Theses and Dissertations

7-2015

Using Peptoids to Build Robust, Efficient Microarray Systems

Dhaval Sunil Shah
University of Arkansas, Fayetteville

Recommended Citation

Using Peptoids to Build Robust, Efficient Microarray Systems

Using Peptoids to Build Robust, Efficient Microarray Systems

A thesis submitted in partial fulfillment
of the requirements of the degree of
Master of Science in Chemical Engineering

by

Dhaval Sunil Shah
University of Arkansas
Bachelor of Science in Chemistry and Physics, 2007

July 2015
University of Arkansas

This thesis is approved for recommendation to the Graduate Council

_____

Dr. Shannon Servoss
Thesis Director

_____   _____

Dr. Robert Beitle   Dr. Christa Hestekin
Committee Member   Committee Member

_____   _____

Dr. Julie Stenken   Dr. David Zaharoff
Committee Member   Committee Member

Abstract

Recent studies have shown microarrays to be indispensable for various biological applications, allowing for high-throughput processing and screening of biological samples such as RNA, DNA, proteins and peptides using a small sample volume (< 1 µL). Peptoids (poly-N-substituted glycine oligomers) can be used as a substitute for antibodies as capture molecules, as well as coatings for slides in antibody microarrays. The ease of synthesis of peptoids, high customizability with desired bioactivity, and speed of synthesis allows us to build a diagnostic system with a large dynamic range that can detect biomolecules from a minimal sample size. In this study, peptoid-based antibody mimics are designed to have both structural and functional features similar to those of antibodies, including a stable constant region (scaffolding) and a variable region for protein recognition. Peptoids previously screened via combinatorial library synthesis to be specific to bind Mdm-2 (mouse double minute 2 homolog) and GST (gluthathione S-transferase), have been synthesized. The protein recognition peptoids have been conjugated to PEG (polyethylene glycol) molecules with modified end groups; an amine group on one end that allows for immobilization and orientation on the slide, and an azide group on the other end that will allow for attachment to the peptoid through "click chemistry". The number of capture molecules printed on the slides can be increased by making the available surface area of the slide larger via coating with microspheres. We have determined that partially water soluble peptoids that are also helical, can self-assemble into microspheres. Sequences have been developed that can consistently produce uniform microsphere coatings on slides that increase the overall surface area. A high surface area corresponds to a higher number of binding sites, and therefore a more sensitive system. The work done has shown that slides may be successfully coated in order to potentially improve the detection system.

Acknowledgements

I owe this work to the patient and helpful mentoring provided by my advisor Dr. Shannon Servoss and the rest of the committee members. My family has been extremely supportive throughout this process and I would be remiss if I didn't thank the numerous graduate students and undergraduate students who helped support this work with their support, both moral and technical.

Table of Contents

List of Figures

List of Tables

1. Background

1.1 Case for Early Detection of Diseases Such as Cancer

Early screening for cancer attempts to detect the presence of cancer before the presence of symptoms. Symptoms of cancer vary with the type of cancer, and often present themselves as symptoms of other diseases, which can make accurate diagnoses very difficult. A large proportion of patients diagnosed with lung, colorectal, and breast cancers already have localized or metastasized malignant masses at the time of diagnosis [1]. The survival rates of patients of advanced cancer are significantly lower than those of patients whose cancers are diagnosed early [1]. Cancer can be a debilitating condition, not only for the patient but also for the patient's family and loved ones. It also comes with a huge economic burden. The National Institutes of Health (NIH) estimated that the total cost of cancer including medical expenses and cost of lost productivity at $171.6 billion in 2008 [2]. As important as it is to research effective methods for treating advanced stages of the ailment, there is a strong case for developing early detection methods. The combination of earlier diagnosis with current available treatment methods can have a profound impact on patient survival.

The method used for screening may vary based on the type of the disease. Universal screening of high-risk populations can be an effective approach to control. For screening to be practical, these tests should have a high accuracy of detection without concomitant false positives, treatment after early detection should improve prognosis, and the harms and costs of screening should be alleviated by the benefits afforded [3]. It can include blood analysis, urine testing, and various imaging techniques. Despite the obvious benefits of screening, there are some significant drawbacks. False positives lead to invasive procedures, increasing the chances of other complications and false negatives would allow the condition to progress unchecked.

Success of early detection can be dependent on identification of appropriate biomarkers. Biomarkers are identifiable and measurable molecules whose presence and concentration correlate to the physiological state and behavior of the organism [4-6]. They include proteins, carbohydrates, mutated genes, RNA, and small metabolites, which can be found in blood, urine, and other body fluids [7]. It is possible to rapidly screen for changes in gene expression in cancer cells due to a rapid expansion in genomic-based technologies [8]. Biomarkers can be present in the body much earlier than any symptoms are displayed, or tumors being visible [9-12]. Biomarkers for various cancers have been elucidated, including those for pancreatic cancer [11, 13, 14], breast cancer [15, 16], and bladder cancer [17]. Cancer, however, is a heterogeneous condition, and therefore, single biomarkers would not provide sufficient information about the cancerous tissue type, stage, and progression of the disease if present. Therefore, panels of biomarkers are needed in order to reliably test for the cancer of interest.

Identification of reliable biomarkers is only part of the equation. Following biomarker identification, reliable assays need to be developed in order to detect these biomarkers from patient samples and quantify them. Detection of these proteins can not only help effective diagnosis but also help determine the most appropriate treatment [10]. Unfortunately, it is difficult to analyze various proteins with small sample volumes in a clinical setting. Microarrays may be used for such assays.

1.2 ELISA Microarrays

ELISA (Enzyme Linked Immunosorbent Assay) is one of the most widely used techniques for determining the presence and amount of a specific protein in a biological sample [18]. It is one of

multiple antibody arrays that can be used for rapid detection of biomolecules in numerous samples [18, 19]. A microarray is a two-dimensional array on a solid substrate that can assay multiple analytes from biological material in parallel, while using a miniscule amount of biological probes [20-22]. There are various types of microarrays including DNA microarrays, protein microarrays, ELISA microarrays (a sub-type of protein microarrays, that typically uses antibodies for molecule detection), and carbohydrate arrays. ELISA microarray technology holds immense potential for the profiling of proteins specific to several diseases and conditions [23-25].

The assay employs printing of various individual antibodies that are specific for the desired targets in an ordered pattern onto a solid support. The antibody binds specifically to the analyte of interest (a biomarker, in this case) that might be present in the sample. The binding can be indicated by fluorescence when the identifying antibody is conjugated to a fluorescent marker. This fluorescence indicates the presence of the target molecule, and its intensity may be used to quantify the concentration, which is done by comparing the intensity against a standard curve. Sandwich ELISA (a subtype of ELISA) microarray, as seen in Figure 1 employs a pair of antibodies, both of which have a high affinity for the target protein.

3
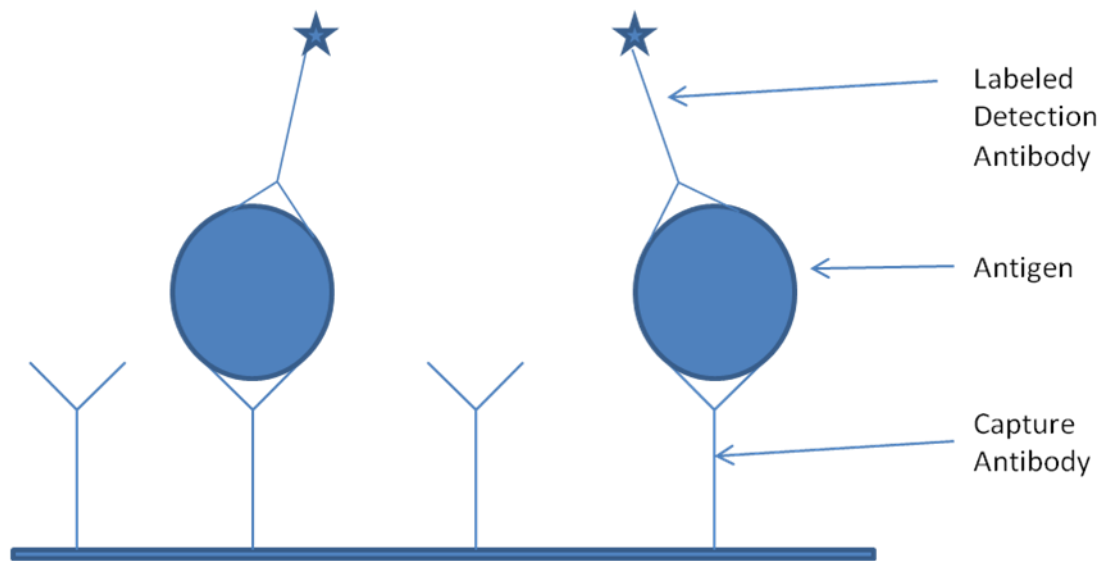
Figure 1 Schematic of Sandwich ELISA Microarray

The antibodies have different epitope specificities, allowing both antibodies to be able to bind strongly with the same molecule non-competitively. The antibody that directly attaches to the substrate is referred to as the capture antibody. The second antibody, known as the detection antibody, is labeled with a fluorescent tag. The signal from this fluorescent tag may be enhanced using an amplification step [10]. The microarrays are incubated with low volumes of sample, and the presence of the analyte is visualized, usually by using fluorescence. Using a calibrant such as GFP (green fluorescence protein) to normalize the data, the analyte concentration can be determined using standard curves of intensity versus concentration [26].

Multiple factors need to be taken into account when designing the microarray:

1) An ideal surface allows for a high signal to noise ratio for the analyte of interest as well as helps in reducing non-specific binding [22, 23, 25]. In order to fabricate a microarray, the surface substrate needs to be engineered so that these desirable properties are obtained.

2) Capture/binding molecules need to be highly specific and have strong binding affinity while displaying minimal cross-reactivity [23].

3) The detection method should have high sensitivity, accuracy, and a wide dynamic range [25].

Peptoids (poly-N-substituted glycines) may be used as coatings as well as capture molecules in slide ELISA-type microarrays.

1.3 Peptoids (Poly-N-substituted Glycines)

Peptoids are peptidomimetic molecules that share a similar structure to peptides and are able to emulate their biological activity [27]. Structurally, they are similar to α-peptides but have the side chain attached to the nitrogen atom of the backbone instead of the α-carbon (Figure 2). This structural modification causes a loss of backbone chirality and removes the ability to form hydrogen bonds, which help stabilize the secondary structure in peptides [28, 29]. Circular dichroism (CD) analysis for a peptoid pentamer displayed minima at 207 nm and 221 nm, and a maximum at 190 nm, which is similar to those observed in the spectrum of a peptide α-helix [29]. Therefore, using chiral side chains in the primary sequence, a helical secondary structure may be induced [30]. Steric hindrances and electrostatic interactions between the side chains stabilize the secondary structure [31].
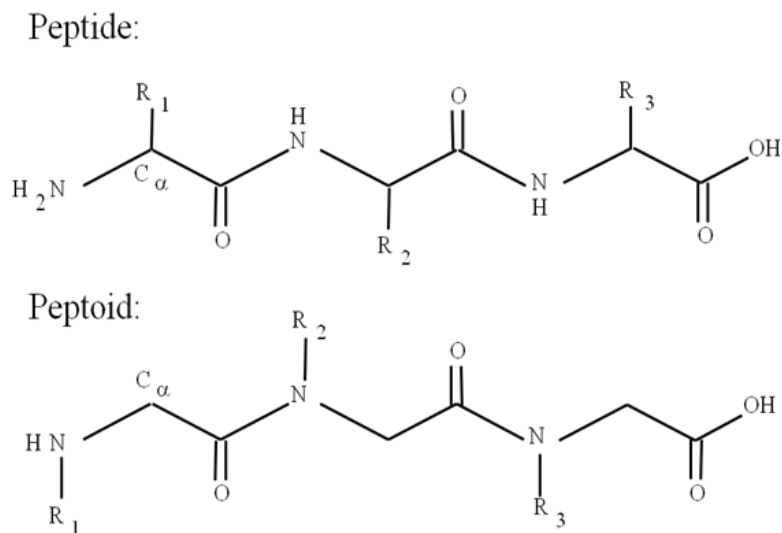
Peptide:

Peptoid:

Figure 2 Structural comparison of peptide and peptoid molecules

Wu et al. studied the chain length dependence of peptoid structure with chiral, aromatic side chains [31]. Their data corroborates that a helical structure is observed in peptoid oligomers that have as few as five monomers [32]. Their research helped establish some general guidelines for the formation of stable peptoid helices:

1) At least 50% of the side chains need to be chiral and aromatic,

2) Inclusion of a chiral, aromatic side chain at the C-terminus helps stabilize the helix,

3) A helical face that is comprised of chiral, aromatic side chains stabilizes the secondary structure [32].

