ + H$: 732.33$ m/z, H-SGWRGGGG-NH$_2$ + 2H$: 366.83$ m/z; B) NSI MS/MS spectrum of H-SGWRGGGG-NH$_2$. H-SGWRGGGG-NH$_2$ + H$: 732.33$ m/z, b7: 658.04 m/z, y7: 645.2 m/z, b6: 601.02 m/z, y6: 588.11 m/z, b5: 544.07 m/z, b4: 487.06 m/z, y5: 402.04 m/z, H-SGWRGGGG-NH$_2$ + 2H$: 366.83$ m/z, b3: 331.14 m/z, y4: 245.90 m/z, b2: 144.84 m/z, y2: 131.87 m/z. [1]
Library Screening

The first OBOC peptide library synthesized was screened to find a ligand to bind to transferrin. Several different combinations of the Tf-AlexaFluor 647 (red fluorescent marker) and Tf-AlexaFluor 488 (green fluorescent marker) were initially used in the screening process. These combinations are as followed: Sample D - red fluorescent marker in the blocker solution; Sample E – red fluorescent marker in PBS without the blocker solution; Sample F – red and green fluorescent marker in the blocker solution; Sample G – green fluorescent marker in the blocker solution; Sample H – no marker, only the blocker solution.

During the screening of Sample D beads it was found that some beads had a more intense fluorescence than others. Some beads from Sample E also fluoresced but more intensely than those in Sample D, which contained the blocker solution. Without the blocker solution, nonspecific binding to transferrin can take place complicating data analysis. Sample F, which contained both the red and green fluorescent marker, was selected to test whether the ligands were binding to transferrin or to the fluorescent marker. It was found that both markers were bound to several beads while some beads only had one marker bound. Sample G was more difficult to screen because it was found that the beads have their highest levels of autofluorescence in the green channel. This would not be used as the marker in future experiments because of the difficulty in differentiating hit beads from ones with high levels of autofluorescence. This point was confirmed with Sample H, which did not contain either marker. The beads showed a natural uniform green fluorescence. From these trials all future screening processes will only use the red fluorescent marker in the
The first screening, Screening 1, of the second peptide library, which contains 16,807 members, was performed using two 25 mg samples. One sample contained the red fluorescent marker and the other no marker, the negative control. The samples were prepared as described in the experimental section using an 83 nM concentration of the red Tf marker. Four beads were found that fluoresced brightly in the red channel and not in the green. These were picked out and separated. The control sample showed beads that fluoresced brightly in the red channel however they also fluoresced in the green channel indicating the natural fluorescence of the beads, Figure 9 (A and B).

The four positive hit beads, one being broken, were treated with 8 M guanidine HCl to denature the transferrin. These hits were washed with DCM but were lost in the cell culture plate due to the incompatibility of the plate materials with DCM. The same sample was screened again and five hits were obtained. These were denatured as mentioned above but it was noticed that a thick white precipitate formed over the beads from a wash with DMF. Because of this only two of the hits, Figure 9, could be cleaved and analyzed. The peptide sequence for positive hits C and D could not be determined.
Figure 9 – Images A 1, A 2, B 1 and B 2 are beads from the control sample. Arrow is pointing to positive hit bead from Screening 1.

Another screening, Screening 2, was performed using one 25 mg sample of beads. Eleven hits were identified and retrieved, but only six were denatured and cleaved. Of these six, one was lost during analysis and the remaining five were analyzed by MS. Figure 10 shows the images of both Hit 1 and Hit 2 from the screening. The mass spectrum for both Hit 1 and Hit 2 are in Figure 16 and 17, respectively.
Figure 10 – Arrow is pointing to positive hit beads from Screening 2. Peptide sequence is shown.

Screening 3 involved two samples from 16,807-member bead library. Thirteen positive hits were identified, retrieved (Figure 11), denatured and cleaved. When looking at the images before the analysis process, one hit was not analyzed because it did not fluoresce as brightly as desired. All twelve beads were analyzed but only five positive beads were able to be sequenced. The mass spectrum data are shown below for each hit 3, 4, 5, 6, and 7 in Figure 18, 19, 20, 21, and 22, respectively.
Twelve positive hits were identified and retrieved from two samples during Screening 4. MS data could only be obtained on three of these hits, Figure 7. The mass spectrum data for these three hits, Hit 8, Hit 9, and Hit 10, are shown in Figure 23, 24, and 25. The others were either lost during the washing process or analysis or did not fluoresce as brightly when the images were reviewed.
The last screening, Screening 5, was performed to obtain negative beads to be used in comparison with positive ones. The beads that fluoresced in both the red and green channel were identified as negative beads. Seven beads were identified and retrieved from this screening but tandem MS data was attained for only three of the beads, Figure 13. The mass spectrum data is shown in Figure 26, 27, and 28.

