8-2012

Peptoid Based Slide Coatings for Disease Detection via ELISA Microarray Analysis

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PEPTOID BASED SLIDE COATINGS FOR DISEASE DETECTION VIA ELISA MICROARRAY ANALYSIS

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

By

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University of Arkansas
Bachelor of Science in Chemical Engineering, 2010

August 2012
University of Arkansas

ABSTRACT

Poly-N-substituted glycines (peptoids) are a very versatile family of synthetic molecules that can be customized for any number of applications. In this study, we chose to use peptoids as a foundation for sandwich ELISA microarray analysis with a long term goal of creating an early detection device for complex diseases such as cancer. The peptoids were designed to self-assemble into microspheres to be used in coatings on the surface of the microarray substrates to increase the surface area available for antibody attachment. This increased antibody density would lead to an increase in the microarray analysis sensitivity and dynamic range. Studies were completed to determine the sequence characteristics and application process conditions necessary to form robust, uniform surface microsphere coatings. Polarity characteristics throughout the peptoid sequences were studied to determine how the choice and placement of charged functional groups effects self-assembly. The affect of secondary structure on the formation of microspheres was also studied, as we believe aromatic stacking mechanisms between the molecule's helical faces help with microsphere stabilization. Through this research, it was found that peptoids with a combination of chiral and aromatic side-chains accounting for at least two-thirds of the residues contain the helical and hydrophobic characteristics necessary to form self-assembled microspheres. An additional study was completed to investigate the effects of application process conditions on the uniformity of the coatings. We have shown that peptoid based microspheres have the ability to form uniform surface coatings under normal processing conditions, and are an exciting new avenue for early disease detection.

This thesis is approved for recommendation to the Graduate Council.

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ACKNOWLEDGMENTS

First, I would like to acknowledge my advisor, Dr. Shannon Servoss, her knowledge and guidance have helped to keep me on track towards my research goals. My gratitude also goes out to my committee members, Dr. Keith Roper, Dr. Ryan Tian, and Dr. Xianghong Qian along with the professors in the Chemical Engineering department for their advice and assistance along the way.

I would like to acknowledge the University of Arkansas Nano-Bio Material Characterization Facility and MALDI Mass Spectrometry Facility for their wealth of knowledge and assistance towards my project goals. I would also like to acknowledge Dr. Suresh Kumar and his group for their assistance in analysis and use of circular dichroism equipment. I would also like to acknowledge the Arkansas Biosciences Institute, the University of Arkansas Start-Up Funds, and the GK-12 Fellowship Program as the funding resources for my research.

I would like to thank my family and friends for the strength and enthusiasm they have shared with me, as it has helped me to stay focused on achieving my goals. Lastly, I would like to thank my husband for his constant support and encouragement through this long and grueling process. I have learned a lot through these past few years and he has been at my side through every up and down.

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1. Introduction

Pursuit of an efficient, non-invasive, and economical tool for the early detection of complex diseases, such as cancer, has been a main focus in the health care industry for many years. There is an array of effective methods used clinically to detect these diseases at various stages to increase survival rates, but they also have many risks and side effects associated with them. Cancer, in particular, can be detected through self-checks, magnetic resonance imaging (MRI) and mammograms, just to name a few.[1] In order to be effective in detection, each of these scans requires the disease be at a visible or advanced stage. Cancer has been a challenge to detect biologically, or through a serum sample, in the early stages as you must be able to scan for a number of biomarkers (*i.e.* proteins) simultaneously to achieve a high level of sensitivity and specificity.[2] These early-stage biomarkers present another challenge as they are found in minute concentrations in serum samples. It is believed that these low abundance proteins would likely be the best marker for the early stages of cancer if they could be readily detected.[2-6]

Sandwich enzyme-linked immunosorbent assay (ELISA) microarray technology has potential for use in cancer detection as it can simultaneously detect multiple proteins (up to 50) and uses a very small sample volume (15 µL after dilution).[2, 7, 8] Although the theoretical level of sensitivity of sandwich ELISA microarrays has not yet been achieved, we believe through optimization of coating, printing, and detection methods, these levels can be reached.[2, 9-12] One aspect of the detection process that can be altered to increase sensitivity is the density of immobilized antibodies. A three-dimensional surface coating would allow for a higher antibody attachment capacity due to increased surface area.[13]

We have found that poly-N-substituted glycines (peptoids) designed with a combination of chiral aromatic side-chains accounting for at least two-thirds of the residues contain the helical and hydrophobic characteristics necessary to form self-assembled microspheres. These microspheres can be used to form uniform slide coatings when dried on a solid substrate surface from an aqueous solution, and have the potential to increase microarray detection efficiency by increasing the overall antibody attachment density. The peptoids have been designed and synthesized with a robust helical structure in mind and then analyzed and purified before creating a solution with which to coat clean glass slides. We recommend using peptoid based slide coatings as the platform from which to build a reliable test for disease detection. These peptoid coated slides can be used in conjunction with the technology of sandwich ELISA microarray analysis to strengthen the detection signal and in turn increase the efficiency of our current disease detection methods. We believe peptoid-based slide coatings will increase the efficiency of disease detection, in turn decreasing the time and money spent towards yearly screenings and disease treatment.

2. Background

2.1 ELISA Microarray

The use of peptoid-based slide coatings with sandwich enzyme-linked immunosorbent assay (ELISA) microarrays is a promising start to detecting small concentrations of protein biomarkers in biological solutions.[2, 8, 14, 15] Sandwich ELISA consists of a number of material interactions, as illustrated in Figure 1. A capture antibody is printed onto a glass slide followed by incubation with a biological sample where, if present, the diseased antigen will attach to the capture antibody. A detection antibody that is specific to the disease antigen is then added to the slides, and if a perfect 'sandwich' attachment is made, the detection antibody will fluoresce when scanned with detection software.[2]

This form of ELISA allows for not only the detection of the targeted antigen in solution, but quantification of the concentration of the antigen in solution.[16] Sandwich ELISA is believed to be

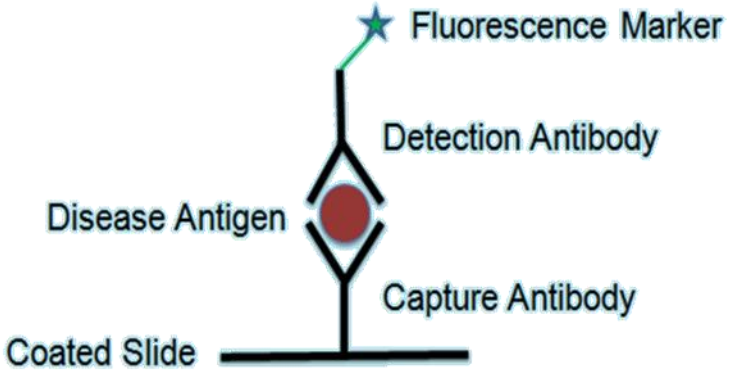


Figure 1. Schematic diagram of sandwich (ELISA) microarray analysis

the most sensitive method available for routinely measuring levels of multiple proteins.[2] While 96-well plate ELISA is currently the gold standard for protein detection, it is inefficient and time consuming when trying to measure levels of multiple proteins and utilizing small sample volumes.[2, 13, 17] Although sandwich ELISA microarray has very high sensitivity levels when compared to other scanning methods, its theoretically predicted levels have yet to be reached.[2,9-12]

2.1.1 Available Coatings

Antibody slide investigations were performed by Angenendt, *et al.* to determine the performance of a number of commercially available slides based on detection limits, slide variation, and signal to spotted concentration ratios. Overall they found that slides that bound antibodies through covalent interactions performed better with respect to signal intensity than those with non-covalently binding surfaces.[8] We hope to incorporate these findings into our future microsphere coatings so they can directly bind with the antibodies and increase signal intensities.