Peptoids are synthesized in a modular, sequence-specific manner on a solid scaffold support (such as Rink amide resin) using an automated peptide synthesizer that has been slightly modified to accommodate the change in chemistry [33]. The submonomer synthesis technique allows for addition of a diverse group of side chains as primary amines [33]. Figure 3 depicts a

schematic of the synthesis.
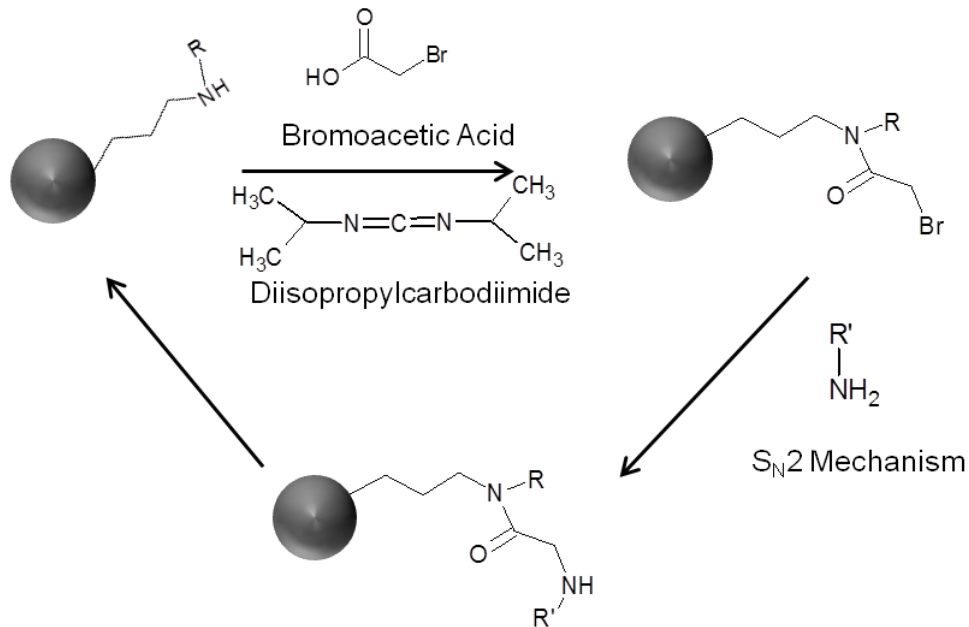
Figure 3 Schematic representation of peptoid synthesis on Rink-amide resin

1.3.1 Peptoids in Microarrays

Peptoid synthesis is modular, and their biological activity is sequence based. Since peptoids form

stable molecules, and can be modified to exercise control over their physical and chemical

properties, it is possible to customize their binding activity for proteins of interest such as certain

biomarkers [33-36]. Therefore, for a sandwich ELISA assay, peptoids can prove to be effective

replacements for antibodies.

A direct sequence transformation from peptide to peptoids may not yield the desired activity or

specificity [27]. Peptomers and peptoids have been synthesized on membranes allowing the

design of sequences with the same biological activity as a peptide of interest [37, 38]. There are

many ways to quickly and easily isolate protein ligands from large peptoid libraries, allowing for

quickly developing a library of peptoids that are specific to bind the desired analyte [39, 40].

Working with a large combinatorial library, Alluri et al. were able to isolate multiple peptoids (immobilized on beads) that bound with high affinity and distinct specificity to the proteins, Glutathione-S-transferase (GST, kD = 62 µM) and human mouse double minute 2 (Mdm2 is a negative regulator of p53 function, making it a viable target for anti-cancer drugs, kD = 37µM) [39]. These peptoids can be used as capture molecules in place of antibodies on a slide ELISA assay. These capture molecules can be used for detection of low concentrations of proteins, hence allowing for early detection.

1.3.2 Advantages of Using Peptoids

Peptoid affinity reagents can possess a significant advantage over antibodies in microarrays. A typical antibody molecule is roughly Y-shaped, with three equal-sized portions, connected by a flexible link. Antibodies are fairly large molecules, with an average molecular weight of roughly 150,000 Da [26, 51]. On the other hand, a peptoid with analogous binding affinity can be as small as 945 Da [39], which is several orders of magnitude smaller in size. This allows for many more peptoids to be printed within a given area than antibodies, even when attached to a linear linker as large as 10 kDa. Taking into account the smaller size as well as linear shape of the molecule, this translates into an enormous increase in binding sites on the slide, and therefore should correlate with a greater dynamic range. Another advantage of having increased binding sites is the possibility of having smaller spots, and therefore allowing a larger variety of affinity peptoids to be printed, and thus, screening for a larger pool of analytes from a single sample. Of course, care would have to be taken to avoid issues with cross reactivity.

1.3.3 Modifying Slide Surface with Peptoids

In addition to engineering a peptoid analog for antibodies to certain analytes, it is also possible to improve the system by modifying the surface chemistry of the glass slide. One way to accomplish that is to increase the available surface area on the slide by coating with microspheres, which would allow for more capture molecules to be printed (Figure 3).
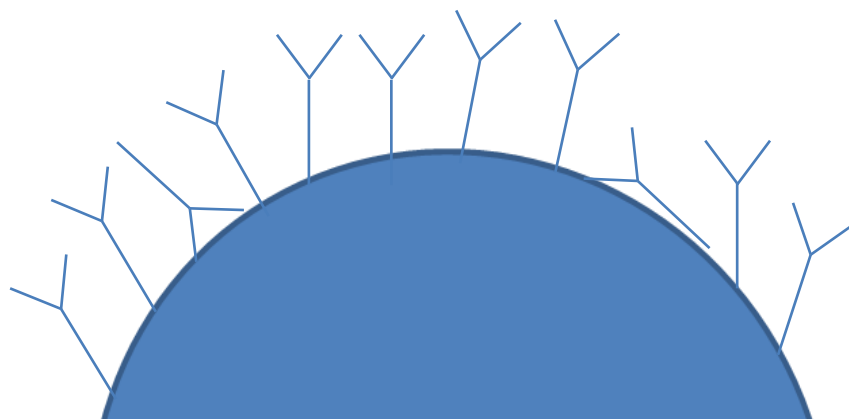
Figure 4 More antibodies can be attached to a curved surface as compared to a flat surface

Many peptides that have short sequences with aromatic side chains tend to self-assemble into microstructures under certain solvent or temperature conditions [41]. This research has been extended to peptoid structures which have been found to self-assemble into a variety of structures [42-44]. The effects of partial water solubility, helicity, charge distribution, and side chain bulk on multiple peptoid sequences were examined in order to study self-assembly of sequences into supramolecular structures [45]. This makes it possible to tune the peptoid structure to form the desired microsphere structure, when under optimal physical and chemical conditions.

2. Determining effect of side chain chemistry and sequence on formation of peptoid microspheres

2.1 Rationale
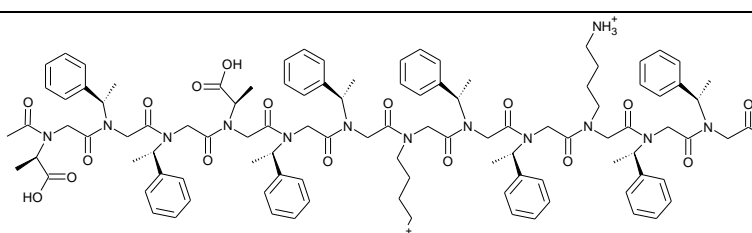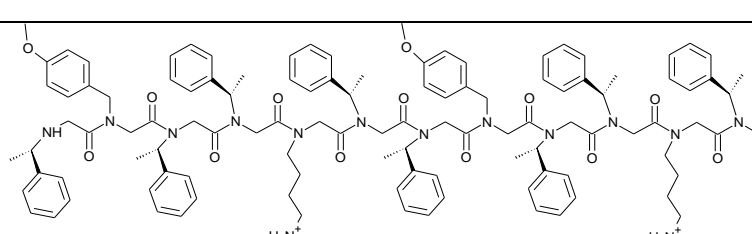
Reches and Gazit synthesized peptide sequences with short aromatic side chains that self-assembled spontaneously into closed-cage nanospheres when diluted from an organic solvent to an aqueous one [41, 46]. The ability of short peptides to form these assemblies allows for the possibility of the same being done with peptoids. Previous studies in the Servoss group have shown that it is possible to design peptoid sequences that self-assemble into spheres under specific conditions. Deposition of the peptoid in a mix of organic/aqueous solution on a silicon microchip leads to the formation of spheres due to the partial water solubility of the molecules in the solvent. We chose to employ peptoids to build these microspheres because they are inexpensive to synthesize, they form stable structures, and their modular synthesis allows for ease of scale-up as well as hig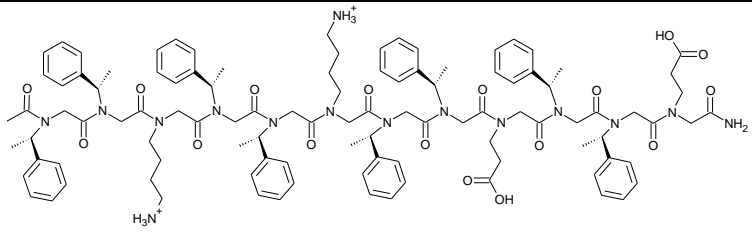h customizability [29, 33]. They are known to self-assemble into multiple different tertiary structures such as crystalline sheets, ribbons, superhelices, and nanosheets assembled from monolayers [41].
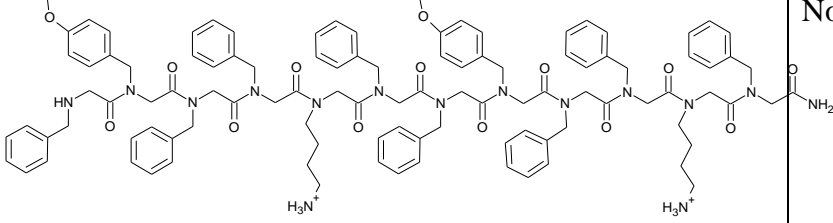
2.2 Preliminary Data

Partially hydrophobic peptoids that have been previously synthesized in the Servoss lab were shown to self-assemble into microspheres when dissolved in an organic/water solution and allowed to air dry on a solid substrate. The peptoid microspheres were formed on both silicon wafers and glass slides. The structures shown in Table 1 were previously studied by Hebert [47]. P4 was designed to determine whether peptoid helicity was a requirement for self-assembly into microspheres. P4 has the same side chains as P3, but lacks the chiral center found on the aromatic side chains that induces helical secondary structure. It is partially soluble in water, but lacks the ability to form a helical structure. The charge states of those peptoids were determined

by pKa values of the amino acid side chains, and the measured pH of the organic/aqueous

solutions used (ranging from 3-4) [47, 48].

Table 1 Peptoid structures with molecular weight (MW), and percent acetonitrile elution from analytical HPLC (% Elution) [50]

Name	Structure	Properties
P1	MW: 1865 Da, % Elution: 80.6	Helical, positive groups near C-terminus, negative groups near N-terminus
rP2	MW: 1865 Da, % Elution: 83.8	Helical, negative groups near C-terminus, positive group near N-terminus
P3	MW: 1919 Da, % Elution: 82.4	Helical with 3rd face containing positively charged and aromatic ether groups
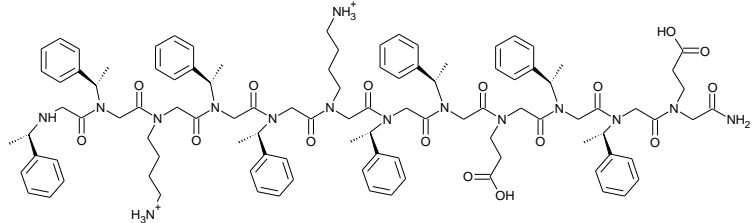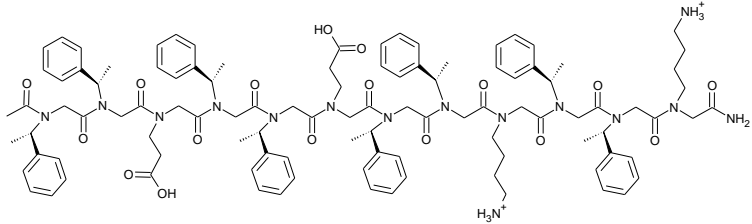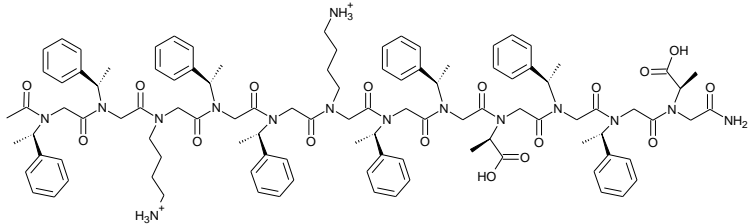
Name	Structure	Properties
P4	 MW: 1807 Da, % Elution: 75.0	Non-Helical

P3 and rP2 formed microspheres when allowed to dry on silicon chips after dissolved in a 50:50 organic:aqueous solvent, while the other sequences did not do so. These experiments examined the effects of hydrophobic and aromatic stacking interactions with the organic/aqueous solution, with the hypothesis that the tertiary structures will be determined by the lowest energy structure that can conceal the hydrophobic faces of the helical peptoid, allowing the polar faces to be on the outside and interact with the aqueous solvent [47].