Figure 13 - Arrow is pointing to the negative bead from Screening 5. Peptide sequence is shown.
The nano manipulator was used to extract the peptide from each of the beads mentioned above. The peptide was analyzed by use of the nanospray. Table 4 lists the sequences for all ten positive hit beads and three negative beads.

<table>
<thead>
<tr>
<th>Bead</th>
<th>Peptide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hit 1</td>
<td>H–WKRYRGGGG-NH₂</td>
</tr>
<tr>
<td>Hit 2</td>
<td>H-WYHYRGGGG-NH₂</td>
</tr>
<tr>
<td>Hit 3</td>
<td>H-WKWYSGGGG-NH₂</td>
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<td>Hit 4</td>
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Figure 16 - A) NSI MS spectrum of H-WKRYRGGGG-NH₂, Hit 1. H-WKRYRGGGG-NH₂ + H⁺: 1070.60 m/z, H-WKRYRGGGG-NH₂ + 2H⁺: 535.98 m/z; B) NSI MS/MS spectrum of H-WKRYRGGGG-NH₂, b₂: 350.04 m/z, b₃: 506.25 m/z, y₆: 565.26 m/z.
Figure 17 - A) NSI MS spectrum of H-WHYRYRGGG-NH₂, Hit 2. H-WHYRYRGGG-NH₂ + H⁺: 1051.87 m/z, H-WHYRYRGGG-NH₂ + 2H⁺: 527.07 m/z; B) NSI MS/MS spectrum of H-WHYRYRGGG-NH₂, b₃: 487.15 m/z, b₄: 650.08 m/z, b₅: 806.22 m/z, y₇: 702.32 m/z, y₆: 565.26 m/z, y₅: 402.26 m/z.
Figure 18 - A) NSI MS spectrum of H-WKWYSGGGG-NH$_2$, Hit 3. H-WKWYSGGGG-NH$_2$ + H$^+$: 996.67 m/z, H-WKWYSGGGG-NH$_2$ + 2 H$^+$: 499.13 m/z; B) N SI MS/MS spectrum of H-WKWYSGGGG-NH$_2$. b$_2$: 314.96 m/z, b$_3$: 499.11 m/z, b$_4$: 663.54 m/z, b$_5$: 750.57 m/z, b$_6$: 807.59 m/z, b$_8$: 921.63 m/z, y$_7$: 682.27 m/z, y$_5$: 332.99 m/z, y$_4$: 245.77 m/z.
Figure 19 - A) NSI MS spectrum of H-GRWWWGGGG-NH₂, Hit 4. H-GRWWWGGGG-NH₂ + H⁺: 1017.56 m/z, H-GRWWWGGGG-NH₂ + 2H⁺: 509.88 m/z; B) NSI MS/MS spectrum of H-GRWWWGGGG-NH₂. b₂: 243.2 m/z, b₃: 399.3 m/z, b₄: 585.38 m/z, b₅: 771.46 m/z, b₆: 828.48 m/z, b₇: 885.5 m/z, y₆: 680.34 m/z, y₇: 804.34 m/z, y₈: 618.26 m/z.
Figure 20 - A) NSI MS spectrum of H-WYKKWGGGG-NH₂, Hit 5. H-WYKKWGGGG-NH₂ + H⁺: 1037.40 \( m/z \), H-WYKKWGGGG-NH₂ + 2H⁺: 519.53 \( m/z \); B) NSI MS/MS spectrum of H-WYKKWGGGG-NH₂. \( b₂ \): 349.04 \( m/z \), \( y₀ \): 851.13 \( m/z \), \( y₇ \): 688.20 \( m/z \), \( y₅ \): 432.07.
Figure 21 - A) NSI MS spectrum of H-RYYGWGGGG-NH$_2$, Hit 6. H-RYYGWGGGG-NH$_2$ + H+: 971.73 m/z, H-RYYGWGGGG-NH$_2$ + 2H+: 486.53 m/z; B) NSI MS/MS spectrum of H-RYYGWGGGG-NH$_2$. b$_2$: 320.07 m/z, b$_4$: 540.07 m/z, b$_5$: 726.13 m/z, b$_6$: 783.13 m/z, b$_7$: 840.13 m/z, b$_8$: 897.20 m/z, y$_7$: 652.07 m/z, y$_4$: 245.93 m/z.
