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Characterization of an Affitoid Variable Region to be used for MDM-2 Detection in ELISA Microarray

Graham Reinhard
University of Arkansas, Fayetteville

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Department of Chemical Engineering
College of Engineering
University of Arkansas
Fayetteville, AR

by

Graham Reinhard

This thesis is approved.

Thesis Advisor:

Dr. Shannon Servoss

Thesis Committee:

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Graham Reinhard, Chemical Engineering

Faculty Advisor: Dr. Shannon Servoss, Chemical Engineering

Abstract

Sandwich enzyme-linked immunosorbent assay (ELISA) microarray using peptoid-based affinity reagents, referred to as affitoids, is potentially a very powerful tool for the early detection of cancer and other complex diseases. Current detection methods for cancer are inadequate because most are imaging techniques and require that the cancer has progressed far enough for growths to be visible, which is often far too late for treatment to be effective. One possible way to overcome this issue is to use a biological detection technique that is able to detect proteins that are secreted specifically by cancerous cells (referred to as biomarkers). These biomarkers can be detected at very low concentrations characteristic of early stage cancer using ELISA. . Peptoids, or poly-N-substituted glycines, are excellent candidates for this purpose due to their inexpensive and simple synthesis, ability to incorporate unique reactive sites, highly stable helical structures, and potential for non-biofouling design. The goal of this study was to synthesize, purify, and test a peptoid for the detection of MDM2, a known cancer biomarker, using an ELISA microarray.

Introduction

The American Cancer Society suggests that possibly the most important factor for determining the prognosis of a cancer patient is how far the cancer has progressed at the time of detection. Current detection methods such as an MRI, mammogram, or CT scan are imaging techniques and require that the cancer has progressed far enough for growths to be visible, which is often far too late for treatment

to be effective. ELISA microarray can solve this problem by detecting various biomarkers, such as proteins secreted by cancer cells. This system is well suited for the task because of its high sensitivity and throughput as well as its relatively low cost. ELISA microarray utilizes two affinity reagents, one to capture specific proteins on a slide and another with a fluorescent marker to signal the presence of the bound antigen.

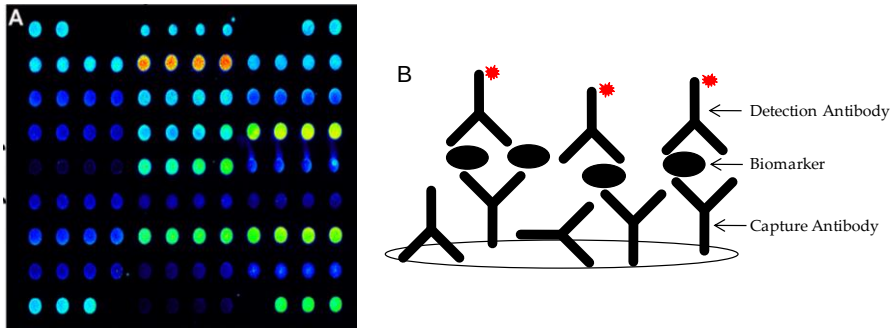

Figure 1. A. Fluorescent scanning of a microarray slide. B. Interactions between affinity reagents and biomarkers.

(Servoss)

While researchers are discovering many potential biomarkers for early stage cancer, validating these biomarkers for use in diagnostic systems is becoming increasingly difficult. Currently, the gold standard reagents for ELISA microarray are antibodies, however there are a limited number of them available and the techniques for affinity reagent development are slow and expensive, greatly limiting the validation of biomarkers.

Peptoids have the potential to solve many of the problems with using the ELISA microarray in disease detection. Peptoids, or poly-N-substituted glycines, have the same backbone as peptides with the side chains attached to the nitrogen rather than the alpha-carbon.

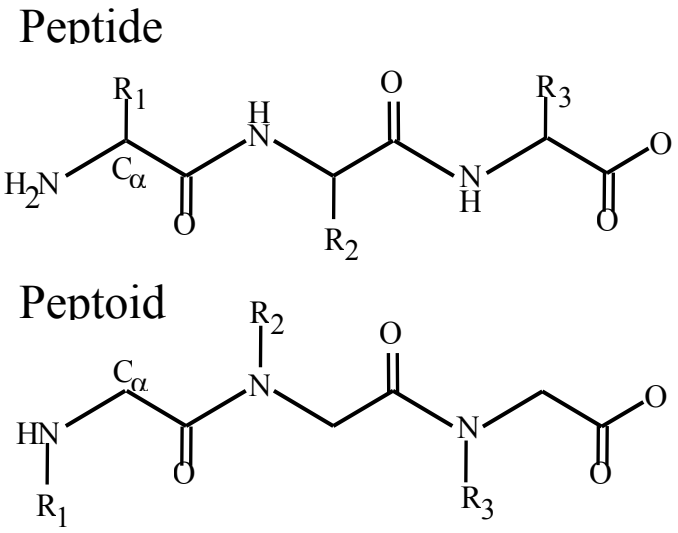

Figure 2. Peptide vs. Peptoid (Simon,1992)