Seurynck-Servoss, *et al.* performed a review of 24, commercially available, slide coatings to evaluate ELISA microarray performance based on spot size and morphology, slide noise, spot background, lower limit of detection, and reproducibility.[7] The review concluded that 2-D surface chemistries, such as lysine- or amine-coated, performed better than the 3-D porous surface coatings, such as hydrogels. Currently available 3-D surface coatings utilize a process where the antibodies diffuse through the porous materials to increase the total number of antibodies attached to the slides. There are many issues with this process when trying to block

the surfaces from nonspecific protein binding due to the slow diffusion rates of the blocking solution.[7]

Our goal is to increase assay sensitivity by creating a non-porous 3-D coating that eliminates the diffusion issues previously observed while simultaneously creating an environment with high binding capacity, retention of antibody structure, low background noise, and the reproducibility needed for antibody immobilization.[2, 7, 13, 18] These coating alterations should result in lower background noise than most 3-D surface coatings and surpass the sensitivity and dynamic range of the currently available 2-D coatings, hopefully reaching the level of sensitivity theoretically determined for sandwich ELISA microarrays.

2.2 Nano/Microstructures

The addition of 3-D microstructures to the surface of ELISA microarray slides will greatly increase the surface area available for attachment of capture antibodies. The designed peptoid sequences will assemble into microspheres that have a diameter (0.8-5 μm) ~100 times greater than that of the IgG antibodies (< 50 nm) used during ELISA microarray analysis.[19, 20] These microstructures will be built using the bottom up method which includes beginning with a base design and building a final structure from that design. This method will hopefully allow us to start by designing the peptoid from scratch to create a specific size and shape of microsphere.[21]

Micro/nanostructures are widely studied for how, when and why they spontaneously self-assemble under certain conditions. It has been found that these structures tend to self-assemble

due to a number of interactions including electrostatic, hydrogen bonding, hydrophobic, and aromatic stacking.[21] Peptides with short sequences, consisting of aromatics, seem to spontaneously form microstructures when an organic solution is diluted to make an aqueous one, or by alternating heating and cooling of an aqueous solution.[21] Other types of microstructures have been shown to form when a colloid containing the sample is deposited on a substrate and the solvent begins to evaporate.[22] The capillary forces exerted during evaporation pull the structures together to form supramolecular assemblies.[21-23] We believe the peptoid microspheres we are studying self-assemble due to hydrophobic and aromatic stacking mechanisms that will help to produce tightly packed, uniform coatings.

2.2.1 Structure Assembly and Utilization

Many different types of materials have been shown to self-assemble on the nanoscale. Nanotubes have been shown by Ghadiri to be customized using peptides and assembled through the same aromatic stacking mechanism that we used with peptoids.[24] These nanotubes were envisioned to be functionalized for use in electronic applications through charge transfer along the outside of the tubes.[24] The self-assembly of metallic-based surface patterns can give ample information about the substrate on which it has formed and the self-assembled sample itself.[25] This phenomenon was demonstrated by Plass during his investigation of lead vapor deposition on copper.[25]

Although the structural patterns discussed thus far have numerous applications, our goal is to create self-assembled nano/microspheres to increase the attachment surface area for our

microarray slides. Jarai-Szabo, Astilean, and Neda described a very simple technique for nanosphere self-assembly through drying of a colloidal poly-styrene suspension onto a substrate in which drying capillary forces pulled the spheres together to yield dense nanosphere arrays.[22] They were also able to develop a model that predicts the organizational patterns of the nanospheres.[22] Gazit described peptide-based nanospheres designed with short aromatic side-chains self-assembled spontaneously when their organic solvent solution was diluted to an aqueous one, much like the formation of nanotubes.[21] We utilized this method for our peptoid-based microsphere formation, as we assumed they would react similarly in solution because their structural design is closely related to that of peptides.

The self-assembly and utilization techniques for nano/microstructures mentioned thus far are just a fraction of the possibilities available for applications. Schatz discussed the "playground" available to researchers through the utilization of such self-assembled structures.[23] We believe through the combination of these self-assembled structures, peptoid customization, and microarray analysis techniques we will be able to create a reliable early disease detection platform.

2.3 Peptoids

Peptoids are biomimetic polymers based on oligo-N-substituted glycine backbones, where the side-chains are appended to the amide groups rather than the α-carbons, as in peptides.[26] The change in side-chain location eliminates the need for backbone protection steps during synthesis and greatly simplifies the synthesis protocol.[26] The most commonly used process to assemble

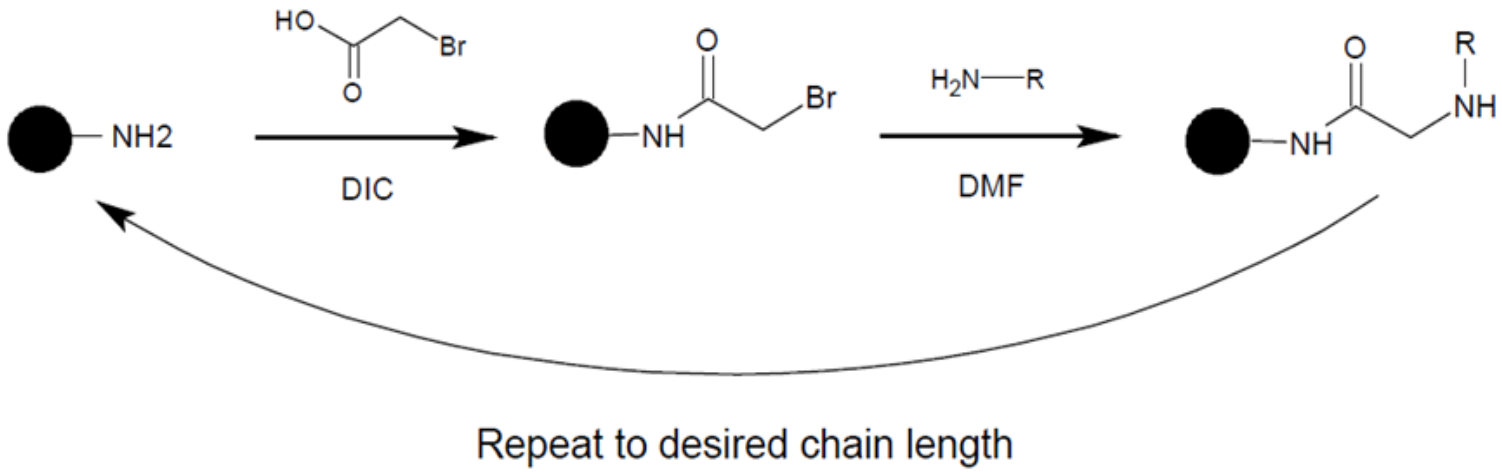


Figure 2. Schematic diagram depicting solid-phase synthesis of a peptoid by submonomer addition method

peptoids is through submonomer additions,[26] as shown in Figure 2. Peptoids can be rapidly synthesized using automated, solid-phase equipment where a multitude of functional groups can be attached as side-chains through the addition of any free amine.[26, 27] The possibilities for side chain combinations are practically limitless, as these sequences create synthetic molecules and are not limited to the naturally occurring amino acids, as with peptides.[27] Although peptoids lack the ability to form the backbone hydrogen bonds that peptides use to induce a stable helical fold, it has been shown that peptoids with chiral, aromatic side-chains can form robust secondary helical structures.[28-33] It is believed the stabilization of the molecule is achieved through aromatic steric interactions along with electrostatic repulsion between the π clouds of the side-chains and the backbone carbonyls.[28, 29] These stable helices can be formed with peptoids as short as five residues in length and strongly resemble the structure of polyproline type 1 helices with a periodicity of three residues per turn and a pitch of ~6 Å.[34] The ability of peptoids to form stable secondary structures leads us to believe they will be the ideal platform on which to build our detection process.