2.3 Charge Placement and Side Chain structure

The effect of charge placement, and side chain structure on formation of microspheres had to be examined more closely. The following peptoids were designed to examine these factors (Table 2).

Table 2 Peptoid structures with molecular weight (MW), and percent acetonitrile elution from analytical HPLC (% Elution) [50]

Name	Structure	Properties
wP1	 MW: 1823 Da % Elution: 76.0	Acetylated version of P1 (reduced hydrophobicity)
rP1	 MW: 1865 Da, % Elution: 81.1	Reversed sequence as P1
P2	 MW: 1865 Da, % Elution: 83.9	Reversed sequence as rP2

Sequences "wP1" and "P1" are similar with the only difference being that the N-terminus of P1 is acetylated. This modification to the structure reduced hydrophobicity of P1 and complete solubility in water. Sequences wP1, rP1, P2 and rP2 were designed in order to compare them to P1 and P2 in order to determine the effect of charge distribution and side chain structure on the desired self-assembly into microspheres.

2.4 Materials and Methods

The peptoid sequences were synthesized using the submonomer protocol on an ABI 433A (Biolytic Lab Performance Inc, Fremont, CA) automated peptide synthesizer. Rink amide resin (substitution ration ~0.56 mmol/g) was initially swelled with dimethylformamide (DMF, Fisher Scientific), and the Fmoc protecting group on the resin was removed using a 20% solution of piperidine (Sigma Aldrich) in DMF. The secondary amine was acylated with the addition of 1.2 M bromoacetic acid (BAA, Sigma Aldrich) in DMF, catalyzed with N, N'-diisopropyl carbodiimide (DIC), and mixing and vortexing for 1 hour. The reaction vessel was drained, and resin rinsed with DMF to eliminate leftover reagent. The desired side chain needed for the sequence was added by delivering 1 M of the primary amine in DMF, and incubating with intermittent vortexing for 90 minutes. The bromoacetylation and amine addition steps were repeated until all side chains were added. The N-termini of P1, rP1, P2, and rP2 were acetylated with 50-fold molar excess of acetic anhydride in order to increase their hydrophobicity. After the synthesis was completed, the resin was washed three times with dichloromethane (DCM, Fisher Scientific) in order to remove residual impurities and allow it to dry. The product was cleaved from the dried resin using a mixture of 95% trifluoroacetic acid TFA, 2.5% water, and 2.5% triisopropylsilane (TIS) for 10 minutes. The acid was removed using a Heidolph Laborota 4001 rotating evaporator (Elk Grove Village, IL), and the samples suspended in a 50:50 solution of acetonitrile:water to achieve a concentration of ~3 mg/mL. The peptoids were purified using a Waters Delta 60 preparative HPLC (Milford, MA), with a Duragel G C18 150x20 mm column (Peeke Scientific, Novato, CA), at a gradient of ~1%/min (gradient was adjusted for optimal

separation) using solvents A and B (A: water, 5% acetonitrile, 0.01% TFA; B: acetonitrile, 5%water, 0.01% TFA). Purity of >97% was confirmed by analytical HPLC (Waters Alliance, Milford, MA) using a Duragel C18 150 x 2,1 mm column (Peeke Scientific, Novato, CA), and a linear gradient of 5-95% acetonitrile in water, over 30 min. Molecular weight (MW) of purified product was measured using MALDI-TOF mass spectrometry (Bruker Daltonics Ultraflex II, Billerica, MA). The measured molecular weight matched the expected mass of the molecule, hence confirming successful synthesis. Purified solutions were dried using a Labconco lyophilzer (Kansas City, MO).

Circular dichroism (CD) was performed using a Jasco J-715 (Easton, MD) at room temperature, with a path length of 0.1 cm and a scanning speed of 20 nm/min. The samples used for CD were dissolved in pure methanol at a concentration of ~100-120 μM. Each spectrum was the average of twenty accumulations.

SEM was performed using a Phillips XL-30 environmental scanning microscope (FEI, Hillsboro, OR). Microsphere peptoid solutions were placed on 1cm x 1cm silicon wafer chips allowing the solution to coat the surface completely with a volume between 20-50 μL, and left to dry in open air by placing the chips in a Petri dish, and letting the solution evaporate on the lab bench top at ambient conditions.

2.5 Results and Discussion

P3 was designed to show that it is feasible to coat slides for biosensor applications. It was synthesized with alternating positively charged and aromatic ether groups on the third face of the helix. The aromatic groups increase bulkiness, and contribute to the partial water solubility. All sequences except P4 were designed to have a helical secondary structure. Their helicity was

confirmed by circular dichroism (Figure 5). The spectra of the sequences present poly-proline type-1-like helical secondary structures, with characteristic minima at 200 nm and 220 nm. P2 and rP2 formed microspheres with average diameters of 0.46 ± 0.19 μm and 0.34 ± 0.19 μm respectively. P1, rP1, P4 and wP1 did not form microspheres. The SEM images of P1, rP2, P3, and P4 (Figures 6-9) were taken from samples prepared by Hebert as part of her Master's thesis [48].
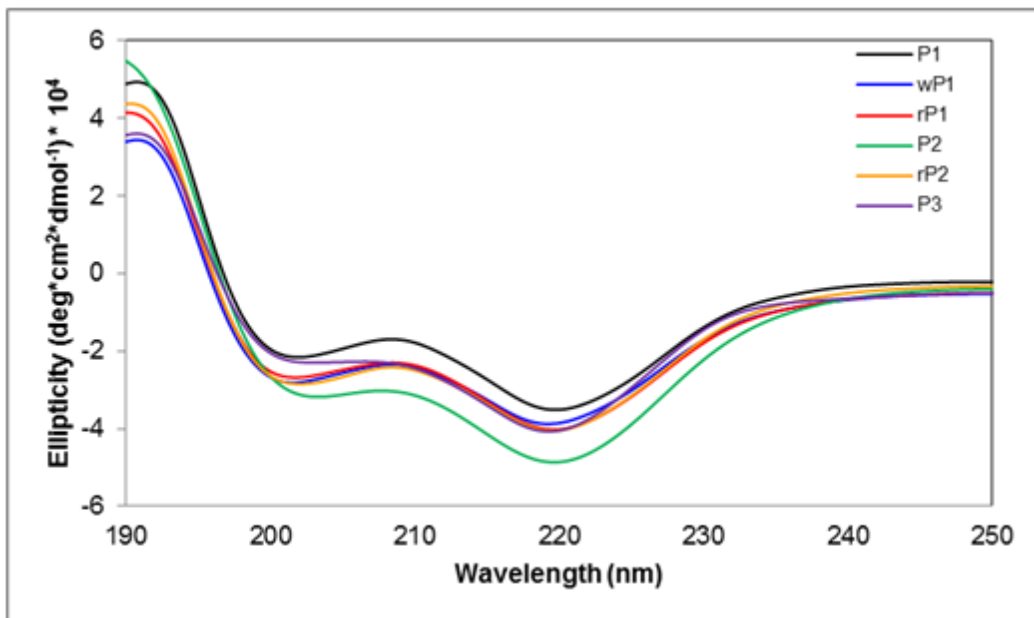
Figure 5 Circular dichroism spectra of P1 (black), wp1 (blue), rP1 (red), P2 (green), rP2 (yellow), and P3 (purple). The spectra show a poly-proline type-1-like helical secondary structures [50]
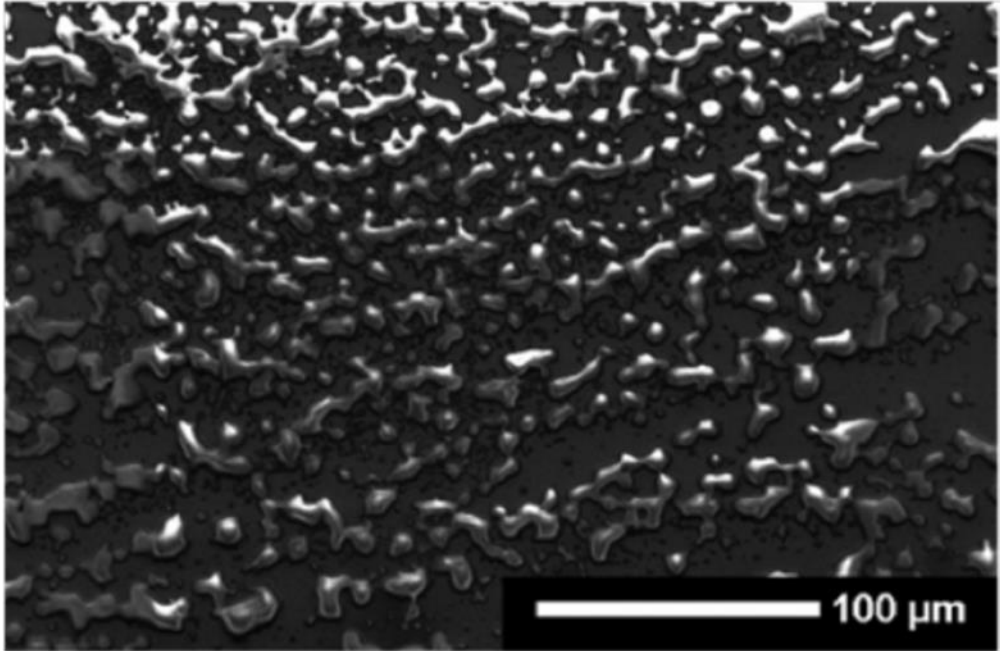
Figure 6 SEM image of wP1, 3 mg/mL, 4:1 ethanol/water [50]
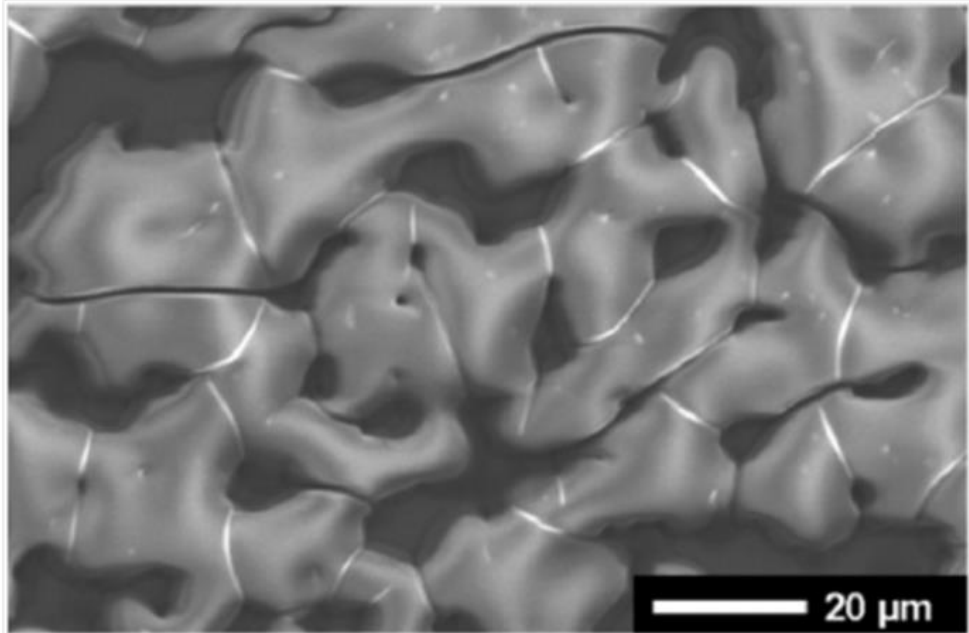
Figure 7 SEM image of P4, 3 mg/mL in 4:1 ethanol/water [50]
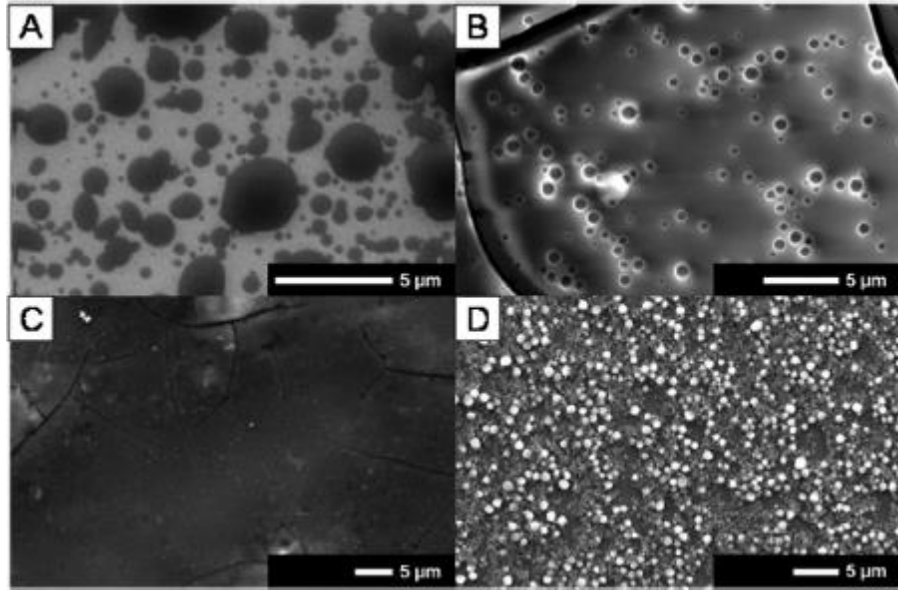
Figure 8 SEM images of P1 (A), P2 (B), rP1 (C), and rP2 (D). All samples dissolved in 4:1 ethanol/water at a concentration of 3 mg/mL [50]
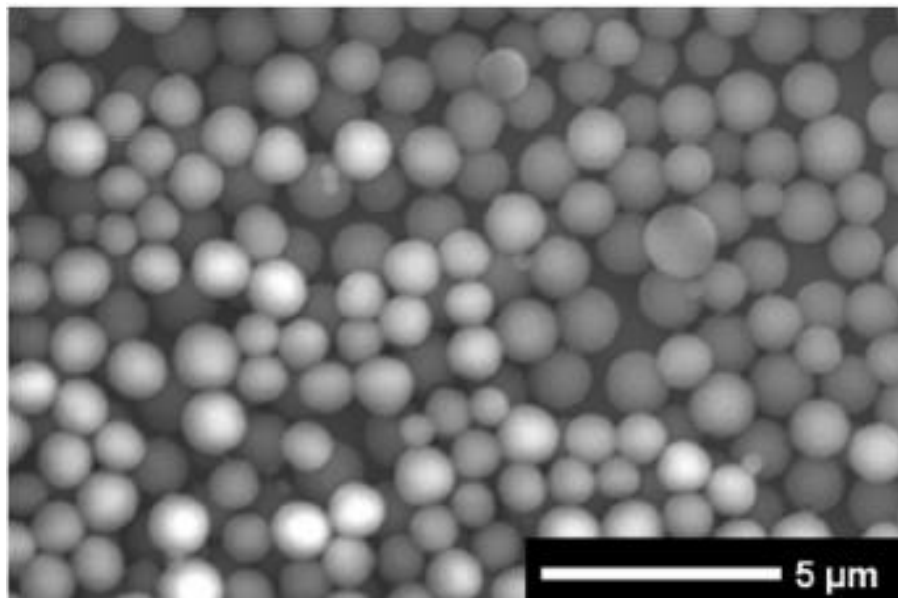
Figure 9 SEM images of P3. 3 mg/mL, 4:1 ethanol/water Scale bar: 20 µm [50]