Several characteristics make peptoids potentially superior affinity reagents for use in an ELISA microarray. Compared to peptides, they are cheaper and simpler to synthesize and therefore better able to meet increasing demand for biomarker validation. Also, because of the diverse array of available peptoid side chains, they are capably of incorporating a wide variety of active sites for protein interaction. Finally, peptoids are able to form stable alpha helices. By using a Polyethylene Glycol (PEG) scaffold to support and orient this robust secondary structure, the possibility exists to significantly increase the sensitivity of the ELISA microarray.

For this study, MDM2 protein was selected as the antigen to be detected. MDM-2 is an important negative regulator of the p53 tumor suppressor [Ito, 2002]. Studies have confirmed that MDM-2 over expression promotes the transformation of primary rodent fibroblasts and eventually leads to tumor formation in mice [Zhao, 2003]. This is not only the case in mice however; several human tumor types have been shown to have increased levels of Mdm2, including soft tissue sarcomas and osteosarcomas as well as breast tumors [Yokoyama, 1998]. From studies performed by Alluri et al. a

peptoid, JPT2, has been identified with affinity for MDM2. The goals of this project were to synthesize and purify JPT2, then compare its effectiveness for detecting MDM2 against standard peptide affinity reagents.

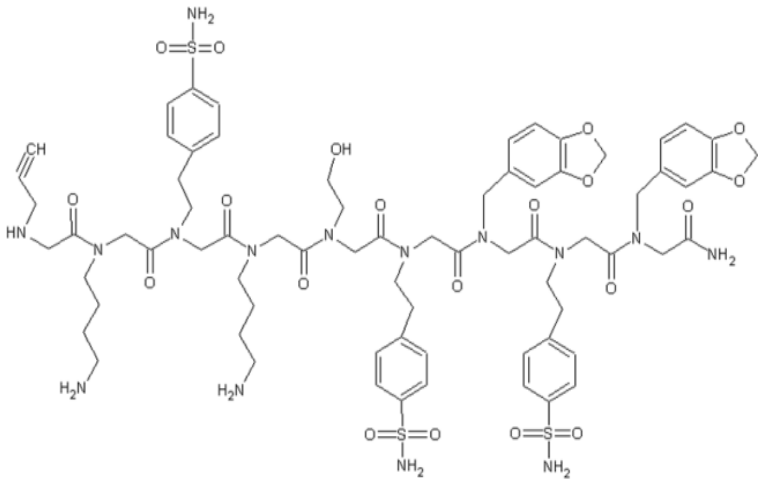

Figure 3. JPT2: Mw 1572.15

(Alluri, 2003)

Methods

Synthesis:

JPT2 was synthesized on rink amide resin using an automated peptide synthesizer with modified chemistry as described in Zuckerman, 1998. The rink amide resin was first placed in a filtered reaction vessel and suspended in DMF for 2 minutes at a concentration of 40 mL/mmol to swell the resin. The Fmoc protection group on the resin was then removed using a 20% piperidine in DMF solution at 40 mL/mmol of resin for 12 minutes. The resin was then washed for 30 seconds with DMF at 40 mL/mmol

five times to prepare for the synthesis of the peptoid chain. The submonomer addition cycle for peptoid synthesis is illustrated in Figure 4.

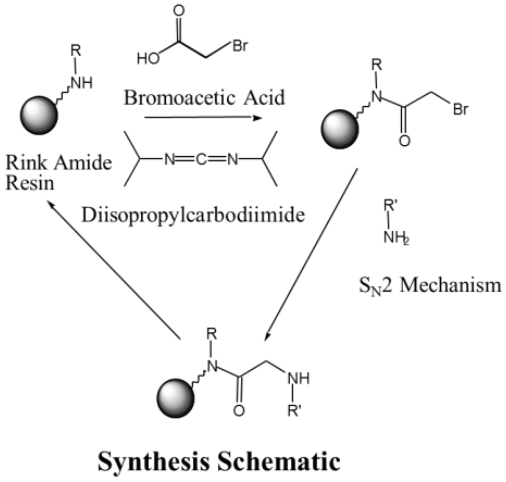

Figure 4.
Zuckerman *et. al.*, Journal of the American Chemical Society, 1992

The resin was first bromoacetyated by suspending it in 1.2 M bromoacetic acid at 17 mL/mmol of resin along with DIC at 3.2 mL/mmol of resin. The resin was then washed for 30 seconds three times with DMF as before. The desired side chain monomer of the peptoid was then attached via Sn2 nucleophilic substitution. 17 mL/mmol of the desired monomer at 1 M concentration was added to the resin and suspended for 20 minutes to complete the coupling. The resin was then washed four times in DMF and the bromoacetylation and Sn2 displacement cycle was repeated until a peptoid of the desired sequence was obtained.