2.3.1 Secondary Structure

Extensive work has been completed in the area of peptoid secondary structure formation. Mohle and Hofmann studied the effects of single side-chain N-substitution on secondary structure formation and found that even minimal N-substitution induced helical structure.[35] Through the use of molecular modeling, Armand, *et al.* predicted that fully N-substituted glycines with chiral functional group centers could successfully form stable helical secondary structures, and later confirmed these predictions by nuclear magnetic resonance (NMR).[34, 36] Kirshenbaum, *et al*.

later observed peptoid oligomers with circular dichroism (CD) spectra closely resembling that of peptide α-helices. They were also able to achieve stable formation of these peptide α-helices-like structures for peptoids as short as five residues in length.[33]

Broad-based studies began to emerge within this expanding new field of research. Wu, *et al*. investigated the effects of sequence specifications and chain length on the formation of secondary structure for peptoids with chiral aromatic side-chains. Through their sequence specifications study they found that at least half of the side-chains in the peptoid sequence must be α-chiral, aromatic in order to form a stable helical structure.[30] These findings have allowed many researchers to functionalize their peptoid designs while remaining confident of the final secondary structure of the molecules. Through their chain length studies, Wu, *et al*. confirmed Mohle and Hofmann's initial findings that peptoids with as few as five chiral residues can form stable helical structures as long as they are made up of α-chiral, aromatic side-chains.[29,35] They also found that although in short peptoids the chain length has a large effect on the CD spectra, the chain length no longer has an effect on the secondary structure after about 12 residues.[29] The peptoids described in this paper are all made up of 12 residues, allowing us to assume all secondary structure is fully formed and stable.

Wu, *et al.* also studied the formation of helices utilizing peptoids with α-chiral aliphatic side-chains. Although aliphatic compounds are non-aromatic and therefore do not have the ability for π-π interactions shown to help stabilize peptoid helices, these peptoids were shown to successfully form stabile helices.[31] Through this aliphatic peptoid study Wu, *et al.* were able to successfully present the first crystallized structure of any peptoid.[31] These peptoids both formed

structures and depicted CD spectra resembling polyproline type 1-like helices.[31] This is different from α-chiral aromatic side-chain containing peptoids, as they form polyproline type I-like helices, but adopt CD spectra similar to peptide α-helices.[31, 32]

Although the majority of the research with peptoids discussed above has referred to peptoids forming stable, robust secondary helical structure, all results have been based on the CD spectra and NMR studies resembling that of peptide helices. These peptide helices have been crystallized and analyzed to view the actual conformation of the individual structures. Stringer, Crapster, Guzei and Blackwell were the first to report completing this same analysis using peptoids with α-chiral, aromatics side-chains. Their findings confirmed the NMR results by Armand, *et al.* that the peptoid forms a stable right-handed helix with an average of three monomers per turn and a pitch of ~6 Å.[34]

2.3.2 Functional Applications

Peptoids are an ideal candidate for use in a number of applications as their choices for side-chain functionality is almost limitless,[27] submonomer solid-phase synthesis allows for quick and inexpensive manufacturing,[26] and stable helical secondary structure leads to possibilities for tertiary structure functionality.[28-33] Peptoids have been proposed for use in biomimicry of peptides and proteins found in nature.[37] Biomimetic peptoids were designed and found to be promising for both lung surfactant protein B and C by the Barron group, both of which could be used to replace the natural lung surfactant of patients with respiratory distress syndrome.[38-43]

Peptoids have also been designed to mimic the characteristics of antimicrobial peptides leading to antibiotic agents that are biostable and protease-resistant.[44,45] Peptoids are also being utilized for their ability to recognize differences in neighboring molecules. Wu, *et al*. showed peptoids have potential as chiral separators through use of their amide and aromatics side-chains that allow for hydrogen bonding between the peptoids and the enantiomers in solution.[46] Huang, *et al.* also took advantage of the protease-resistant characteristics of peptoids to create a lipid-peptoid structure for DNA transfer within the body.[47] These lipid-peptoid structures are hoped to replace the currently used viruses for transport of DNA into cells and greatly reduce the risks factors for patients of gene therapy.[47]

It is obvious that peptoids have many possible applications, and we believe our self-assembled microsphere coatings will add to this growing list. Not only do peptoids possess the specific characteristic of robust secondary helical structure necessary for the assembly of our coatings,[28-33] but they are highly customizable for future applications.[26, 27] Peptoids will help to form the ideal coating for our disease detection device.

2.4 Microsphere Motivation

In this research we utilized the unique characteristics of ELISA microarray analysis, peptoids, and microstructure formation to create a stable foundation on which to build a reliable tool for early disease detection. We chose to use peptoids as our basis for assembling uniform nanospheres due to their low cost and the fact that they can be highly customizable. Peptoids have just recently become an interest of study for their ability to self-assemble into organized

tertiary structures. Seo, Barron, and Zuckermann reported that peptoids had the potential for numerous applications in the field of nanoscience through their investigation of the structure inducing chiral centers introduced to peptoid side-chains.[28] Since this observation of assembly potential, many self-assembled peptoid structures have been created including superhelicies,[48] crystalline sheets,[49] ribbons,[50] and monolayers that can further form into stable nanosheets.[51] The use of peptoids as the basis for our self-assembled microspheres allows us to utilize their unique characteristics in order to create a robust, stable disease detection device that can be customized to fit numerous applications.

Helical, hydrophobic peptoids have been shown to create microspheres when dissolved in an organic/water mixture and then dried on a substrate (Dr. Modi Wetzler, Personal Communication 2008). These helical peptoid microspheres have been verified in our lab as displayed in Figure 8B & C.

We hypothesize that peptoid microspheres form due to a combination of hydrophobic and aromatic stacking interactions when introduced to an aqueous/organic solution. Due to their hydrophobic nature, the individual peptoids group together when water is introduced to the organic environment. Aromatic stacking interactions occur between the Nspe groups on two of the helical faces of each peptoid, as depicted in Figure 10A.[48,52] These interactions are an attempt to conceal the hydrophobic faces of the helical molecules from the water. Since there are two complete faces of hydrophobic residues on each molecule, this stacking can create organization in multiple directions simultaneously.

Further organization of the peptoids can be achieved through incorporation of polar functional groups (NLys, NGlu, Ala [t-Bu]) on the last helical face of each molecule. Although many of the polar faces will be buried inside the spheres as the individual peptoids have a length of ~72Å and the microspheres have a diameter near 1µm, leading to the conclusion that many peptoids are coming together to form these spheres, some the polar faces will form a hydrophilic barrier around the outside of the sphere. The peptoids form a spherical structure since it is the lowest energy structure in nature that can conceal the hydrophobic faces and allow only polar faces to interact with the aqueous solvent.

3. Materials and Methods

3.1 Materials

4-methoxybenzylamine (Nmba), (S)-methylbenzylamine (Nspe), and benzylamine (Nba) were purchased from Acros Organics (Pittsburgh, PA). Tert-Butyl N-(4-aminobutyl)carbamate (NLys) was purchased from CNH Technologies INC. (Woburn, MA). 2-carboxyethylamine (NGlu) was purchased from EMD Chemicals (Gibbstown, NJ) and H-Ala-OtBu·HCL (Ala(t-Bu)) was purchased from BACHEM (Torrance, CA), which underwent a free-base reaction to remove HCl before peptoid synthesis. Acetic anhydride was purchased from Alfa Aesar (Ward Hill, MA). Sequencial grade N,N-Diisopropylethylamine (DIEA) for use during N-terminus acetylation was purchased from Pierce (Rockford, IL). MBHA rink amide resin was purchased from NovaBiochem (Gibbstown, NJ). Test grade silicon wafers were purchased from University Wafer (South Boston, MA) and ultra clean glass microarray slides were purchased from Thermo Scientific (Pittsburgh, PA). All other reagents used during synthesis, purification and sample preparation were purchased from VWR (Radnor, PA) and used without any further purification.