The results suggest that for the concentrations and solvent used, partial water solubility, charge

placement and side chain bulk play an important role on the formation of peptoid supramolecular

structures. The non-helical control sequence (P4) did not form spheres. P4 was designed as a non-helical peptoid with a sequence similar to the helical P3. This shows that helical content is a contributing factor in the formation of microspheres. The stacking of the aromatic groups on the two faces of the peptoid helix is most likely responsible for the formation of spheres, as seen in figure 9. The formation of such structures has been previously linked to such stacking in peptides as well as peptoids [41, 46, 48].
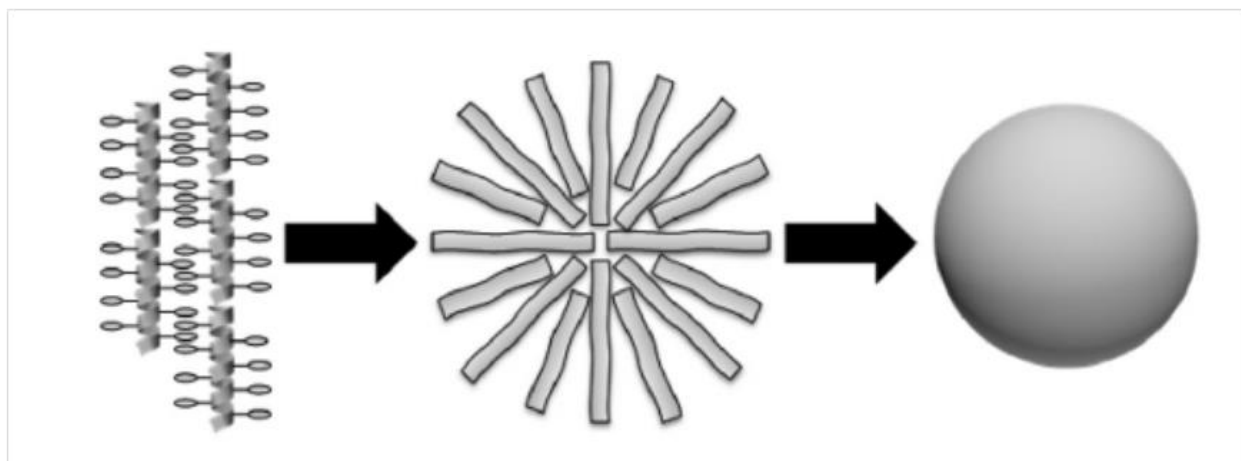
Figure 10 Schematic Representation of peptoid microsphere formation [50]

The microspheres formed range in size (diameter) from ~0.3 – 3 μm. This is much larger than the size of a single peptoid helix (~24 Å). We hypothesize that the molecules are accumulating with one another along the length of the helical axis, which then assemble into stable microspheres through aromatic stacking and hydrophobic effects. When dissolved in the ethanol/water solvent, aromatic groups will be hidden in the core of the spheres, while most of the polar groups will be exposed to the solvent in order to form the lowest energy structure. Microspheres formed by P3 were approximately ten times larger than those formed by rP2. The MW difference between the two peptoids is 54 Da. The fewer number of peptoids in the rP2 spheres can be explained by the interactions between the charged groups and the polar solvent.

Based on the results, we have learned what parameters can be adjusted in order to tune the formation of microspheres. This can be done by selecting side chains with the desired properties on the third face of the peptoid helix. Using negatively and positively charged groups, where the negative side chain is chiral, compels the formation of small microspheres. Using bulkier, aromatic side chains leads to the formation of larger microspheres. Combining these side chain permutations with varying peptoid lengths makes it possible to tune the formation of peptoid microspheres with the desired diameter and surface chemistry for multiple applications. One such application would be as a coating for slide microarrays in order to increase available surface area with customized chemistries.

3. Effect of Solvent Selection on Slide Coatings [46]

3.1 Rationale

We have been able to successfully synthesize peptoid sequences that self-assemble into microspheres. Multiple sequences were synthesized and we were able to study the effect of helical structure, charge placement, and side chain bulk on the formation of the microspheres as well as the size of the spheres formed. One application of these self-assembling microspheres would be to use them as coatings for slide microarrays. An ideal coating should provide:

a) A high surface area allowing for an increased number of binding sites

b) Resistance to biofouling, and

c) Easily customizable chemistries.

Peptoid (poly-N-substituted glycines) sequences that self-assemble into microspheres are a suitable candidate for the desired coatings. The effect of multiple protocols on the uniformity of

peptoid microsphere coatings was examined. Type of solvent, administration technique, and drying methods were varied. The variation in uniformity of the microsphere coating was examined to determine the ideal coating technique.

3.2 Preliminary Work

Previous work had been done by Hebert et al. on the effect of application technique and drying method used. Two peptoid sequences, rP2 and P3 (figure 10) that formed microspheres in previous experiments were chosen for the study.
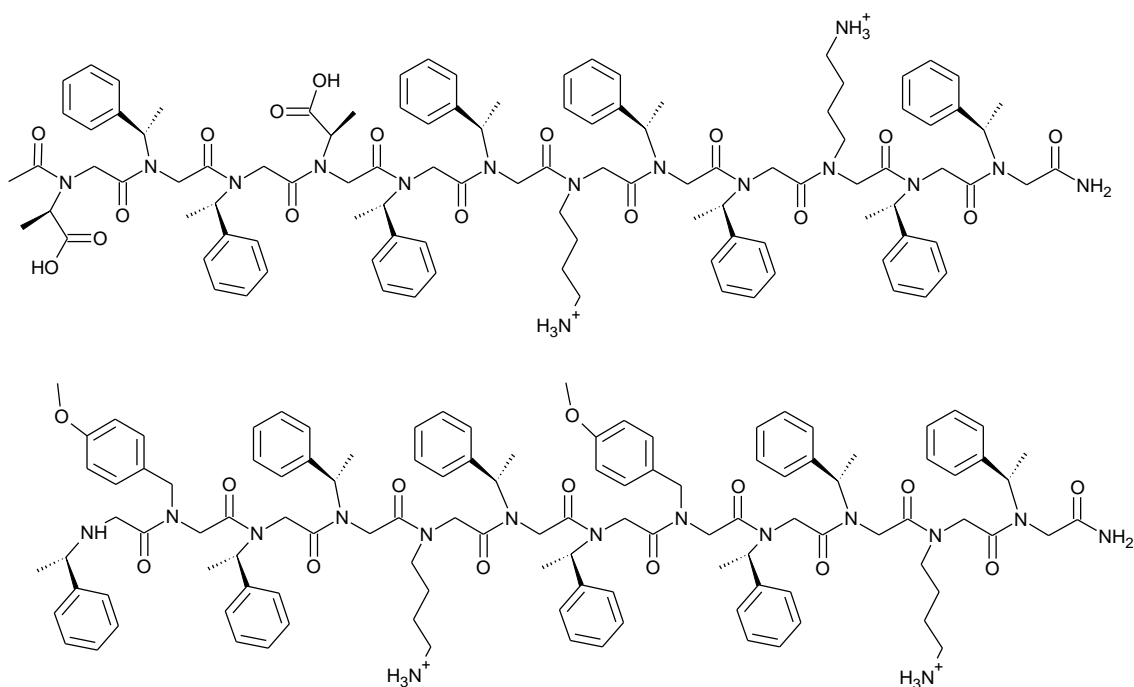
Figure 11 Chemical structures of peptoids rP2 (top) and P3 (bottom) [46]

Sequences rP2 and P3 both have two chiral, aromatic functional groups that induce a secondary helical structure. The third face contains negatively or positively charged groups in rP2, and aromatic ether groups and positively charged side chains in P3.

3.3 Methods and Materials

Peptoids were synthesized, characterized and purified using the same methods and materials as elucidated in section 2.4. The dried, purified peptoids were dissolved in a 4:1 organic:water solution, the organic solvents being methanol, ethanol, isopropanol, and acetonitrile. The solutions were dried on silicon wafer chips (~1cm x 1cm) using a combination of administration and drying techniques enumerated in Tables 3 and 4 [46,48].

Table 3 Administration Techniques Used [46]

	Administration Technique	**Description**
1	Pipette Spot	Small volume (~5 μL) placed in middle of chip using a pipette.
2	Full Coverage	~20-50 μL applied to chip via pipette and fully coating the surface.
3	Dip	Submerging chip in the sample and removing it vertically.

Table 4 Drying Methods Used [46]

	Drying Method	Description
1	Open Air	Chips placed in an uncovered Petri dish and allowed to dry at room temperature.
2	Humidity Chamber	Chips placed in a closed container with a constant humidity of 60% and allowed to dry.
3	Vacuum Chamber	Chips placed in a sealed chamber and house vacuum applied to it until dry.

Three different protic solvents and one non-protic solvent were employed in order to examine their effectiveness in inducing uniform, robust coatings. Protic solvents help stabilize the secondary helical structure of peptoids via hydrogen bonding. Therefore, it was expected that they would yield more stable structures. To test the solvent performance, both peptoids were dissolved at a concentration of 3 mg/mL in the solvents shown in Table 5.

Table 5 Solvent Conditions

	Organic Solvent	Solvent Type	Organic:Aqueous ratio

1	Methanol	Protic	4:1
2	Ethanol	Protic	4:1
3	Isopropanol	Protic	4:1
4	Acetonitrile	Aprotic	4:1

The coatings were analyzed using a Phillips XL-30 (FEI, Hillsboro, OR) environmental scanning electron microscope (ESEM) in order to visualize the chip morphology and establish what methods yield the best (most uniform and robust) coating on the chip. This study was a continuation and addendum to work performed by Hebert in 2012 [47]. Hebert's work concluded that substrate coating method as well as drying had a large effect on how uniform the coating was. Consistent coating was obtained by spot technique and full coverage [48]. The administration, and drying methods were explored by Hebert.