Cleavage:

After synthesis, the JPT2 was then cleaved from the rink amide resin. To do this, a portion of the resin was added to a 95% trifluoroacetic acid, 2.5% TIS, and 2.5% water solution. This mixture was then

agitated on a belly dancer machine for exactly 10 minutes. The solution was then immediately filtered from the resin using a filter column and reduced to an oil form using a Rotovap. After removing the solvent, the remaining peptoid residue was dissolved in 50:50 acetonitrile and water to obtain a 3 mg/mL concentration. The presence of JPT2 in the crude peptoid from the cleavage was then verified using analytical HPLC and MALDI mass spectroscopy.

Purification:

JPT2 was purified using Preparative HPLC. A mildly hydrophobic C18 column was used as the stationary phase and a gradient of water and acetonitrile with 0.1% TFA was used as the mobile phase. Initial HPLC runs were performed using a 5-95% acetonitrile in water gradient at 1% per minute with a 25 minute column equilibration time. These conditions, however, were inadequate. Because the peptoid proved to be more hydrophilic than expected, a 0-95% acetonitrile concentration was used instead which provided good separation. Desired fractions from multiple HPLC runs were then combined, measured for purity using an analytical HPLC, and analyzed for identity using MALDI mass spectroscopy.

Antibody only Assay:

In order to obtain a basis for comparison for JPT2, it was first required to obtain a working assay with the peptide antibodies only. A monoclonal capture antibody and a biotinylated detection antibody for MDM2 were obtained for use in this assay. Along with MDM2, Alexa 647 and PBS were used as positive and negative controls respectively and Green Fluorescent Protein (GFP) was used as a standard for comparison. Using a GeSiM NanoPLotter 2.1 microarray printer, capture antibodies were printed at a concentration of 0.8 mg/mL into 8 rows of two onto the microarray slides for a total of 16 chips per slide. MDM2 and GFP antigens were then added to the slides starting at 50,000 pg/mL in 1 mg/mL casein solution in PBS. 7 fourfold dilutions for MDM2 were used to cover a wide range of protein concentrations for testing but the concentration of GFP antigen was held constant. The slides were then

incubated with the antigen for at least 17 hours on the belly dancer. After being rinsed in PBS-T, biotinylated detection antibodies for MDM2 and GFP were added to the slides at a concentration of25 ng/mL and incubated for 2 hours. After another PBS-T rinse, the slides were incubated for 30 minutes with a Streptavadin-HRP solution at 1 µg/mL in PBS-T, rinsed and incubated with a biotinyltyramide solution for exactly 10 minutes. Finally, the slides were incubated with Alexa 647 conjugate at 1 µg/mL in PBS-T for 30 minutes before being rinsed and dried in a centrifuge. The finished slides were then scanned using a Genepix® scanner.

Results

Synthesis and Purification

As shown in Figure 5, MALDI mass spectroscopy confirmed the presence of JPT2 in the raw peptoid post cleavage. The sample showed the highest absorbance at a molecular weight of 1572 au, indicating a successful synthesis.