3.2 Methods

3.2.1 Peptoid Synthesis and Purification

Peptoids were synthesized via the submonomer addition method[26] on rink amide resin using an 433A Applied Biosystems Peptide Synthesizer (Carlsbad, CA) that had been refurbished from an

ABI 431A synthesizer. Synthesis was controlled with manually altered peptide chemistry and programming. The N-termini of peptoids 1 and 2 were acetylated with a 50 fold molar excess of acetic anhydride as the last step of synthesis. Once synthesized, all peptoids were cleaved from the resin by bathing in a solution of 95% triflouroacetic acid (TFA), 2.5% triisopropyl silane, and 2.5% water for a period of 2 - 10 minutes (previous studies in our lab and others have shown that extended cleavage times can lead to the removal of the aromatic side chains). The acid was removed via a Heidolph Laborota 4001 rotating evaporator (Elk Grove Village, IL) and the samples were diluted to a concentration of ~3 mg/ml in a 50:50 solution of acetonitrile:water. Peptoids were purified using a Waters Delta 600 preparative high performance liquid chromatography (HPLC;Milford, MA) with a Duragel G C18 150 x 20.1 mm column (Peeke Scientific, Novato, CA) and gradients of ~1% acetonitrile increase per minute over a range which included the peptoid elution percentages listed in Table 1. These gradients were created through the combination of solvents A and B (solvent A: water, 5% acetonitrile, 0.01% TFA; solvent B: acetonitrile, 5% water, 0.01% TFA). Peptoids were confirmed to be >97% pure via reversed-phased analytical HPLC (Waters Alliance, Milford, MA) using a Duragel G C18 150 x 2.1 mm column (Peeke Scientific, Novato, CA) and a linear gradient of 5 to 95% solvent B (acetonitrile, 0.1% TFA) in solvent A (water, 0.1% TFA) over 30 min. The purified molecular weights were confirmed to match theoretical values using matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (Table 1).

3.2.2 Sample Preparation

Purified peptoid solutions were dried to a powder using a Labconco lyophilizer (Kansas City, MO). The dried peptoids were dissolved in a solution of 4:1 ethanol:water with a peptoid concentration of 3 mg/ml for use during initial surface morphology studies. The peptoids were also dissolved in a solution of pure methanol at a concentration of 1 mg/ml for use in secondary structure analysis. Dried peptoids were also dissolved in 4:1 methanol:water and propanol:water solvent solutions at a concentration of 3 mg/ml for use in the extended application process study.

3.2.3 Circular Dichroism

CD spectrometry analysis was performed on a Jasco J-715 instrument (Easton, MD) at room temperature with a scanning speed of 10 nm/min and a path length of 0.1 mm. The peptoid samples were analyzed in a pure methanol solution at ~0.5 μM concentration. Each resulting spectra is the cumulative average of twenty scanning accumulations over a wavelength range of 190-250 nm.

3.2.4 Surface Morphology Analysis

Peptoids were administered to silicon wafer chips (~ 1 cm x 1 cm) and allowed to dry according to the appropriate experimental specifications. Scanning electron microscope (SEM) images were taken of all peptoid microsphere coatings discussed in this paper. Although ELISA microarray analysis is performed using large glass slides, SEM must have a conductive surface

in order to display a satisfactory image of the surface morphology. Silicon wafer chips were used during this study to achieve high resolution images of the peptoid microsphere coatings. Once coated and dried, the chips were visually analyzed using a Phillips XL-30 environmental scanning electron microscope (FEI, Hillsboro, OR) to determine the surface morphology and ability to uniformly form a self-assembled peptoid microsphere surface coating. Selected images were analyzed using ImageJ software to obtain microsphere size and distribution data.

4. Peptoid Microsphere Rationale

4.1 Methods

Peptoid solutions were administered manually by pipette to 1cm x 1cm silicon wafer chips using a full coverage coating technique, which consisted of fully coating the top of the chip with a sample volume between 20 and 50 µL, and open air drying technique which consisted of the coated wafers being placed in an open Petri dish and then left to dry on the bench top without any added airflow.

4.2 Peptoid Sequence Rational

Peptoids 1 - 3 (P1 - P3) were designed with 2/3 chiral aromatic functional groups that have been shown to induce secondary helical structure[28-33] and to allow for the necessary hydrophobic characteristics leading to partial water solubility (Figure 4). Due to the patterning of the individual functional group types (structures displayed in Figure 3) these peptoids have two distinct chiral aromatic faces with the third face functionalized with polar and/or hydrophobic side-chains. Peptoid 4 (P4), on the other hand, was designed with achiral aromatic side-chains (Figure 4) in hopes of creating a partially water soluble, non-helical peptoid for structural comparisons.

P1 and P2 were designed to investigate the effect of polarity on the formation of self-assembled microspheres. P1 was designed as depicted in Table 1 with the amine containing (positively

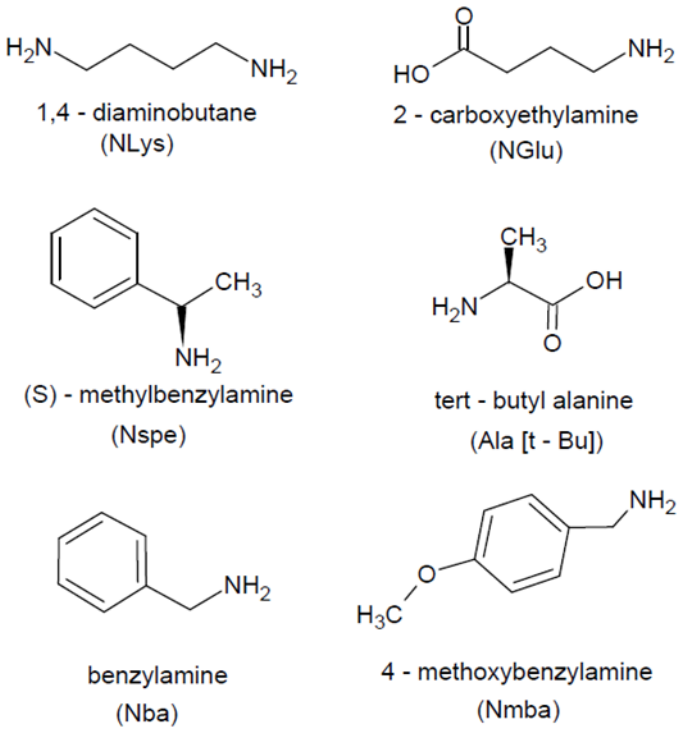
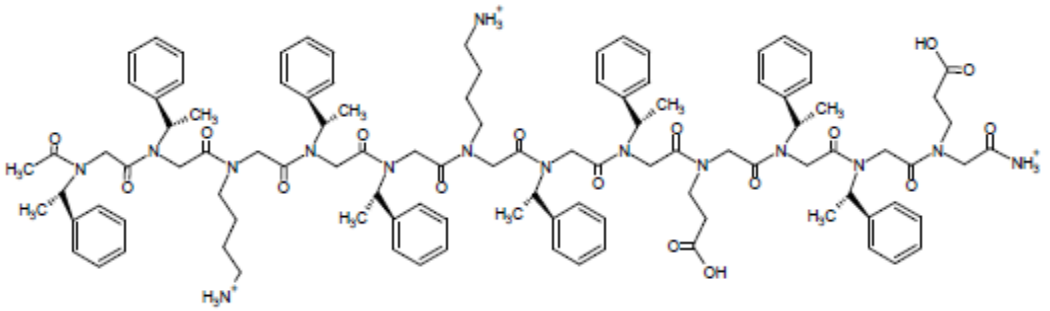


Figure 3.Structures of side-chains used for synthesis of peptoids 1-4 and their referenced designations.