3.4 Results and Discussion

Based on previous studies, we are aware that both sequences should self-assemble into microspheres. The SEM images of P1, rP2, P3, and P4 were taken from samples prepared by Hebert as part of her Master's thesis [48]. P3 forms spheres about 10 times larger (3.2 ± 1.6 μm) than those formed by rP2 (0.34 ± 0.19 μm). We believe that this is due to the negatively and positively charged side chains on rP2 to form a tighter assembly.
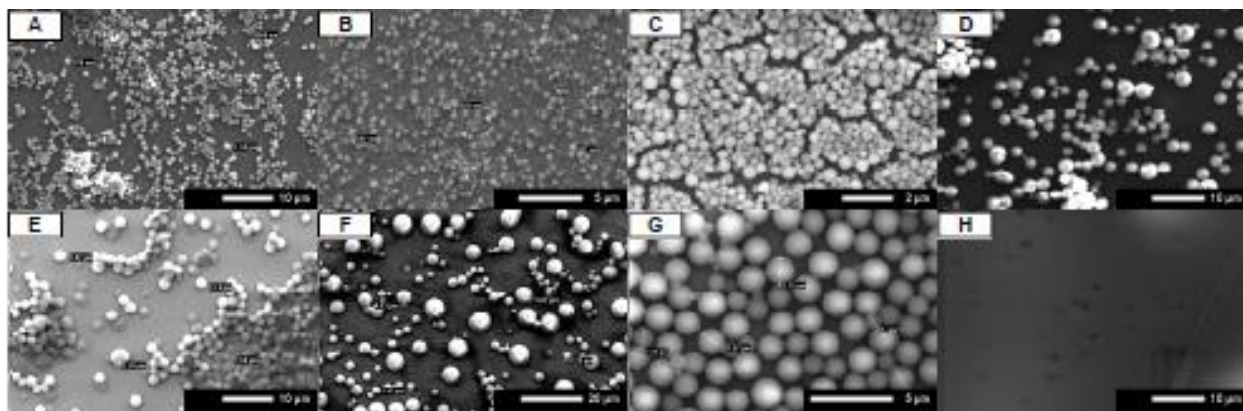
Figure 12 SEM images of rP2 (A-D), and P3 (E-H) in the 4:1 organic/water solution: methanol (A and E), ethanol (B and F), isopropanol (C and G), and Acetonitrile (D and H) [45]

The SEM images in figure 11 show that both peptoids form microspheres in all the protic solvents, which reinforces our hypothesis. Uniform coverage is seen with both ethanol and isopropanol. The spheres formed with methanol are not uniformly spread on the surface. rP2 forms spheres in acetonitrile as well but the coverage is spotty and the spheres don't have a consistent size. P3 did not form any spheres in acetonitrile. Therefore, we can confirm that protic solvents yield a uniform coating of the peptoid microspheres
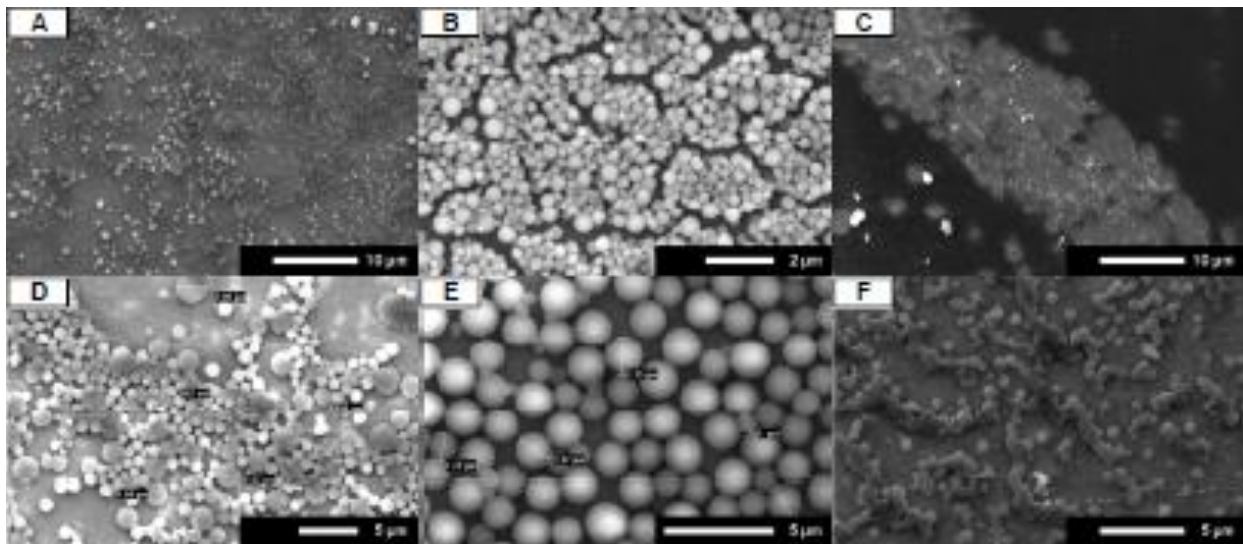
Figure 13 SEM images for rP2 (A-C) and P3 (D-F). Administration techniques were pipette spot (A and D), full coverage (B and E), and dipping (C and F). The peptoids dissolved in 4:1 isopropanol:water and allowed to dry in an open dish at ambient conditions [45]

The SEM images (figure 12) demonstrate a noticeable difference in uniformity of microsphere coverage between the administration techniques used for both the peptoid sequences. Both pipette spot as well as full coverage resulted information of microspheres. However, only the full coverage method yielded a consistent, uniform coating for both rP2 and P3. The dip method did not produce distinct microspheres let alone yield a uniform coating. This suggests that the peptoid spheres do not attach to the surface until the solvent starts evaporating from the surface. Therefore, we can determine that the full coverage technique is ideal for the formation of a uniform microsphere coating.
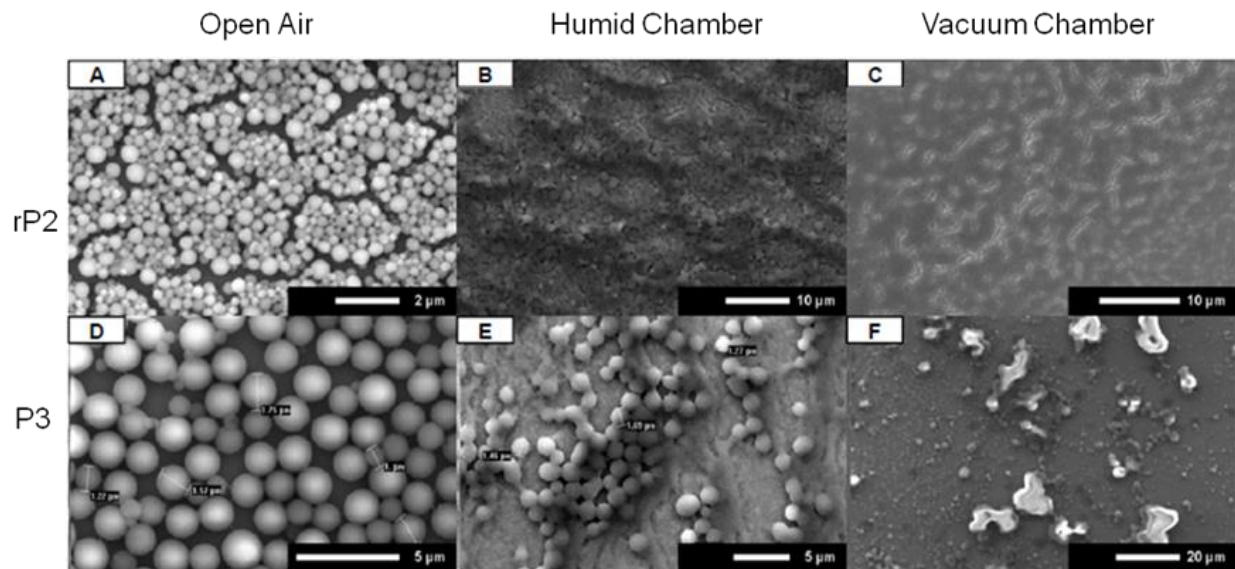
Figure 14 SEM images for rP2 (A-C), and P3 (D-F), using the drying techniques: open air (A and D), humidity chamber (B and E), Vacuum Chamber (C and F). The peptoids were dissolved at 3 mg/mL in 4:1 isopropanol:water and administered via full coverage [45]

The open air environment had a drying time of about 1 hour, the humidity chamber (60%) had a drying time of 3-4 hours, while the vacuum chamber had a drying time of ~ 5 minutes. Both rP2 and P3 formed uniform coatings in open air conditions. The vacuum chamber (fast drying) as well as the humidity chamber (slow drying) yielded poor formation of uniform coatings. It was observed that in the humidity chamber, both peptoid solutions started drying inwards from outermost edges of the chip. The increased microsphere density at the center supports that observation. The rapid drying in the vacuum chamber led to a spotty coating with rP2, and no microspheres were formed with P3 at all. Therefore, we can associate open air drying with an ideal coating.

In addition to the work done by Hebert, the drying times for the peptoids were measured, and a more robust vacuum system for drying was built to test the effect of the vacuum. The work on the aprotic solvent mixture was also performed independently with the full coverage method

using all three drying methods in table 4 in order to establish the importance of solvent selection. The conclusions obtained in this study were used to optimize the techniques used to obtain the data in section 2.4.

Based on these results, we can conclude that the best coatings are obtained with using a protic solvent, applying the peptoid solution using the full coverage technique, and drying in open air.

4. Peptoids as Capture Affinity Reagents

4.1 Rationale

Current biomarker validation technology uses DNA, single-stranded oligonucleotides and antibody (monoclonal IgG) probes immobilized on slides. Single-chain antibodies have been shown to be a more effective alternative to monoclonal IgG because their smaller size allows for a higher density placement on arrays [29, 48]. They possess the variable light and heavy-chain regions from IgG that are connected by a short linker. However, since they lack disulfide linkages and constant domain, they usually have a lower structural stability, which is a significant drawback for immobilization on a slide [50]. This issue may be addressed by the use of N-substituted glycines (peptoids) [29] as capture agents on the microarray surface.

It is possible to design peptoid sequences that exhibit binding activity similar to that of antibodies. Alluri *et al.* have discovered sequences that bind to GST (glutathione-S-transferase) and mdm-2 (mouse double minute-2), when the peptoids were immobilized on a bead. Our goal is to develop a peptoid based biomolecule detection system that can be used to efficiently and rapidly screen for biomarkers. Therefore, in addition to microsphere coatings, we can also

employ peptoid based molecules that can be used for immobilizing an analyte of interest on a slide in order to perform a qualitative as well as quantitative assay.