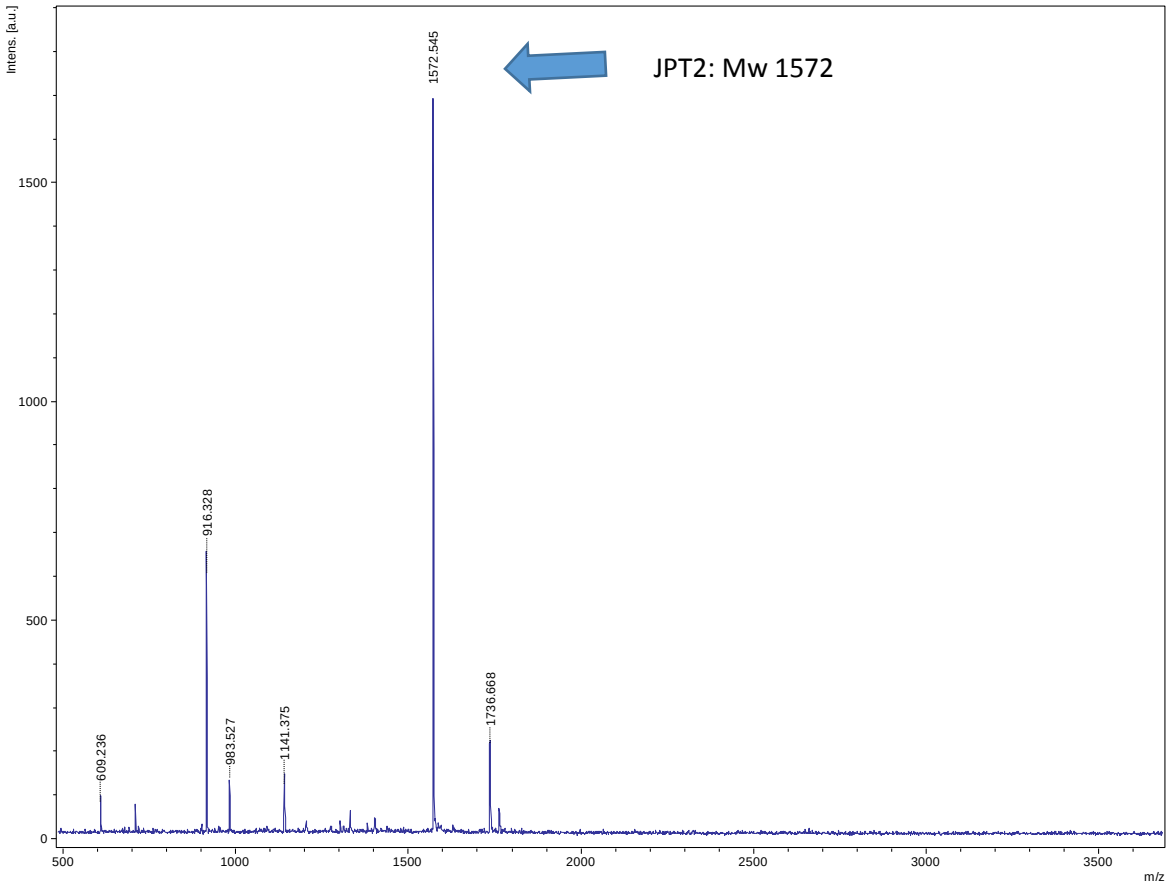

Figure 5. MALDI Spectrum of Raw Peptoid.

Initial prep runs at 5-95% acetonitrile in water with a 25 minute equilibration time did not provide a good separation. Because the peptoid was so hydrophilic, most of the desired product eluted with the solvent peak as shown in Figure 6. A small amount of JPT2 did elute at 35% acetonitrile (30 minutes) as predicted by the analytical HPLC results.