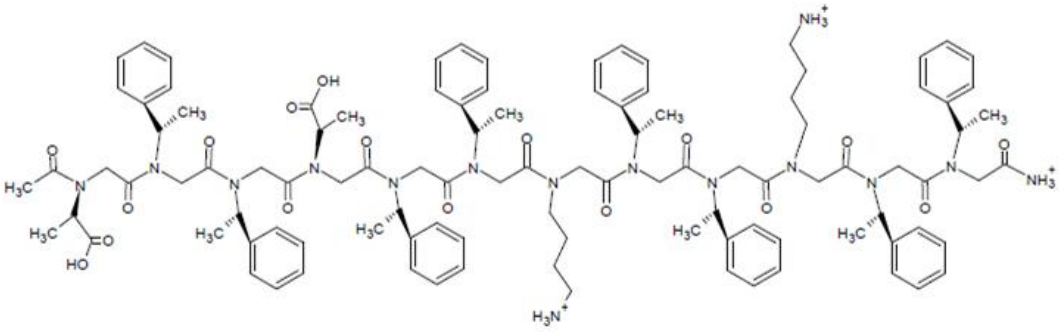
Table 1. Peptoid designations, sequences, molecular weights, and HPLC elution percents of acetonitrile. *red – possible negative charged side chains; green – possible positive charged side chains; purple – chiral aromatic side chains; blue – achiral side chains

	Sequence (N→C)*	Molecular Weight (Da)	% HPLC Elution
P1	(Nspe-Nspe-NLys)$_2$-(Nspe-Nspe-NGlu)$_2$	1863	74
P2	(Nspe-Nspe-Ala[t-Bu])$_2$-(Nspe-Nspe-NLys)$_2$	1864	77
P3	(Nspe-NLys-Nspe-Nspe-Nmba-Nspe)$_2$	1917	78
P4	(Nba-NLys-Nba-Nba-Nmba-Nba)$_2$	1806	72

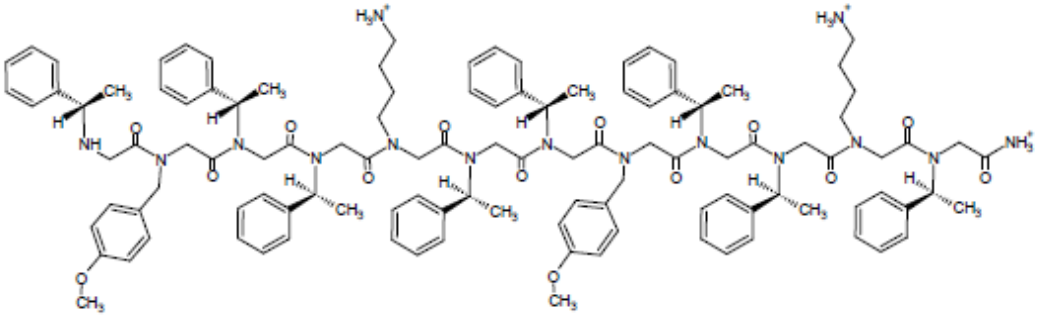
P1



P2



P3



P4

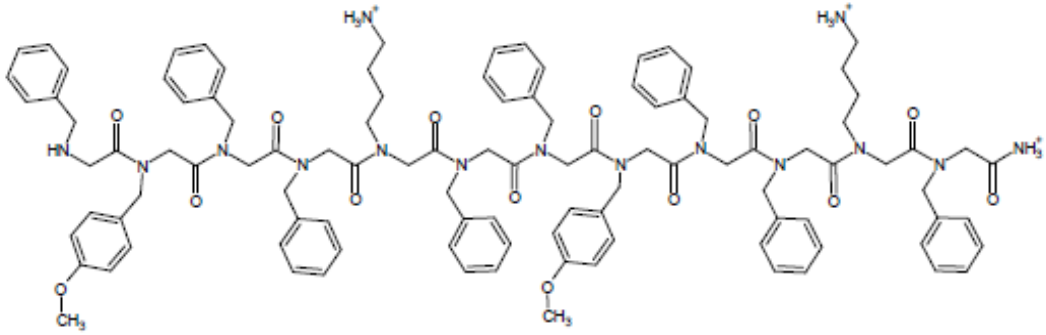


Figure 4. Chemical structure sketches for peptoids 1-4 depicting functional group sequence and protonation states

charged) functional groups oriented near the C-terminus and carboxyl containing functional groups oriented near the N-terminus. P2 on the other hand was designed with its positively and negatively charged functional groups oriented near the N and C-termini respectively, also depicted in Table 1. The charged side-chains for both P1 and P2 are located on the third face of the helix. The charge distribution differences between P1 and P2 led to conclusions depicting the effects that charged functional groups can have on the formation of peptoid microstructures.

The charge states of P1 and P2 were determined based on acid dissociation (pKa) values for each representative amino acid of the chosen functional groups (Table 2), and the measured pH of the coating solutions (pH ranges from 3 to 4). All peptoids formed acidic solutions because of preceding cleavage protocols, causing them to become TFA-salts. These assumed and measured values were then used in conjunction with the Henderson-Hasselbach equation:

$$pH = pKa + \log\left(\frac{[A^-]}{[HA]}\right)$$

to determine the ratio of protonated to non-protonated forms of each individual side chain.[53] This process was chosen based on the same convention used for peptides and proteins, and allowed us to roughly determine the charged state of each peptoid.

This rough estimation of functional group pKa values could be further refined by investigating the effects on ionization characteristics due to the organic solvent and proximity of neighboring polar groups. Hestekin, *et al*. has shown that organic solvents can increase the pKa of a polar

Table 2. Peptoid designations coupled with amino acid equivalents and pKa values used in conjunction with the Henderson-Hasselbalch equation.

Side-chain Designation	Amino Acid Equivalent	pKa
NLys	Lysine	10
NGlu	Glutamic Acid	4.4
Ala[t-Bu]	C-terminus	3.1
N-terminus	N-terminus	8

molecule an average of two points on the pH scale.[54] They also found that an increase in percentage of organic solvent in an aqueous solution leads to a larger change in pKa.[54] Due to these peptoids having a secondary helical structure, one complete face of the helix will contain the potentially charged functional groups that could be influenced by proximity effects. The Debye length for each charged ion would need to be investigated to determine how these neighboring charges will interact.[55] The Zuckermann group has shown that peptoids containing multiple ionic functional groups can have identical groups with different pKa values.[48, 49] This is caused by the first charged group making it more difficult to ionize the second group of the same (potential) charge type due to their close proximity.[48, 49] Dong, *et al.* have shown in two separate studies that proximity effects can also greatly affect the pKa and in turn the charge characteristics of the -COOH groups of the peptoids. They concluded that acidic groups such as -COOH attached to a backbone are much weaker than their monomer forms in solution.[56, 57]

Both organic solvent and proximity effects could have a large impact on the charging characteristics of P1 and P2. The organic solvent would most likely only affect the deprotonation of any hydroxyl groups in the sequences. Raising the pKa of the NGlu and Ala[t-Bu] functional

groups would make them less likely to be charged in the coating solutions. The proximity effects, on the other hand, would have an impact on all of the polar functional groups for both peptoids. The Debye length would need to be studied for each individual group to determine the possibility of positive and negative charging throughout the molecule. Although an organic solvent present would raise the pKa of the hydroxyl groups, having an oppositely charged group within its Debye length could lower the pKa, making it more easily deprotonated. Cross-interactions between the pKa differences caused by both solvent and proximity effects would need to be investigated to conclude the exact charging characteristics of these two peptoids in solution.

P3 and P4 were designed to investigate the effect of secondary helical structure on the formation of self-assembled microspheres. P3 was designed with the necessary chiral aromatic functional groups to form a robust secondary helical structure, along with two positively charged functional groups to simulate the surface chemistry of the currently used poly-L-lysine coated slides and two PEG-like functional groups to reduce non-specific protein binding on the substrate surface,[58] as depicted in Table 1. P4, on the other hand, was designed with achiral aromatic functional groups to remove the ability to form a secondary helical structure. This extreme difference in secondary structure allowed us to observe how the helical content of the peptoid molecule assists in the assembly and stabilization of the peptoid microspheres.

The charging characteristics for P3 and P4 were determined using the same parameters discussed above regarding P1 and P2. Organic solvent effects should not have a large impact on the ionization of P3 and P4 since both P3 and P4 contain only strongly protonated functional groups.

On the other hand, the proximity of these charged groups to one another could create strain throughout the molecule and make it difficult to have all of the groups protonated at once. Again, the Debye length of the ions would need to be investigated to undoubtedly determine the charge characteristics of each individual functional group.

4.3 Results and Discussion

P1 was originally designed with the sequence displayed in Table 1, but without acetylation of the N-terminus (referred to as $P1_O$). $P1_O$ has a molecular weight and percent HPLC elution 43 Da and 2% less than the data displayed in Table 1 for P1, respectively. SEM images for $P1_O$ depicted only globular formations on the surface and no formation of organized microspheres (Figure 5A). This led us to believe that $P1_O$ did not possess the hydrophobic characteristics necessary to self-assemble, and the N-terminus was acetylated to increase the hydrophobicity.