4.2 Peptoid DS1 to bind GST

Peptoid DS1 (Fig. 15) is a slightly modified sequence from the GST-binding peptoid discovered by Alluri et al. [40]. The modification is addition of an alkyne group (by addition of a propargylamine side chain) to the N-terminus. This allows for the addition of a functionalized amine-PEG-azide group via copper catalyzed click-chemistry (technique in appendix B). The PEG is added to the peptoid in order to provide a linker to the slide, helping reduce steric hindrances from the molecule being too close to the surface of the slide when printed. Also, the linker helps orient the peptoid in the appropriate direction.
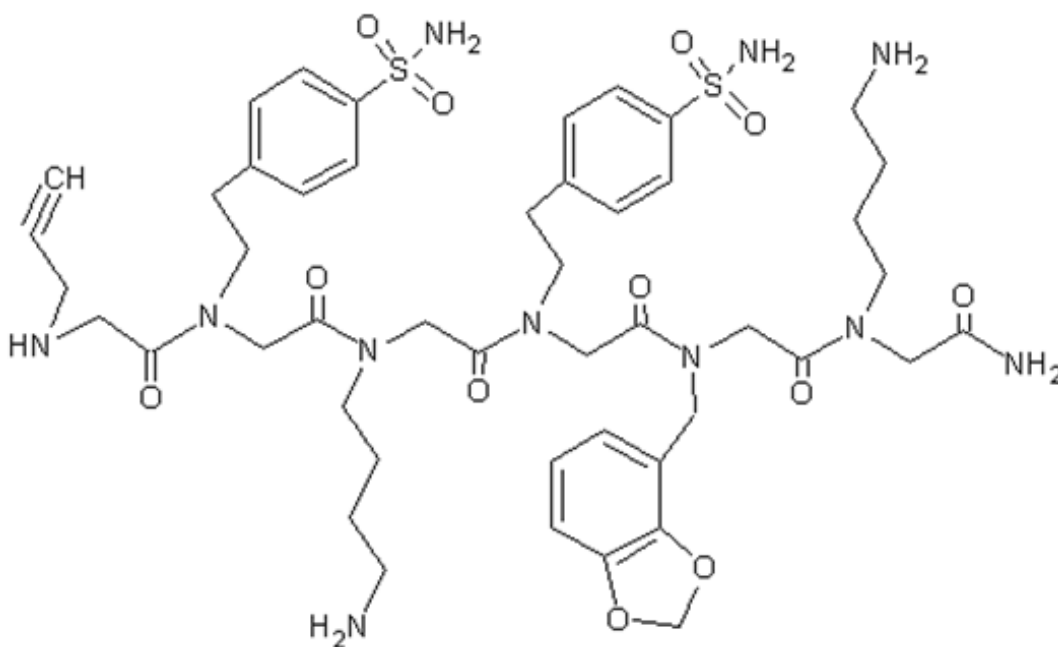
Figure 15 Peptoid DS1 designed to bind specifically to GST, MW: 1040 Da
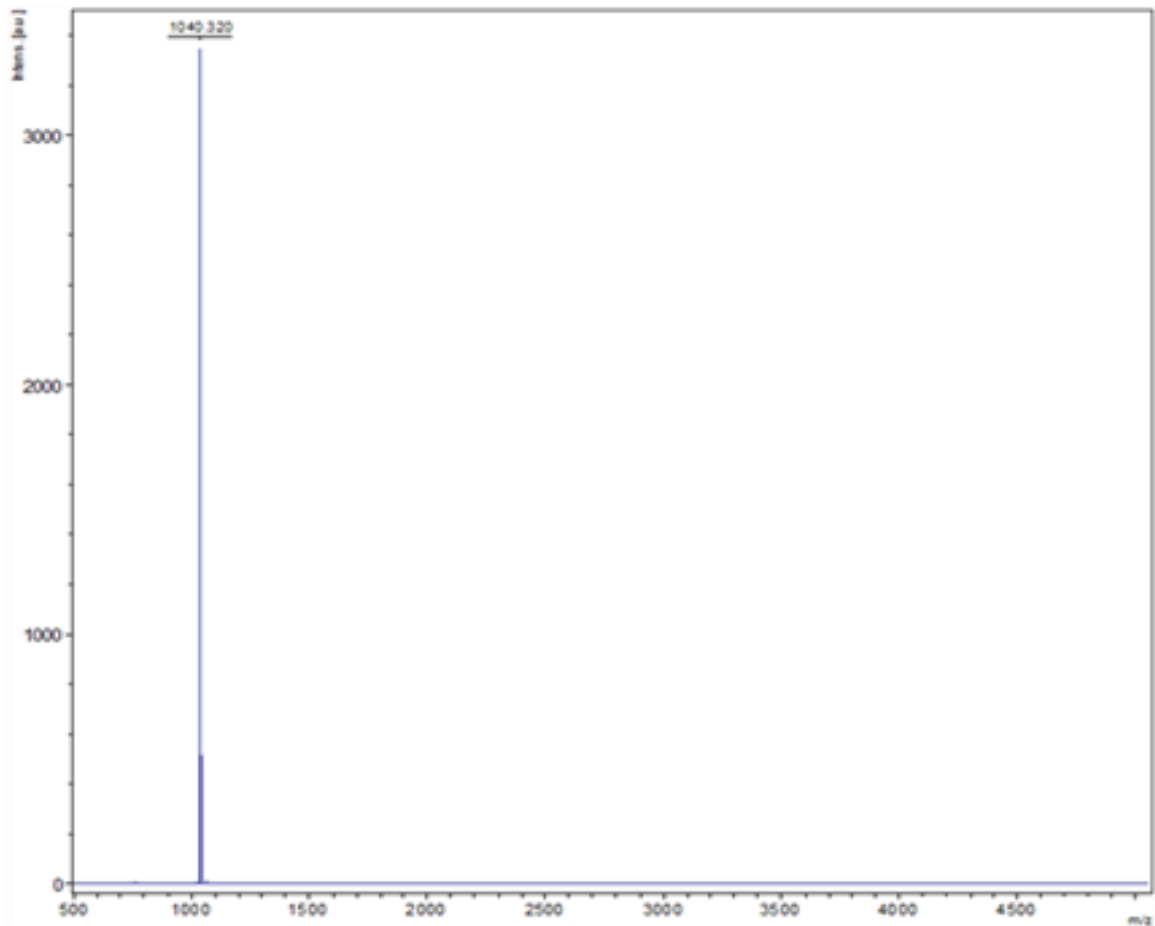
Figure 16 MALDI spectrum of peptoid DS1, MW: 1040 Da

Figure 16 shows the MALDI spectrum of purified peptoid DS1 indicating the expected

molecular weight of 1040 Da. Figure 17 is the analytical HPLC trace (214 nm) of a 1 mg/mL

solution in 50:50 acetonitrile/water solvent, run at a gradient of 5-95% acetonitrile over 30

minutes on a C-18 column.
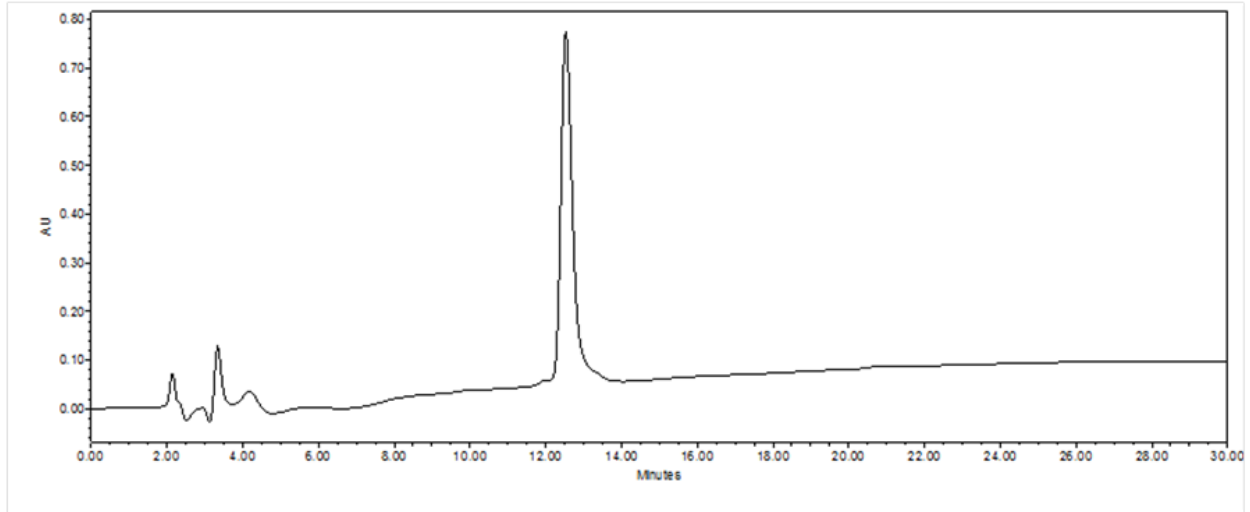
Figure 17 Analytical HPLC trace of Peptoid DS1 (5-95% Acetonitrile, 30 mins, C-18)
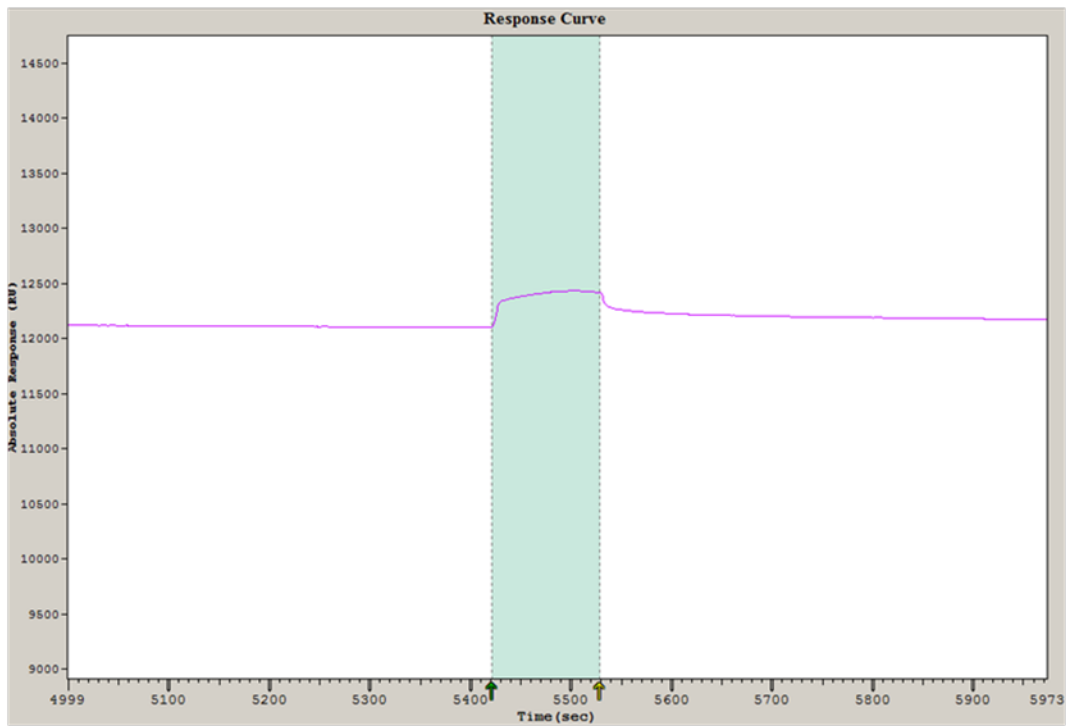
Figure 18 SPR response curve of channel with immobilized GST after addition of PEG-DS1

Positive binding was confirmed (indicated by the increase in response units as seen in Figure 18) when the PEG-DS1 construct (45 μg) was allowed to flow over the GST-bound surface. Therefore, we know that the construct is able to bind to the protein of interest.

4.2.1 Methods

The peptoid with the alkyne end group (from propargylamine), and the PEG molecule were individually dissolved in a 1:1 solution of *t*-BuOH/water to a concentration of roughly 4 mM. To a round bottom flask equipped with a magnetic stirrer, equimolar amounts of both compounds were added, along with 2x molar solution of L-(+)-sodium ascorbate, and 0.5x molar solution of $CuSO_4.5H_2O$. The reaction mixture was stirred overnight under nitrogen. When the reaction was completed, the reaction mixture was dialyzed in Slide-A-Lyzer (molecular weight cut off: 10 kDa) dialysis cassettes (Pierce, Rockford, IL) for 3 days in a 9:1 water/ethanol solution (1 L). The solvent was changed after 2 h, 4h, 1 day, and 2 days. The sample remaining in the dialysis cassette was lyophilized, and resuspended to a concentration of 0.8 mg/mL in PBS (NERL Diagnostics).

The PEG-DS1 construct was purified after click chemistry by dialysis using a membrane with a molecular weight cut off (MWCO) of 2000 Da. A COOH-2 SPR chop (SensiQ, Oklahoma City, OK) was activated by flowing 100 mM N-Hydroxysuccinimide (NHS) and 50 mM ethyl(dimethylaminopropyl) carbodiimide (EDC), both in in HEPES buffer. GST (10 μg) was then bound to the chip by allowing it to flow over the activated chip.

4.3 Testing PEG-DS1 on Microarray Slides

The PEG-DS1 construct was then tested in a slide-microarray setting. Lysine coated glass slides were treated with a homobifunctional crosslinker in order to create a binding surface for the

peptoid. For this purpose, DSS (disuccinimidyl suberate, Pierce, Rockford) was used. The amount of DSS needed for a final volume of 0.2 mg/mL was dissolved in 0.5-1.0 of DMSO (dimethyl sulfoxide, Fisher Scientific) in order to facilitate its solubility in methanol. The appropriate volume of HPLC-grade methanol (Fisher Scientific) was placed in an incubation chamber, and the DSS-DMSO solution was added to it.

Slides were placed in a slide rack, and immersed in the solution for 5 minutes at room temperature. The slides were then rinsed twice in fresh HPLC-grade methanol and centrifuged dry at 500xg for 30 seconds in an Eppendorf centrifuge 5430 (Beckman Coulter, Indianapolis, IN) designed to spin multi-welled microplates.

4.3.1 Experimental Method

For this experiment, we were comparing the PEG-DS1 constructs to commercially available anti-GST monoclonal antibodies (Rockland Immunochemicals, Gilbertville, PA). PBS (phosphate saline buffer) was used as a negative control. A-647 (Alexis-647) was used as a positive control. Unconjugated peptoids, and just the PEG scaffolding were also used as controls in order to confirm sequence-specific binding. Anti-GFP (green fluorescent protein) antibody was printed on the slide as well in order to be able to use GFP as a quantitative calibrant.

All capture molecules were suspended in PBS to a concentration of 0.8 mg/mL. 15 µL of each solution was dispensed into a 384-well flat bottom plate in the order they were to be printed. For spotting the molecules on the slide, the non-contact printer GeSiM NanoPlotter 2.1 (GeSiM, Bautzner Landstraße, Germany) was used. An external humidifier was employed in order to maintain humidity at ~60%, and the plate cooler was set at 15 ºC, which was 1-3 ºC above the

dew point. This was done in order to prevent condensation on the slides and evaporation of capture reagents.

After printing, the slides were left in the printer at 60% humidity for one hour in order to allow for completion of the immobilization reaction. Following that, the slides were imprinted with a wax printer in order to create hydrophobic barriers between blocks of arrays. The slides were then immediately immersed in a 10 mg/mL casein in PBS block solution (Bio-Rad, Hercules, CA) for an hour at room temperature in order to block non-specific binding sites.