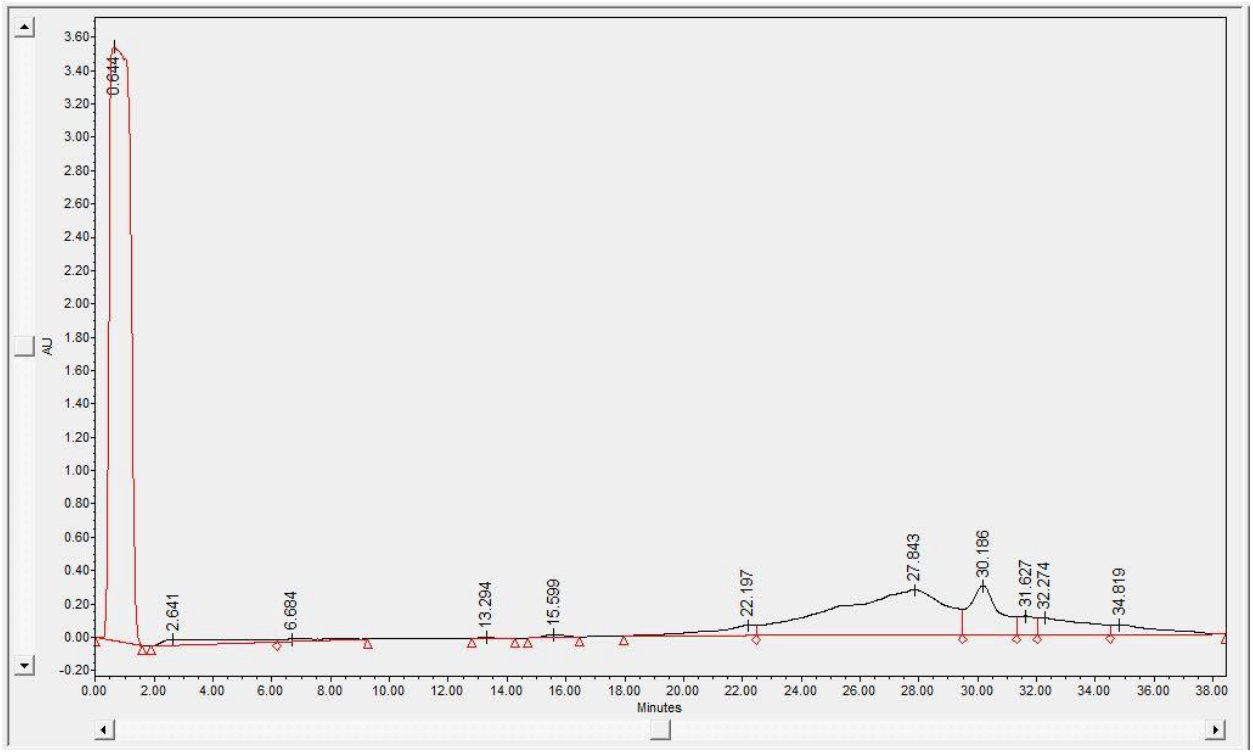

Figure 6. HPLC spectrum. 5-95% acetonitrile +0.1% TFA at 1% per minute, 25 min equilibration.

By increasing the column equilibration time to one hour, a significantly better separation of the peptoid was achieved. This spectrum is shown in Figure 7. Although the JPT2 was confirmed to be in the dominant peak eluting at 28 minutes, the high absorbance of the solvent peak indicated that much of the peptoid was still eluting early.

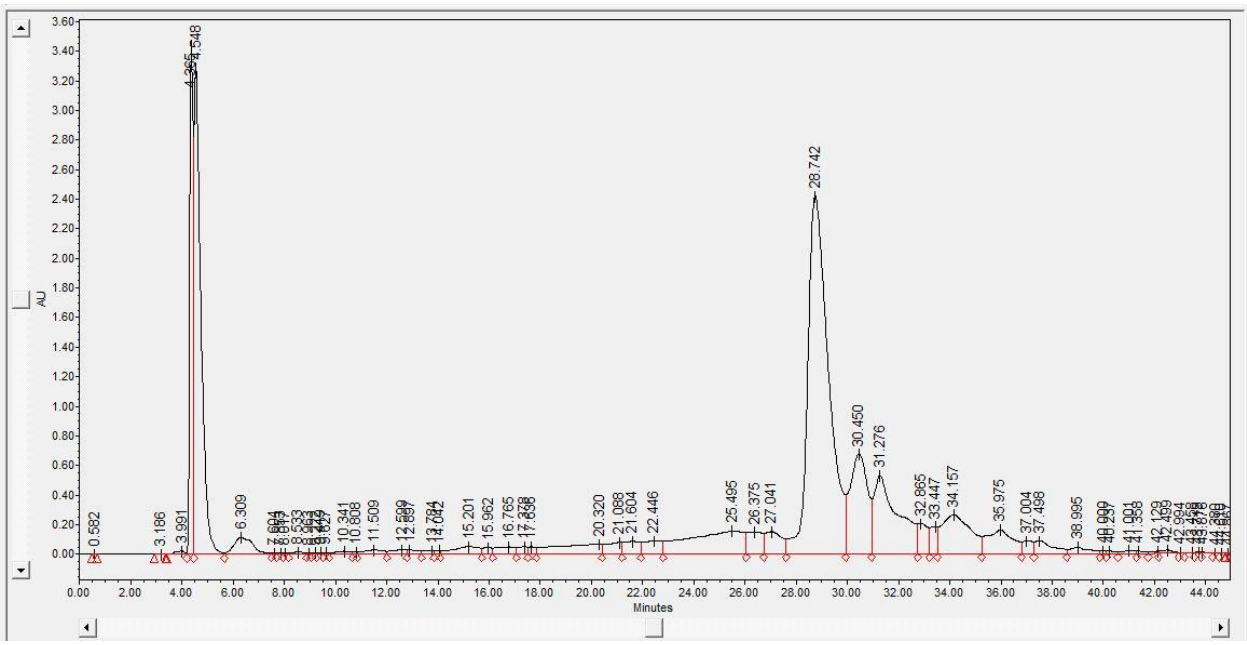

Figure 7. HPLC spectrum. 5-95% +0.1% TFA at 1% per minute, 60 min equilibration.

By using a 0-95% acetonitrile concentration in water and by cleaning and equilibrating the injection loop of the instrument prior to each run the separation was improved further. These changes allowed for better interaction between the peptoid and the solid phase, and a nearly ideal separation of the peptoid sample was achieved as shown in Figure 8. As expected, the peptoid eluted in the major peak at approximately 35% acetonitrile (33 min).