The acetylation was completed by returning the remaining resin to the automated synthesizer and washing it in a solution of acetic acid. The acetylation increased the molecular weight and elution percentages to those listed in Table 1. This observed increase in HPLC elution percentage indicates an increase in the hydrophobicity, or decrease in water solubility, of the peptoid. P1 was then re-imaged using SEM, and was found to microspheres, but only sparsely (Figure 5B). Although some spherical globules are observed in the P1 coating, no uniform formation of the expected, tightly formed microspheres were observed.

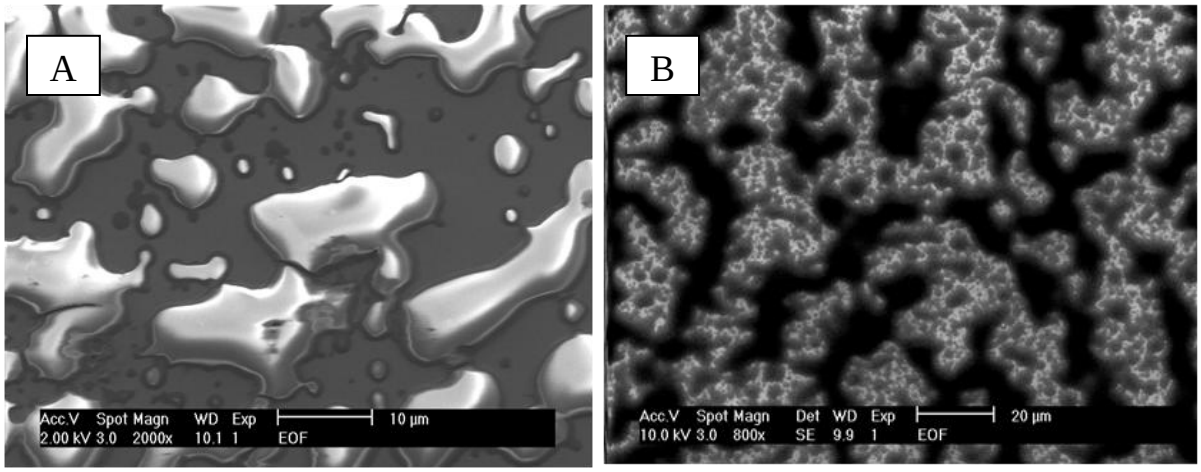


Figure 5. SEM images of peptoid 1 pre (A, scale bar = 10 µm) and post (B, scale bar = 20 µm) acetylation at a concentration of 3 mg/ml in a 4:1 ethanol:water solution and applied to the silicon substrate through full coverage coating and open-air drying techniques

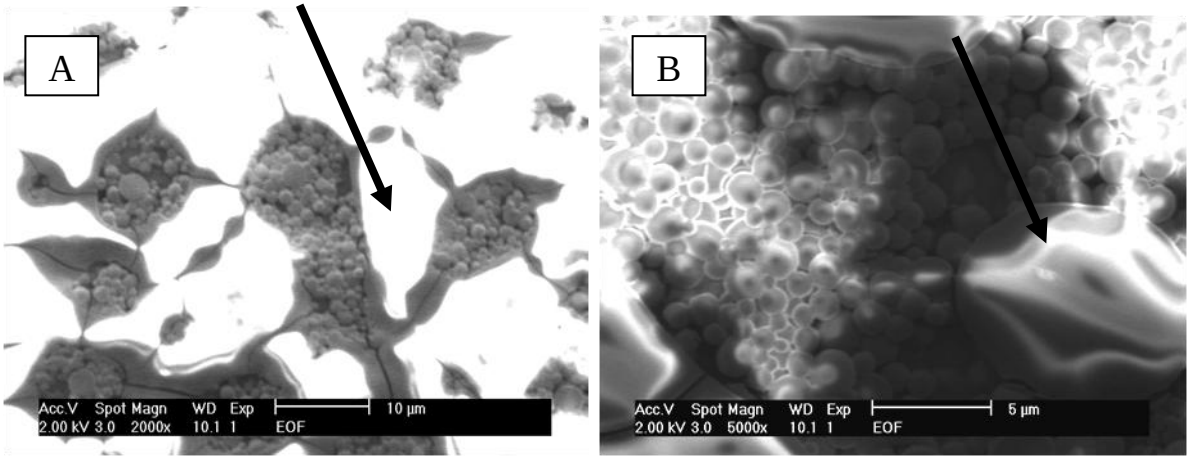


Figure 6. Initial broad view SEM image of peptoid 2 (A, scale bar = 10 µm) and a zoomed-in image (B, scale bar = 5 µm) at a concentration of 3 mg/ml and a 4:1 ethanol:water solvent mixture applied through the pipette spot coating and open-air drying techniques

P3 was designed with a single charge-type present in the sequence to reduce the potential for protein unfolding on the surface of the future detection slide. P3 also included PEG-like functional groups to reduce non-specific protein binding on the surface. Initial SEM images of P3 were analyzed after coating a silicon wafer with a 3 mg/ml sample in a solution of 4:1 ethanol:water using the pipette spot coating and open-air drying techniques (Figure 6). Tightly packed, self-assembled peptoid microspheres were observed on the surface of the substrate. Although microspheres were observed, the level of uniformity of the coating itself was found to be very low. Layering and cracking issues were observed throughout the substrate and are indicated by black arrows in Figure 6.

The formation and uniformity problems discovered through the initial analysis of the P1 and P3 coatings led us to design two separate studies to determine causes of and solutions for these observed issues. A study of the effect of polarity and secondary structure was designed to determine the effects different characteristics of the peptoid sequence have on its ability to self-assemble into tightly packed microspheres. An application process study was designed to address the fact that P3 successfully formed microspheres, but was not able to create a uniform surface coating. This application process study analyzed an array of processing techniques that consisted of three different solvent solutions, coating techniques and drying techniques.

5. Peptoid Sequence Polarity and Secondary Structure Study

5.1 Results and Discussion

This study was designed to investigate our hypothesis that a peptoid with a stable secondary helical structure and partial water-solubility will self-assemble into microspheres when introduced to an organic/water solution. As discussed in Chapter 4, although P1 was designed to have a secondary helical structure and hydrophobic aromatic functional groups, it was not able to successfully form microspheres. This observation led to the inclusion of this sequence polarity study, as we believe the location of the polar functional groups throughout the molecule affects the ability of the peptoid to self-assemble into microspheres.

5.1.1 Seconday Helical Structure

Peptoids have been shown to form stable, robust, polyproline type 1-like helices when they contain at least half chiral aromatic functional groups as a part of their sequence.[29, 30] When properly designed, the chiral aromatic functional groups align to form one of the helical "faces" and help to stabilize the structure.[28-30] Although peptoids do not possess the ability to stabilize their structure through hydrogen bonding due to their lack of free amines in the backbone, they are believed to form these stable helices through steric hindrances of their aromatic functional groups and electronic repulsions throughout the molecule.[28-30]