Figure 22 - A) NSI MS spectrum of H-WGRSWGGGG-NH$_2$, Hit 7. H-WGRSWGGGG-NH$_2$ + H$: 918.67$ m/z, H-WGRSWGGGG-NH$_2$ + 2H$: 460.13$ m/z; B) NSI MS/MS spectrum of H-WGRSWGGGG-NH$_2$. $b_1$: 186.34 m/z, $b_4$: 487.20 m/z, $b_5$: 673.13 m/z, $b_6$: 730.07 m/z, $b_7$: 787.13 m/z, $b_8$: 844.07 m/z, $y_5$: 432.18 m/z, $y_4$: 245.93 m/z.
Figure 23 - A) NSI MS spectrum of H-WRYHYHGGGG-NH$_2$, Hit 8. H-WRYHYHGGGG-NH$_2$ + H+: 1051.60 m/z, H-WRYHYHGGGG-NH$_2$ + 2H+: 526.47 m/z; B) NSI MS/MS spectrum of H-WRYHYHGGGG-NH$_2$. b$_4$: 669.13 m/z, b$_5$: 806.07 m/z, b$_6$: 863.20 m/z, y$_5$: 383.07 m/z.
Figure 24 - A) NSI MS spectrum of H-WRWYRGGGG-NH₂, Hit 9. H-WRWYRGGGG-NH₂ + H⁺: 1093.40 m/z, H-WRWYRGGGG-NH₂ + 2H⁺: 547.53 m/z; B) NSI MS/MS spectrum of H-WRWYRGGGG-NH₂. b₂: 343.07 m/z, b₅: 847.3 m/z, y₇: 751.20 m/z, y₆: 565.13 m/z.
Figure 25 - A) NSI MS spectrum of H-WRSSWGGGG-NH₂, Hit 10. H-WRSSWGGGG-NH₂ + H+: 948.67 m/z, H-WRSSWGGGG-NH₂ + 2H+: 475.13 m/z; B) NSI MS/MS spectrum of H-WRSSWGGGG-NH₂, b₂: 343.07 m/z, b₃: 430.07 m/z, b₄: 514.07 m/z, b₅: 703.13 m/z, b₆: 760.13 m/z, b₇: 817.17 m/z, b₈: 874.20 m/z, y₇: 606.07 m/z, y₆: 519.07 m/z, y₄: 245.93 m/z.
Figure 26 - A) NSI MS spectrum of H-HWHWHGGGG-NH$_2$, Negative bead 1. H-HWHWHGGGG-NH$_2$ + H$^+$: 1029.47 m/z, H-HWHWHGGGG-NH$_2$ + 2H$^+$: 515.81 m/z; B) NSI MS/MS spectrum of H-HWHWHGGGG-NH$_2$. b$_2$: 324.00 m/z, b$_4$: 647.07 m/z, b$_5$: 784.00 m/z, y$_6$: 892.13 m/z, y$_7$: 706.06 m/z, y$_8$: 569.07 m/z, y$_6$: 383.07 m/z.
Figure 27  - A) NSI MS spectrum of H-HWSHHGGGG-NH$_2$, Negative bead 2. H-HWSHHGGGG-NH$_2$ + H$: 930.47$ m/z, H-HWSHHGGGG-NH$_2$ + 2H$: 465.93$ m/z; B) NSI MS/MS spectrum of H-HWSHHGGGG-NH$_2$. b$_2$: 324.07 m/z, b$_4$: 548.00 m/z, b$_5$: 685.13 m/z, y$_6$: 793.13 m/z, y$_7$: 607.07 m/z, y$_5$: 383.07 m/z.
Figure 28 - A) NSI MS spectrum of H-HWKWHGGGG-NH₂, Negative bead 3. H-HWKWHGGGG-NH₂ + H⁺: 1020.47 m/z, H-HWKWHGGGG-NH₂ + 2H⁺: 511.00 m/z; B) NSI MS/MS spectrum of H-HWKWHGGGG-NH₂. b₂: 324.07 m/z, b₃: 451.23 m/z, b₄: 638.07 m/z, b₅: 774.37 m/z, y₆: 883.07 m/z, y₇: 697.07 m/z, y₆: 569.00 m/z, y₅: 383.00 m/z, y₄: 245.87 m/z.
Comparison of Positive and Negative Beads