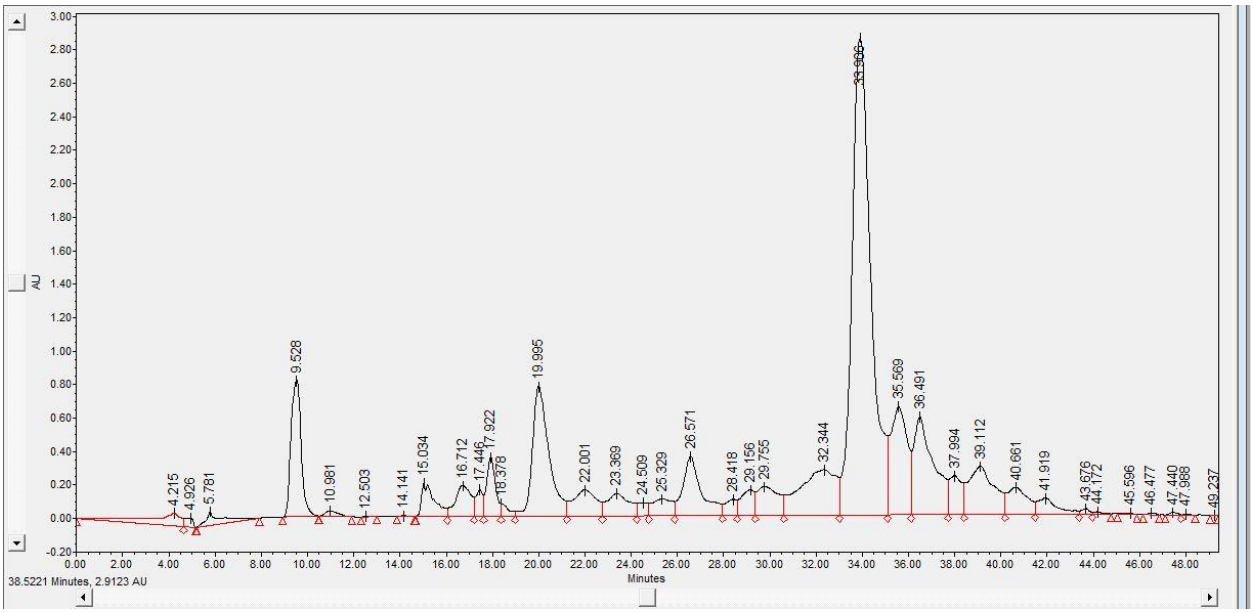

Figure 8. HPLC spectrum. 0-95% acetonitrile +0.1% TFA at 1% per minute, 25 min equilibration.

The fractions from the desired peak were combined and confirmed to contain JPT2 by MALDI. The spectrum for the purified peptoid is shown in Figure 9.