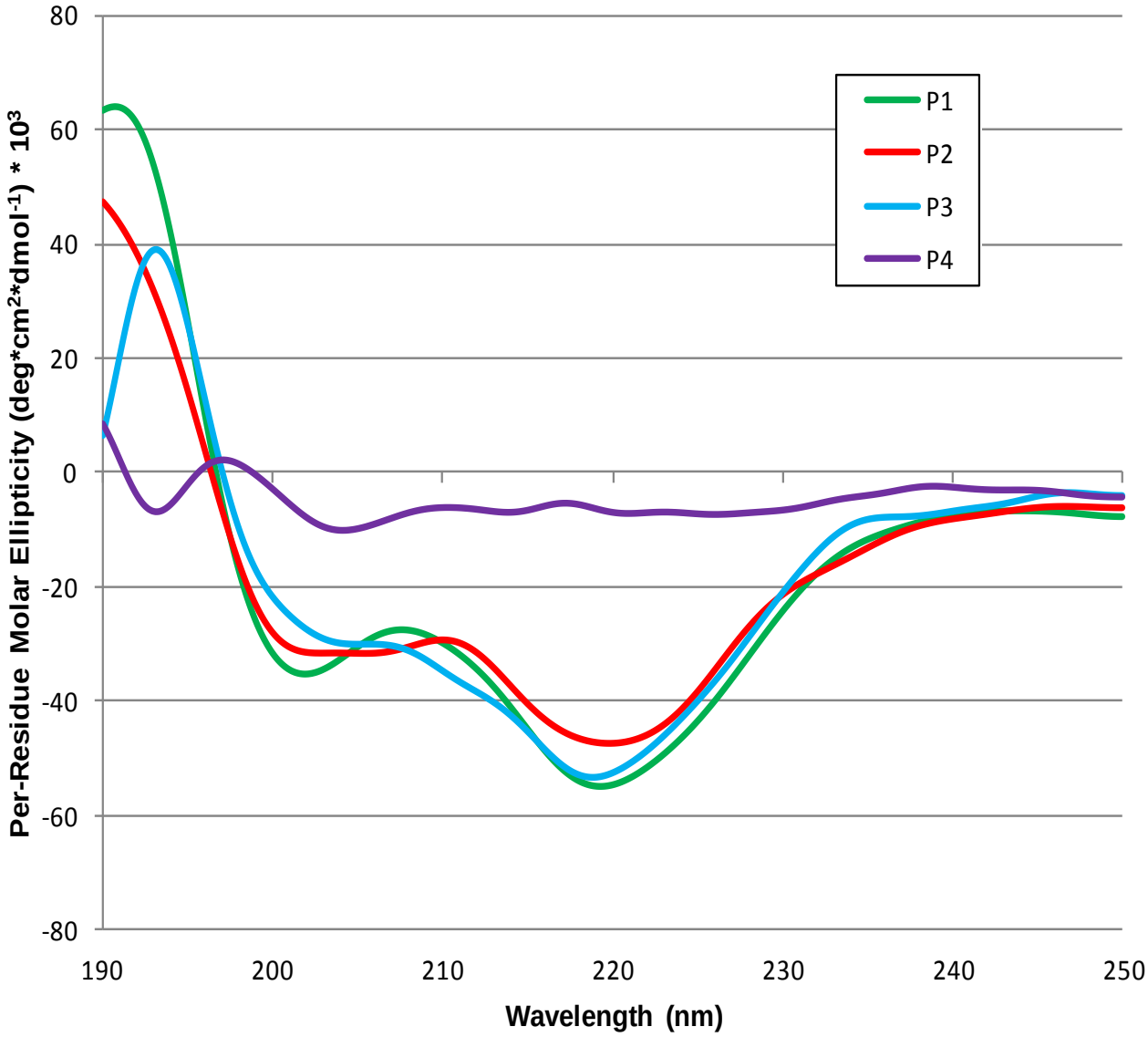


Figure 7. Circular dichroism spectrum of P1 - 4 depicting polyproline type 1-like helical secondary structures for P1 - 3 and a random coiling orientation for P4

A pure, HPLC grade, methanol solvent was used for all peptoid CD structure analyses. Methanol was chosen as the preferred solvent due to its polar protic characteristics. These types of solvents have been shown to help "induce" helix formation in peptoids that contain the 2/3 chiral aromatic functional groups necessary to form the polyproline type 1-like helices.[30-32, 59] Protic solvents are believed to create hydrogen bonds with the functional groups of the peptoid, and therefore increase stability of the peptoid helices in solution.[59]

Secondary helical structure is depicted by the CD spectra for P1, P2, and P3 with a large peak at 190 nm followed by two dips of increasing intensity near 200 and 220 nm.[30, 33, 37] While P2 also depicts this helical structure, its helix shows to be slightly of both P1 and P3, as its main peak at 190nm has a lower intensity. P4, on the other hand, does not depict any type of helical structure according to its CD spectra. The data shows P4 adopts a random coil orientation due to a lack of chiral aromatics in the functional groups present in its sequence that are necessary for helix formation.[30-32]

5.1.2 Self-assembled microsphere formation

Both P2 and P3 consistently assembled into uniform microsphere surface coatings, while P1 and P4 were never able to assemble into tightly formed microspheres (Figure 8). The structural differences between the peptoids that did and did not form microspheres include functional group placement, affecting the water solubility, and chirality, affecting the secondary structure.

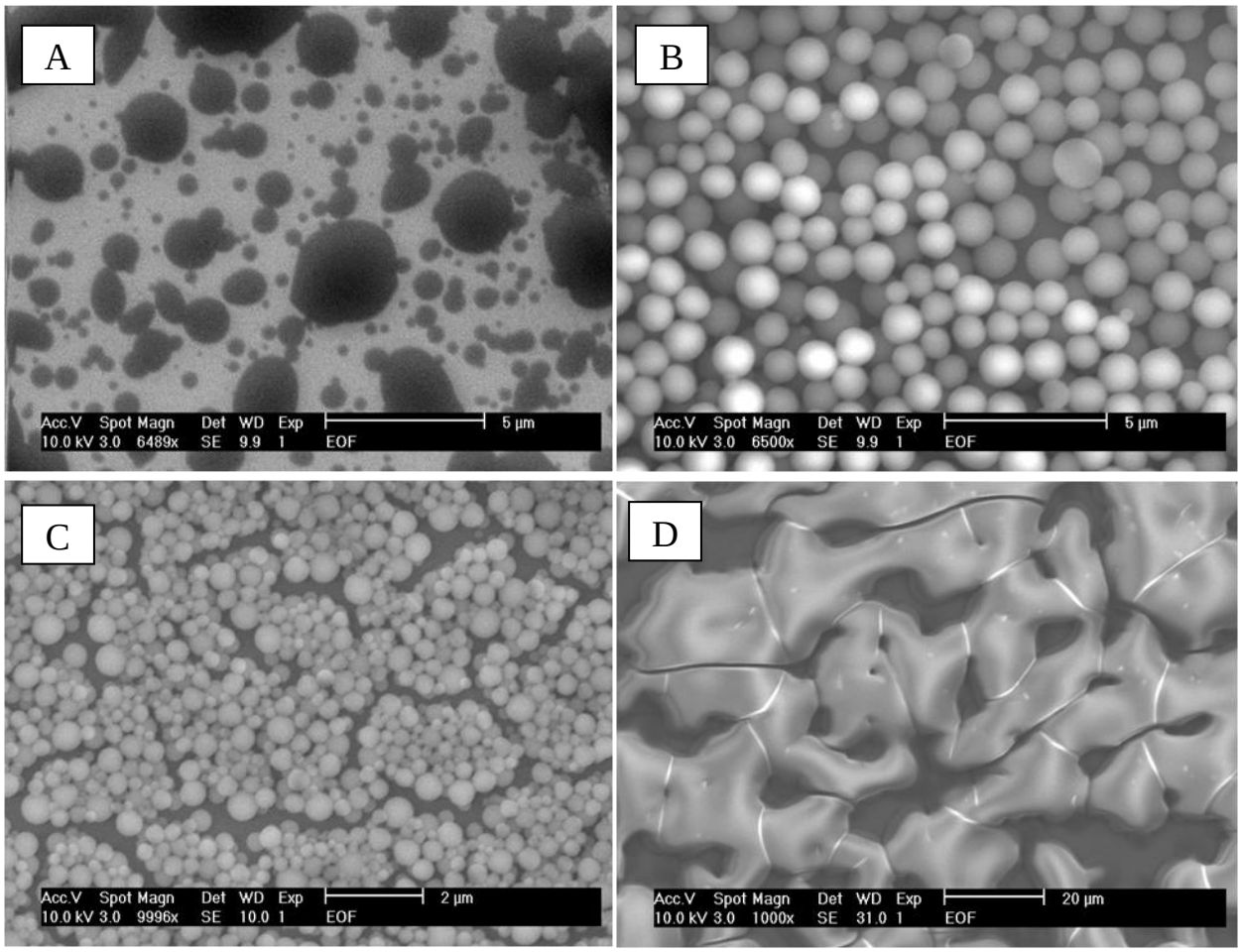


Figure 8. SEM images of self-assembled microspheres consisting of P1 (A, scale bar = 5 µm), P2 (B, scale bar = 5 µm), P3 (C, scale bar = 2 µm), and P4 (D, scale bar = 20 µm) at 3 mg/ml concentrations and 4:1 ethanol:water solvent solutions