The positive hit used for comparison was from Screening 2, hit 1, with the sequence H-WKRYRGGGG-NH$_2$. The negative bead was from Screening 5, negative bead 1, with the sequence H-HWHWHGGGG-NH$_2$. Both of these were synthesized as described in the experimental. Bulk samples of positive and negative hit beads were incubated with an 83 nM concentration of the red fluorescent marker. The positive beads showed increased fluorescence in the red channel; however, it was not significantly different than that of the negative beads, Figure 14. When a 500 nM concentration of the red fluorescent marker was used a greater increase of fluorescence was observed on the positive beads compared to the negative ones, Figure 15. More tests to compare the avidity of transferrin for the hit sequence will be performed.

Figure 14 – Images A1, A2, B1, B2, C1, C2, D1 and D2 show transferrin bound to the target ligand. Images E1, E2, F1 and F2 show the negative control. This was done using an 83 nM transferrin concentration.
Figure 15 – Images A1-D2 show transferrin bound to the target ligand. Images E1, E2, F1 and F2 show the negative control. Images G1, G2, H1 and H2 show a side by side comparison of the positive bead (on left) and negative bead (on right). This was done using a 500 nM transferrin concentration.

Peptide Synthesis on Smaller Beads

Many benefits can be obtained from using smaller beads in a peptide library. First, the use of smaller beads leads to a more diverse library on a per gram basis. Conducting screenings with smaller beads will therefore generate the desired “hits” using much smaller quantities of the library. This will be faster and more cost effective in terms of library synthesis and screening. Second, smaller beads can be screened by using a standard flow cytometer. This will again decrease the screening time and also
prevent the need for a non-standard flow cytometer needed for screening larger beads. A comparison of the bead size is shown in Figure 29.

![Figure 29 – 130, 30, 20, and 10 µm beads, respectively at 100x magnification.](image)

In order to prove that peptide synthesis is possible on 30 µm and 20 µm beads, a four glycine chain was synthesized. During the synthesis of the peptide on the 30 µm beads, all Kaiser tests were as expected for each of the four glycines indicating that they were added successfully. A bulk sample of the peptide analyzed by electrospray showed the presence of four glycine units, Figure 30. Single bead peptide analysis is currently underway.

The Kaiser tests for the synthesis of the four glycine peptide on the 20 µm beads were also as expected; however, no usable data has been collected to date. Bulk and single bead analysis are currently underway.
Figure 30 – ESI MS spectrum of H-GGGG-NH₂ on 30 μm resin beads.

20 μm Peptide Library

The synthesis of the miniaturized peptide library was more tedious than the original library on 130 μm beads. The main difficulty is the use of the smaller vessel containing the smaller pore-size frit. Adequate stirring is challenging even with the smallest stir bars available. Manual stirring was often employed to ensure all reagents accessed the entire bead sample. The library was synthesized starting with 500 mg of 20 μm beads. The reagents and procedures were similar to those used previously. The
The general formula for the library was \( H-X_5X_4X_3X_2X_1GGGG-HMBATentaGel \) where \( X = R, S, W, H, Y, \) or \( K \). This gives 7,776 different peptide combinations in the library. According to the Kaiser tests each amino acid was added and deprotected successfully during each split and mix step. Since these beads are much smaller an micromanipulator ep pendofr Tr ansferMan N K 2at tachment on the Nikon Eclipse T microscope was used to separate out individual beads for single bead analysis. Although many individual beads have been analyzed via nanomanipulation/nanoelectrospray MS, no interpretable data has been obtained. Efforts are underway to sequence individual members of this library.

Conclusions

The majority of the goals set out at the beginning of this project have been met. First an efficient protocol for general peptide synthesis was put in place utilizing microwave irradiation to reduce reaction times. Synthesis times which once took up to 24 hours were decreased to 10 minutes. With this new method an O BOC peptide library was generated straightforwardly in little time meeting two of the eight goals.

A new technique for cleavage of peptides from resin beads was developed by which exposure of an H MBA-linked peptide to high pressure ammonia gas led to efficient cleavage in as little as 5 minutes. Cleavage could be achieved in a single step without the use of any solvents, which is not only faster than all other reported methods employing HMBA as a resin linker, but is also significantly faster than most of her techniques currently in use. The successful extraction of peptides from individual resin beads was achieved. The ability to sequence peptides sequestered on individual resin beads of a prototypical combinatorial library with the aid of nanomanipulation/NSI
MS/MS was also demonstrated. The data generated from individual beads matched that of authentic bulk samples showing this method to be successful. It was also shown that peptides of varying length are amenable to the process and that it is not dependent on having glycine as the anchoring residue. The new techniques are attractive because they reduce the amount of post-screening processing needed to identify hits (the only solvent needed is one for collection of the hit peptide from the resin bead for analysis). This new combination of techniques allows for rapid, reliable, environmentally responsible sequencing of hit beads from combinatorial libraries.

Our screening process was also successful in identifying several ligands that bind transferrin. The avidity of one of those hits was demonstrated qualitatively. More experiments will be conducted with the positive hit sequences generated to quantify relative binding avidity to transferrin.

Initial steps in the synthesis of a miniaturized peptide have been conducted. Synthesis of a four glycine spacer peptide was successful on both 30 µm and 20 µm beads according to Kaiser tests. However, MS data confirming the synthesis has only been collected thus far for bulk samples of the 30 µm beads. The synthesis of the 20 µm peptide library also appears to have been successful according to the Kaiser tests, but no MS data has been collected. Future work is aimed at sequencing peptide contained on individual 30 µm beads, and bulk and individual bead analysis of peptide contained on 20 µm beads. Random individual beads from the 20 µm library will also be analyzed and sequenced.
WORKS CITED