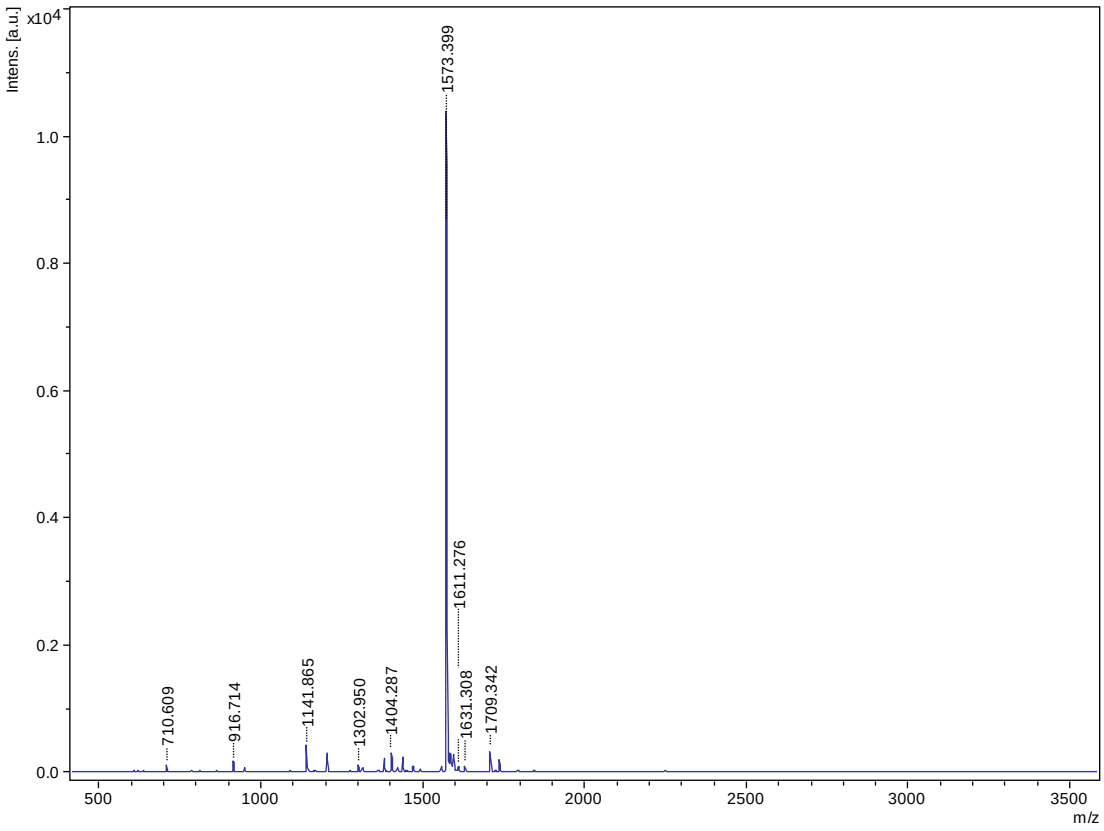

Figure 9. MALDI Spectrum of Purified JPT2

Using analytical HPLC, the purified fractions of JPT2 were shown to have a purity greater than 97% as required by use in later work. The analytical HPLC spectrum is displayed in Figure 10.

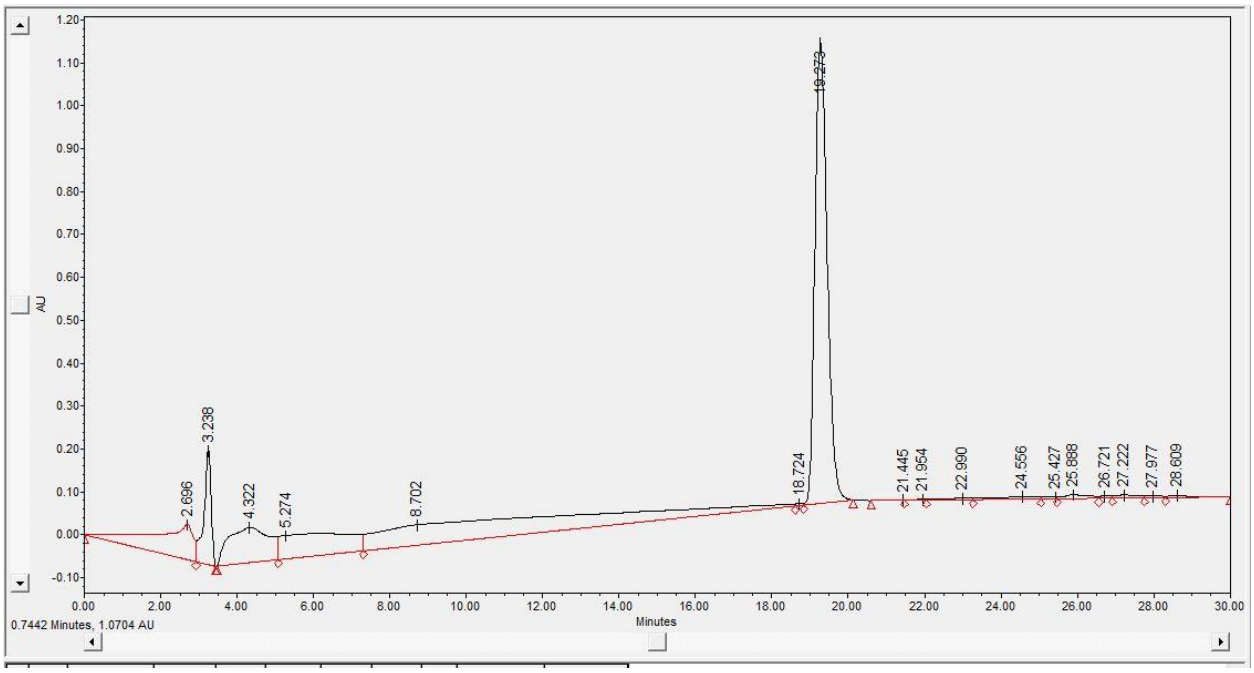

Figure 10. Analytical HPLC spectrum of purified JPT2 > 97% purity.

Antibody Assay

The processed microarray slides were scanned using the GenePix® scanner with two different wavelengths of light and varying laser intensity. Figure 11 is a close up of the scanned result of the chips that were exposed to the highest concentration of MDM2. As shown in the figure, both Alexa 647 as the positive control and GFP as the standard showed fluorescence and PBS as the negative control did not as expected. However, the spot for MDM2 detection did not show any fluorescence at all, even at the highest concentration of antigen.