The slides were then immersed in a 0.05% solution of Tween 20 (Sigma Aldrich) in PBS (PBS-T), and incubated for 2-5 minutes. Extra fluid was aspirated from the chips. Standard solutions of antigen mix (GST, and GFP) were made in 0.1x casein block solution, and diluted fourfold with seven serial dilutions as well as an antigen-free blank. Maximum concentration of GST was varied from 20,000-100,000 pg/mL. The GFP concentration was kept constant throughout all samples, including the blank, so it can be used as a calibrant. 15 µL of antigen sample was added to the chips, each in triplicate. Incubation was performed in a humid chamber under low light-conditions on a Belly Dancer shaker (Cole Palmer, Vernone Hills, IL) for 12-16 hours.

After incubation, the slides were immersed in PBS-T, transferred to fresh PBS-T and incubated for 2-5 minutes. A solution (25 ng/mL) of biotinylated detection polyclonal antibodies specific for GST, GFP, and Mdm-2 antibodies (Rockland Immunochemicals, Gilbertville, PA) was prepared. The slides were incubated for 2 hours on the Belly Dancer under previously described conditions, and then immersed in PBS-T. They were then transferred to fresh PBS-T, and incubated for 2-5 minutes. The blocks on the slides were then treated with StAv-HRP (streptadivin-horse radish peroxidase), diluted 1:99 in PBS-T and incubated for 30 minutes. The

slides were rinsed once again with PBS-T, and then transferred to fresh PBS-T and allowed to incubate for 2 minutes, and then the wash repeated twice.

Excess fluid was aspirated from chips, and then 15 µL of biotinyltyramide (Perkin Elmer), diluted 1:99 in amplification diluent (from commercial kit, Perkin Elmer), and was added to the chips. The slides were incubated for 10 minutes with rapid mixing on the Belly Dancer. The slides were then rinsed and incubated in PBS-T. Alexa-647 conjugate (1 mg/mL in PBS-T) was added to the chips, and the slides were incubated for 30 minutes. They were rinsed and incubated with PBS-T once again, and then rinsed quickly twice in deionized water. The slides were then centrifuge dried at 500 x g for 30 seconds on each side, and then scanned for A647 using an Axon GenePix 4200AL microarray scanner (Molecular Devices, Sunnyvale, CA). ProMAT, a custom software for ELISA analysis, was used to calibrate the intensities of spots, and to fit standard curves.

4.3.2 Results and Discussion

A standard curve for GST was obtained from the slides. However, there was no fluorescence indicative of positive binding at the spots corresponding to the PEG-peptoid construct. Even after repeating with an excessively high concentration of antigen, no fluorescence was detected.

Based on the SPR results and the success Alluri et al. has had with attaching GST to the sequence, it is unlikely that the molecule is not binding to the protein. However, it is possible that the construct is not binding covalently to the slide surface with the desired alignment. The DSS group is used to form a covalent link between the amine groups on the slide surface, and the amine group at the terminal end of the PEG molecule. However, the peptoid sequence has multiple primary amines on its backbone, and it is possible that those amines are attaching to the

DSS linker instead. This would cause misalignment of the capture molecule on the slide. The sequence that is specific to bind GST is most likely inaccessible to the protein. If only a few capture molecules bind to GST, the fluorescence signal obtained would not be strong enough to be detectable over background noise. Therefore, in order to obtain a stronger signal, an alternative immobilization technique may need to be employed. One option would be to modify the slide surface and the terminal end group of the PEG in order to use a different linking chemistry. Thiol groups could be a possible solution.

4.4 Peptoid DS2 to bind Mdm-2

Peptoid DS2 (Figure 19) is a slightly modified version of the Alluri peptoid specific to bind Mdm-2 [40]. The modification is the same as with Peptoid DS1, wherein, a propargylamine (Alfa Aesar) side chain is added to the N-terminus in order to make the peptoid amenable to conjugation with a NH_2-PEG-azide molecule via copper catalyzed click chemistry. The synthesis technique is the same as the one used for Peptoid DS1, albeit with the appropriate side chains and sequence. Successful synthesis was confirmed by MALDI (Figure 20).
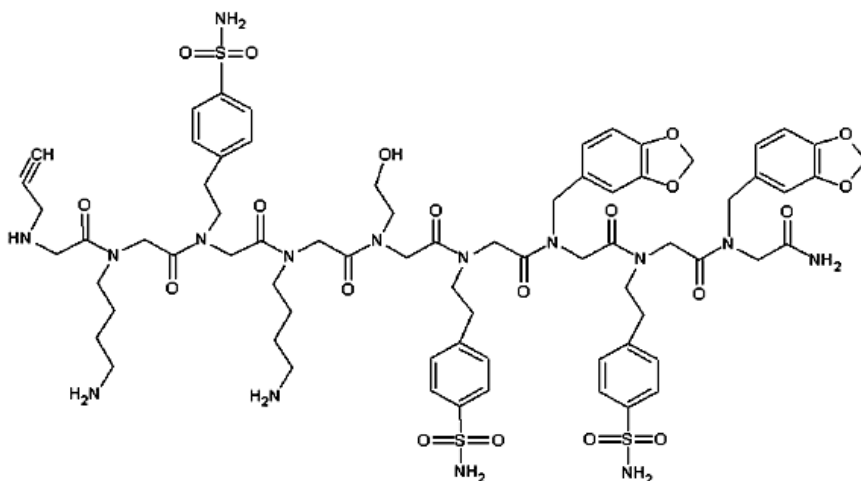

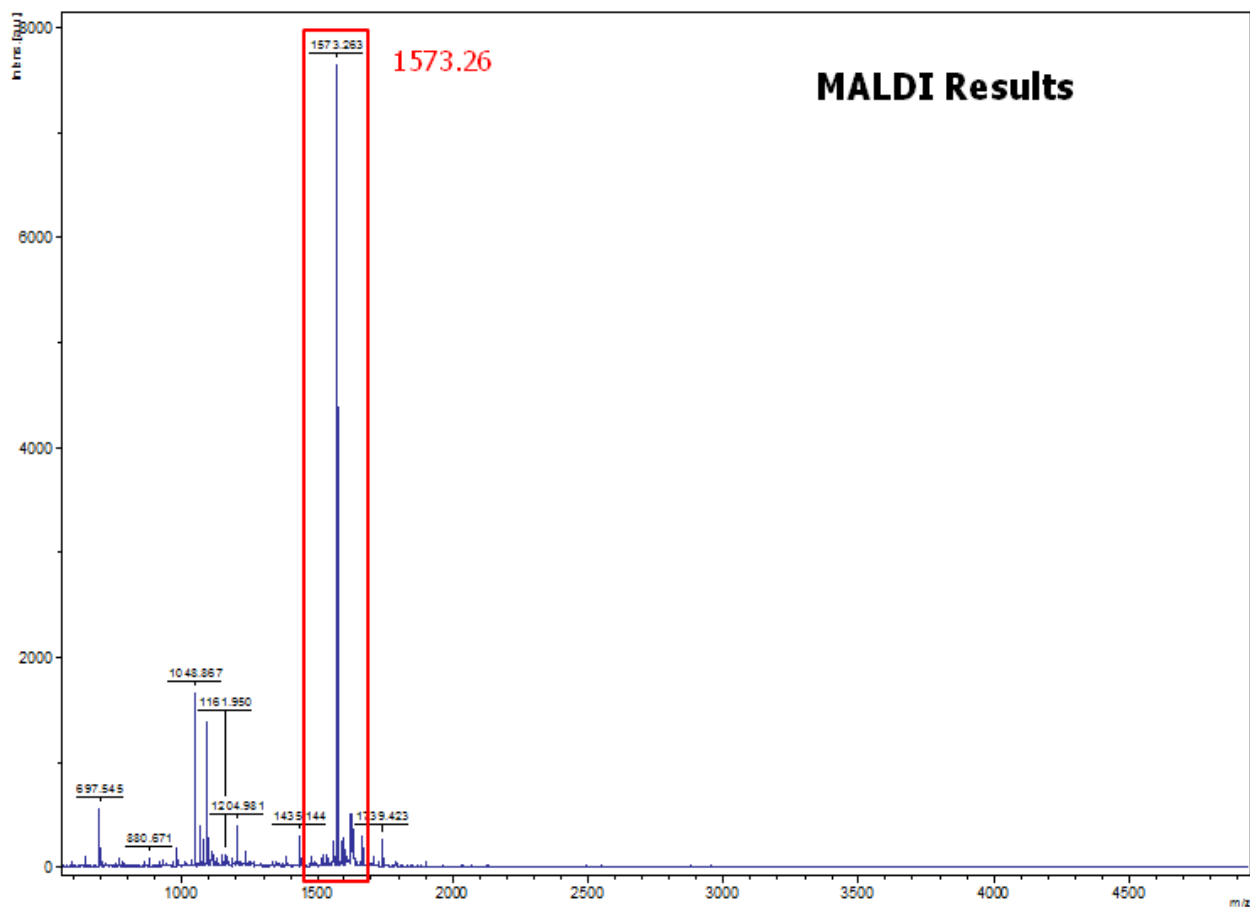**Figure 19 Peptoid DS2: Designed to specifically bind Mdm-2 MW: 1573 Da**

Figure 20 MALDI spectrum for Peptoid DS2

5. Conclusion and Future Direction

We were successfully able to design peptoid sequences that self-assemble into microspheres, and determine the conditions and sequences that enable tuning of these microspheres. We were also able to develop an optimal technique to consistently form uniform peptoid microsphere coatings on silica chips. It was discovered that partial water solubility and secondary structure are essential for the formation of these microspheres from organic/aqueous solutions. The size of these spheres was greatly influenced by the bulkiness and charge of the side chains on the third face of the peptoid helix. Experimental data suggests that these microspheres are formed due to

aromatic stacking on two faces of the helix. The organic:aqueous solvent forces the supramolecular structures to hide the aromatic groups, which are hydrophobic, in the core of the sphere, while the polar groups remain exposed to the solvent. By including both negatively and positively charged side chains in the sequence, where the negative group is chiral, smaller microspheres are yielded. Using bulky aromatic side chains produces larger spheres. By varying the type of side chain and length of sequence, it is possible to design peptoids that can assemble into spheres with the desired size and surface chemistry. This can be invaluable in many applications, including biosensors, coatings, and for increasing the available surface area of a slide ELISA microarray. A 1 μm diameter spot can contain five microspheres of diameter 0.3 μm along the horizontal and vertical axes. While the original spot would have a surface area = $\pi*(.5)^2 = 0.785$ μm^2, just five spheres would change the area to $5*.67*4*\pi*(0.15)^2 = 0.95$ μm^2, assuming 2/3 of the sphere is available for binding, corresponding to a 21% increase in surface area. A more efficient packing would allow eight spheres, and therefore an available area of 1.51 μm^2, resulting in a 92% increase in surface area, which would yield a significantly increased number of potential binding sites.

The peptoids designed to bind GST and mdm-2 need to be tested and validated as viable on a slide microarray. The main concern is that they are not binding to the slide surface with the right alignment. This issue can be addressed by using a different surface chemistry, and modifying the PEG molecule used. Glass slides may be functionalized with a maleimide surface, using N-(p-maleimidophenyl) isocyanate (PMPI), which allows the covalent conjugation to that surface to a thiol group. Slide functionalization may be confirmed using a thiol-based fluorescent dye. Instead of using an amine/azide bifunctional PEG molecule, a thiol/azide PEG may be used as a spacer between the slide surface and the peptoid. Also, in order to compare the peptoid without a

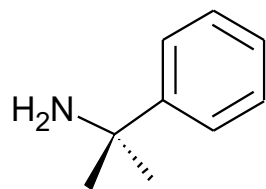PEG spacer, a side chain bearing a thiol group (such as cysteine or cysteamine) may be added to the terminal end to facilitate binding to the maleimide coated surface. This can eliminate the undesired binding of amine groups from the peptoid with the slide surface.
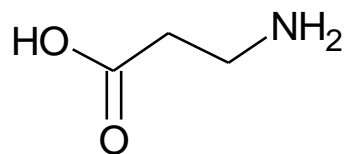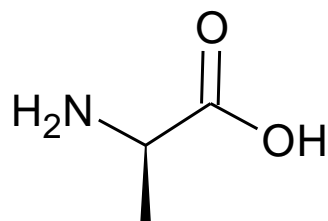
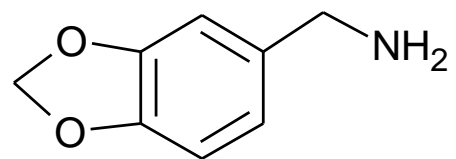### Appendix: Submonomer structures

NLys:
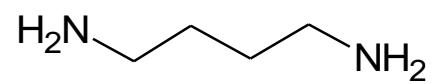
NSpe:

NGlu:

N-Ala [t-Bu]:

N-Pip:

N-bsa:
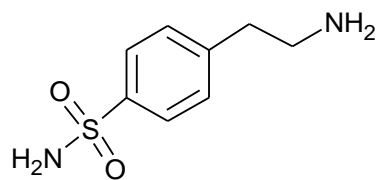
Ethanolamine:
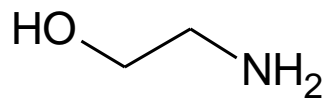
Propargylamine:
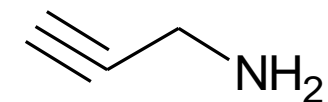
Appendix B

Click chemistry protocol:

The peptoid with the alkyne end group (from propargylamine), and the PEG molecule were individually dissolved in a 1:1 solution of t-BuOH/water to a concentration of roughly 4 mM. To a round bottom flask equipped with a magnetic stirrer, equimolar amounts of both compounds were added, along with 2x molar solution of L-(+)-sodium ascorbate, and 0.5x molar solution of

$CuSO_4.5H_2O$. The reaction mixture was stirred overnight under nitrogen. When the reaction was completed, the reaction mixture was dialyzed in Slide-A-Lyzer (MWCO: 10 kDa) dialysis cassette (Pierce, Rockford, IL) for 3 days in a 9:1 water/ethanol solution (1 L). The solvent was changed after 2 h, 4h, 1 day, and 2 days. The sample remaning in the dialysis cassette was lyophilized, and resuspended to a concentration of 0.8 mg/mL in PBS.

References

1. National Cancer Institute. Surveillance Epidemiology and End Results Program

2. American Cancer Society. Cancer Facts & Figures 2013. Atlanta, GA.

3. Wilson K.M.G, Jungner G. (1968) Principles and practice of screening for disease. Geneva: World Health Organization. Public Health Papers, #34

4. Liotta, L. and Petricoin, E. (2000) *Molecular profiling of human cancer*, Nat. Rev. Genet. **1**, 28-56

5. Negm, R.S., Verma M, Srivastava S (2002) *The promise of biomarkers in cancer screening and detection*. Trends Mol. Med. **8**, 288-293

6. Ludwing, J.A. and Weistein, J.N. (2005) *Biomarkers in cancer staging, prognosis and treatment selection.* Nat. Rev. Cancer **5**, 845-856

7. Phan, J.H., Moffitt, R.A. Stokes, T.H., Liu, J, Young, A.N., Nie, S, & Wang, M.D. (2009*) Convergence of biomarkers, bioinformatics and nanotechnology for individualized cancer treatment*. Trends in biotechnology, **27** (6), 350-8

8. Kiviat, N.B. & Critchlow, C.W. (2002) *Novel approaches to identification of biomarkers for detection of early stage cancer*. Dis. Markers **18**, 73-81

9. Anderson, K.S., Sibani, S., Wallstrom, G., Qiu, J., Mendoza, E.A., Raphael, J., Hainsworth, E., Montor W.R., Wong, J., Park, J.G. et al. (2011), *The Early Detection of Breast Cancer*, J Proteome Res. **10** (1): 85-96

10. Tainsky M.A. (2009) Genomic *and Proteomic Biomarkers for Cancer: A Multitude of Opportunities*, Biochim Biophys Acta*;* **1796** (2) 176-193

11. Sun, C., Rosehndahl, A.H., Ansari, D., Andersson, R. (2011) *Proteome-based biomarkers in pancreatic cancer*, World J Gastroenterol, **17**(44) 4845-4852

12. Zangar, Richard C.; Daly, Don S.; White, Amanda M. (2006). *ELISA microarray technology as a high-throughput system for cancer biomarker validation*, Expert Rev. Proteomics, **3**, 37-44

13. Jimeno, A., & Hidalgo, M. (2006). *Molecular biomarkers: their increasing role in the diagnosis, characterization, and therapy guidance in pancreatic cancer*, Molecular cancer therapeutics*,* **5**(4), 787-96

14. Bachet, J-B., Marechal, R., & Van Laethem, J.L. (2011). *Treatment of Pancreatic Cancer: What Can We Really Predict Today?* Cancers, **3**(4), 675-699

15. Li, J., Zhang, Z., Rosenzweig, J., Wang, Y.Y., & Chang, D.W. (2002). *Proteomics and bioinformatics approaches for identification of serum biomarkers to detect breast cancer*, Clinical chemistry, **48**(8), 1296-1304

16. Jahid, M.J., & Ruan, J. (2012). *A Steiner tree-based method for biomarker discovery and classification in breast cancer metastasis*, BMC genomics, **13** (Suppl 6), S8

17. Urquidi, V., Chang, M., Dai, Y., Kim, J., Wolfson, E.D., Goodison, S., & Rosser, C.J. (2012*). IL-8 as a urinary biomarker for the detection of bladder cancer*, BMC Urology **12,** 12

18. Haab, B.B. (2005). *Antibody arrays in cancer research*. Molecular & Cellular Proteomics: MC, **4**(4), 377-383

19. Woodbury, R.L., Varnum, S.M., and Zangar, R.C. (2002) *Elevated HGF levels in sera from breast cancer patients detected using a protein microarray ELISA*, J. Proteome Res. **1**, 233-237

20. Loy, A., and Bodrossy, L. (2006) *Highly parallel microbial diagnostics using oligonucleotide microarrays*. Clin. Chim. Acta **363**, 106-19

21. Sethi, D.; Kumar, A.; Gupta, K.C.; Kumar, P. (2008) *A Facile Method for the Construction of Oligonucleotide Microarrays*, Bioconjugate Chem. **19** 2136-2143

22. Choithani, J.; Vaijayanthi, B.; Kumar, P.; Gupta, K.C. (2007) C*onstruction of oligonucleotide microarrays (biochip) using heterobifunctional reagents,* Methods Mol. Biol. **381**, 133-163

23. Angenendt, P.; Glokler, J.; Murphy, D.; Lehrach, H.; & Cahill, D.J. (2002). *Toward optimized antibody microarrays: a comparison of current microarray support materials*. Analytical biochemistry, **309** (2) 253-260

24. Borrebaeck, C. a K.; & Wingren, C. (2009). *Design of high-density antibody microarrays for disease proteomics: key technological issues*. Journal of proteomics, **72** (6), 928-935

25. Taussig, M.J.; Landegren, U. (2003*) Progress in antibody arrays*, Targets **2** (4) 169-176

26. Astle, J. M., Simpson, L. S., Huang, Y., Reddy, M. M., Connell, S., Wilson, J., & Kodadek, T. (2011). *Seamless Bead to Microarray Screening: Rapid Identification of the Highest Affinity Protein Ligands From Large Combinatorial Libraries*. Chem. Biol. **17** (1): 38-45

27. Hoffmann, B.; Ast, T.; Polakowski, T.; Reineke, U.; and Volkmer, R. (2006) *Transformation of a Biologically Active Peptide into Peptoid Analogs While Retaining*

Biological Activity. Protein & Peptide Letters. **13:** 829-833

28. Miller, S.M.; Simon, R.J.; Ng, S.; Zuckermann, R.N.; Kerr, J.M.; Moos, W.H. (1995). *Comparison of the Proteolytic Susceptibilities of Homologous L-Amino Acid, D-Amino Acid, and N-Substituted Glycine Peptide and Peptoid Oligomers*. Drug Development Research **35**: 20-32

29. Simon, R.J.; Kanie, R.S.; Zuckermann, R.N.; Huebner, V.D.; Jewell, D.A.; Banville, S.; Ng, S.; Wang, L.; Rosenberg, S.; Marlowe, C.K.; Spellmeyer; D.C.; Tan, R.; Frankel, A.D.; Santi, D.V.; Cohen, F.E.; & Bartlett, P.A. (1992*). Peptoids: A modular approach to drug discovery.*

30. Proc. Natl. Acad. Sci. USA **89** 9367-9371

31. Wu, C. W.; Sanborn, T. J.; Huang, K.; Zuckermann, R. N.; & Barron, a E. (2001). *Peptoid oligomers with alpha-chiral, aromatic side chains: sequence requirements for the formation of stable peptoid helices*. Journal of the American Chemical Society, **123**(28), 6778–6784

32. Wu, C.W., et al., *Peptoid oligomers with a-chiral, aromatic side chains: Effects of chain length on secondary structure.* Journal of the American Chemical Society, 2001. **123**(13): p. 2958-2963.

33. Armand, P.; Kirshenbaum, K.; Goldsmith, R. a; Farr-Jones, S.; Barron, a E.; Truong, K. T.; Dill, K. a; et al. (1998*). NMR determination of the major solution conformation of a peptoid pentamer with chiral side chains*. Proceedings of the National Academy of Sciences of the United States of America, **95**(8), 4309–4314

34. Zuckermann, R.N.,;et al., *Efficient method for the preparation of peptoids [oligo(N-substituted glycines)] by submonomer solid-phase synthesis.* J. Am. Chem. Soc., 1992. **114**(26): p. 10646-7.

35. Barron, A.E.; & Zuckermann R.N., *Bioinspired polymeric materials: in-between proteins and plastics.* Current Opinion in Chemical Biology, 1999 **3**: 681-67

36. Gellman, S.H.; *Foldamers: A manifesto*. Accounts of Chemical Research. 1998 **31** (4): 173-180

37. Kirshenbaum, K.; Zuckermann, R.N.; & Dill, K.A.; *Designing polymers that mimic biomolecules.* Current Opinion in Structural Biology 1999 **9**: 530-535

38. Reineke, U.; Volkmer-Engert, R.; & Schneider-Mergener, J. (2001) *Applications of peptide arrays prepared by the SPOT-technology.* Current Opinion in Biotechnology **12**: 59-64

39. Zimmermann, J., Kühne, R., Volkmer-Engert, R., Jarchau, T., Walter, U., Oschkinat, H., & Ball, L. J. (2003). *Design of N-substituted peptomer ligands for EVH1 domains*. The Journal of biological chemistry, *278*(38), 36810–8

40. Fowler, S. a, & Blackwell, H. E. (2009). *Structure-function relationships in peptoids: recent advances toward deciphering the structural requirements for biological function*. Organic & biomolecular chemistry, *7*(8), 1508-24

41. Alluri, P. G., Reddy, M. M., Bachhawat-Sikder, K., Olivos, H. J., & Kodadek, T. (2003). *Isolation of protein ligands from large peptoid libraries*. Journal of the American Chemical Society, *125*(46), 13995–4004

42. Gazit, E. (2007), *Self-assembled peptide nanostructures: the design of molecular binding blocks and their technological utilization.* Chem. Soc. Rev. **36**: 1263-1269

43. Murnen, H.K.; Rosales, A.M.; jaworski, J.N.; Segalman; R.A.; & Zuckermann, R.N. (2010), *Hierarchical Self-Assembly of a Biomimetic Diblock Copolypeptoid into Homochiral Superhelics*. J. Am. Chem. Soc. **132:** 16112-16119

44. Nam, K. T., Shelby, S. a, Choi, P. H., Marciel, A. B., Chen, R., Tan, L., Chu, T. K., et al. (2010). Free-floating ultrathin two-dimensional crystals from sequence-specific peptoid polymers. *Nature materials*, *9*(5), 454–60.

45. Sanii, B., Kudirka, R., Cho, A., Venkateswaran, N., Olivier, G. K., Olson, A. M., et al. (2011*). Shaken , Not Stirred : Collapsing a Peptoid Monolayer To Produce Free Floating, Stable Nanosheets*; J. Am. Chem. Soc. **133**, 20808–20815.

46. Hebert, M.L.; Shah, D.S.; Blake, P.; Servoss, S.L. (2013) *Uniform and Robust Peptoid Microsphere Coatings*. Coatings. **3**: 98-107

47. Reches, M., & Gazit, E. (2004). Formation of Closed-Cage Nanostructures by Self-Assembly of Aromatic Dipeptides. *Nano Letters*, *4*(4), 581–585. doi:10.1021/nl035159z

48. Hebert, Melissa. (2012). Peptoid Based Slide Coatings for Disease Detection bia ELISA Microarray Analysis, Thesis, Master's Science Chemical Engineering, University of Arkansas

49. Holliger, P., & Hudson, P. J. (2005). *Engineered antibody fragments and the rise of single domains*. Nature biotechnology, **23**(9), 1126–36.

50. Hebert, M.L.; Shah, D.S.; Blake, P.; Turner, P.J.; & Servoss, S.L. (2013) *Tunable peptoid microspheres: effect of side chain chemistry and sequence.* Org. Biomol. Chem. **11** 4459-4464

51. Seurynck-Servoss, S.L.; Baird, C.L.; Miller, K.D.; Pefaur, N.B.; Gonsalez, R.M.; Apiyo, D.O.; Engelmann, H.E.; et al. (2008*). Immobilization strategies for single-chain antibody*

microarrays . Proteomics, **8** (11), 2199-2210

52. Janeway, C.A. Jr; Travers P.; Walport, M.; et al. (2001) . *Immunobiology: The Immune System in Health and Disease,* 5th Edition. Garland Science