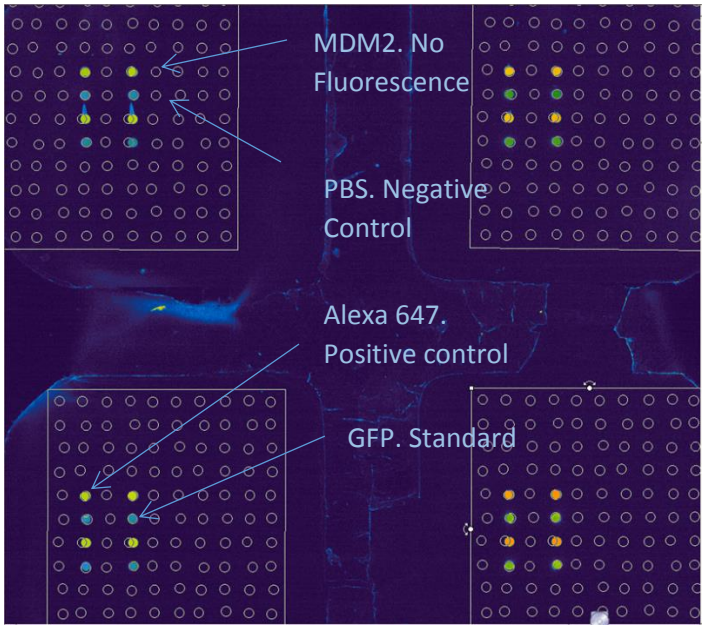

Figure 11. Microarray Fluorescence

Conclusions and Future Work

In this study a peptoid specific to MDM2 was successfully synthesized and purified for use as the variable region of a peptoid affinity reagent in an ELISA microarray. Before it can be used in an assay, it must first be combined with PEG via "click" chemistry to form a complete affitoid with a structural constant region and a functional variable region. This PEG scaffold will help to facilitate antigen binding and it is also believed that the structure will help the affitoid overcome the "floppiness" inherent to these types of peptoids.

Another issue that must be addressed in this project is the antibody assay. In order to have a basis to compare JPT2 peptoid with, a standard curve for MDM2 must be obtained from a working antibody assay. The lack of fluorescence for MDM2 in the antibody assay performed in this study could have several possible causes. To find the source of the problem, each component needs to be tested individually. Printing the antibody itself on the alone and incubating the slide with detection antibody could help determine if the protein-detection antibody interaction is the problem. If this proves not to

be the case the protein concentration could be increased or a polyclonal capture antibody could be used to try to improve the capture antibody-protein interaction.

Once a standard curve of MDM2 concentration versus fluorescence can be obtained from the antibody assay, the JPT2 affitoid can be tested under the same conditions. It is believed that this comparison will demonstrate that peptoids are just as effective if not superior to peptides for use as affinity reagents in an ELISA Microarray. If this is the case, it is likely that peptoids will be able to replace peptides for this application, thereby speeding the validation of disease biomarkers and potentially providing an early detection method for complex diseases such as cancer.

References

1. Alluri PG, Reddy MM, Bachhaurat-Sikder k, Olivos HJ, Kodadek T. “Isolation of Protein Ligands from Large Peptoid Libraries.” Journal of American Chemical Science, 2003: 125 (46): 3995-4004.
2. Dhaval Shah, Shannon Servoss, and James P. Turner. Peptoids As Capture Molecules In Antibody Microarrays. Poster. The University of Arkansas, Ralph E Martin Department of Chemical Engineering, 2010.
3. Ito, Akihiro; Kawaguchi Yoshiharu, Lai Chun-Hsiang, Kovacs Jeffrey J, Higashimoto Yuichiro, Appella Ettore, Yao Tso-Pang (November 2002). "MDM-2-HDAC1-mediated deacetylation of p53 is required for its degradation". EMBO J. 21 (22): 6236–45.
4. Kirshenbaum et al, PNAS, 95, 1998
5. Seo, Jiwon, Annelise Barron, R. Zukerman, “Novel Peptoid Building Blocks: Synthesis of functional Aromatic Helix-inducing submonomers.” Organic Letters, vol. 12, no. 3, pp. 492-295, 2010.
6. Wu et al, JACS, 123, 2001
7. Yokoyama R, Schneider-Stock R, Radig K, Wex T, Roessner A.” Clinicopathologic implications of MDM-2, p53 and K-ras gene alterations in osteosarcomas: MDM-2 amplification and p53 mutations found in progressive tumors. Pathol Res Pract. 1998;194(9):615-21.
8. Zhao, Lili; Samuels Tina, Winckler Sarah, Korgaonkar Chandrashekhar, Tompkins Van, Horne Mary C, Quelle Dawn E (January 2003). "Cyclin G1 has growth inhibitory activity linked to the ARF-MDM-2-p53 and pRb tumor suppressor pathways". Mol. Cancer Res. 1 (3): 195–206.
9. Zuckermann RN, Simon RJ, Kania RS, Huebner VD, Jewell DA, Banville S, Ng S, Wang L, Rosenberg S, Marlowe CK. PNAS 1992; 89 (20): 9367-71.