Table 3. Peptoid size and distribution data collected through ImageJ analysis of SEM images of uniform P2 and P3 surface coatings

	Average Diameter	Standard Deviation
Peptoid 2 (P2)	0.337 µm	0.186 µm
Peptoid 3 (P3)	3.24 µm	1.62 µm

P1 did not self-assemble into a microsphere surface coating (Figure 8A). Through comparison of the elution data displayed in Table 1 it was obvious that P1 was more hydrophilic than P2, and we hypothesize this characteristic was due to the carboxyl containing functional groups being located too closely to the positively charged C-terminus. This orientation of polar groups could lead to the molecules being repelled from their counterparts in solution reducing their ability to self-assemble. P2 was designed to study the effect of carboxyl group placement. Specifically, the polar charged functional group locations were switched relative to P1 to remedy the peptoid from repelling itself in solution. As displayed in Table 1, these changes in polar functional group distribution did increase the hydrophobicity of the molecule by increasing its elution point by 3%. P2 showed consistent self-assembly into microspheres when introduced at a concentration of 3 mg/ml to a 4:1 ethanol:water solvent solution, and coated onto a silicon wafer chip using the full coverage coating and open air drying techniques, as shown in Figure 8B.

P3 was shown to have a helical secondary structure and possess the hydrophobic characteristics necessary to self-assemble into microspheres. These microspheres are shown in Figure 8C, and were consistently formed by P3 when it was processed using the same solvent, coating, and

drying conditions as P2. The microspheres formed by P3were much larger in diameter then those of P2, as determined by ImageJ analysis and depicted in Table 3.

We hypothesize that P2 was able to form much tighter microspheres that P3 due to organization of the individual peptoids, as depicted in Figure 10C. P3 contains not only a single polarity (NLys), but two extra aromatic groups (Nmba) located on its polar face, both characteristics that could cause P3 to form larger spheres. The single polarity would repel other peptoids of the same sequence that are otherwise being assembled and stabilized due to their hydrophobicity and aromatic stacking mechanisms (Figure 10A). The additional aromatic groups cause P3 to be more bulky than P2 and could possibly lead to a larger structure size. We are unsure of whether the microspheres formed by P2 and P3 contain the same number of peptoids, and further testing would need to be completed to draw conclusions regarding the interactions leading to these distinct size differences.

P4 was designed to have the exact sequence of P3 with the chirality excluded from its functional groups. Due to this lack of chirality, CD analysis showed random coiling rather than helix formation (Figure 7). Even though P4 was only partially water soluble, a characteristic necessary to form microspheres, without secondary helical structure it did not depict any surface microspheres when introduced to the organic:water solution (Figure 8D). These observations confirmed that both partial water solubility and secondary helical structure characteristics are necessary for peptoids to self-assemble into tightly packed, uniform microspheres.

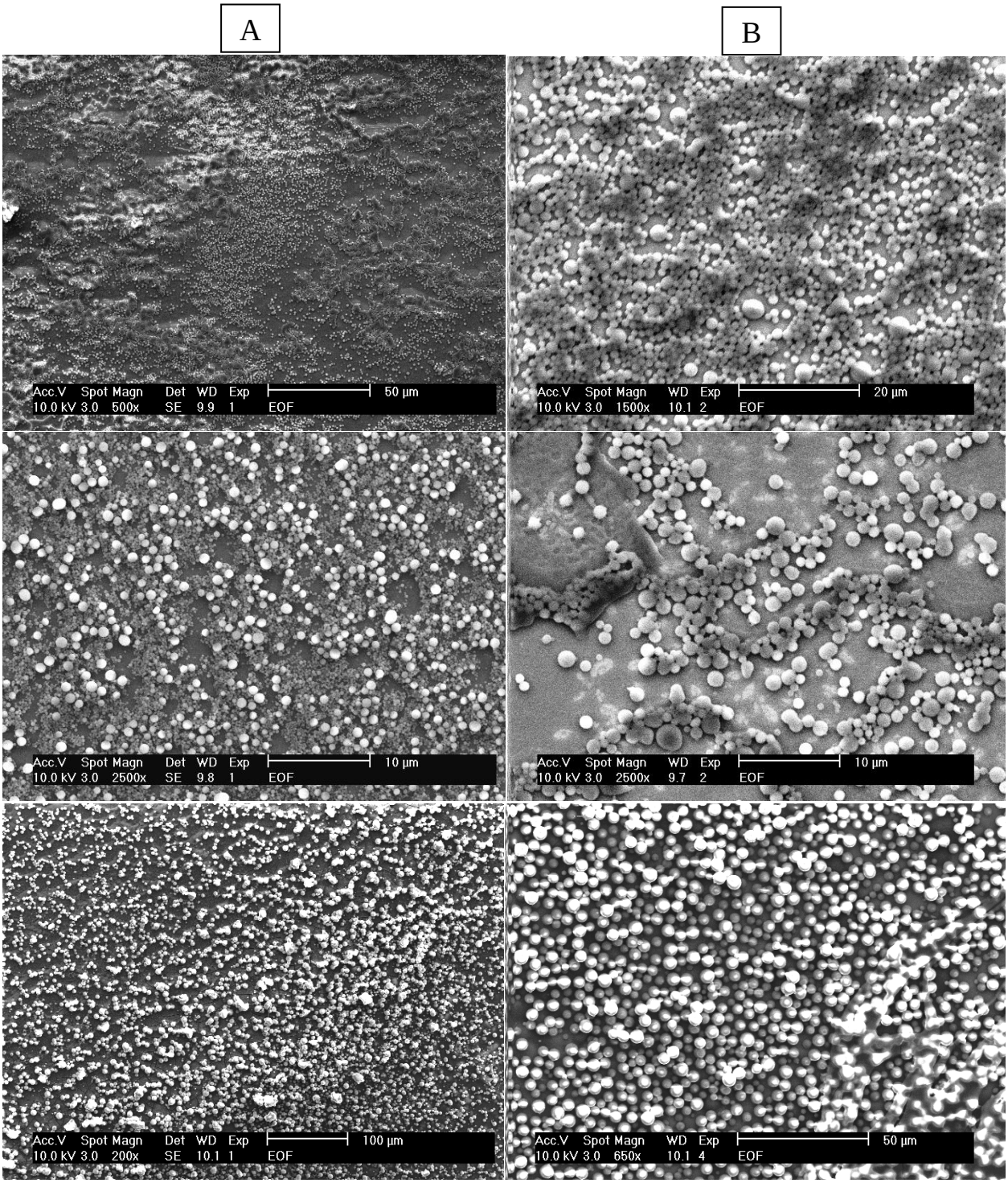


Figure 9. SEM images of microsphere coatings formed using P2 (column A) and P3 (column B)

5.2 Conclusions

Peptoids that are partially water-soluble, and have secondary helical structure, possess the characteristics necessary to self-assemble when introduced to an organic/water solution and form microsphere surface coatings when dried on solid substrates. Through sequence polarity and secondary structure observations, it was found that peptoids self-assemble through the interplay between electrostatic and hydrophobic interactions. Groupings are formed by the peptoids due to partial water solubility characteristics. If these groupings are made up of peptoids that possess a secondary helical structure, created through the use of bulky chiral, aromatic functional groups, the groupings will self-assemble into stable microspheres through aromatic stacking of the Nspe functional groups on their helical faces (Figure 10A).

The location and orientation of polar functional groups throughout the structure of a peptoid has a large effect on the ability of the peptoid to self-assemble into microspheres. This conclusion was obvious through the analysis of P1 and P2. P1 was not able to successfully assemble into microspheres even though it possessed the necessary chiral, aromatic functional groups that would give the molecule secondary helical and partial water solubility characteristics. Although P1 did adopt a robust secondary helical structure, it was found to be too hydrophilic to form uniform microspheres. We believe this is due to its hydroxyl containing functional groups being located near the C-terminus, which is positively charged in the coating solution. We hypothesize that this polarity organization cause neighboring peptoids of the same structure to repel one another (Figure 10B) and lead to the loosely formed spherical globules we witnessed and are displayed in Figure 8A.

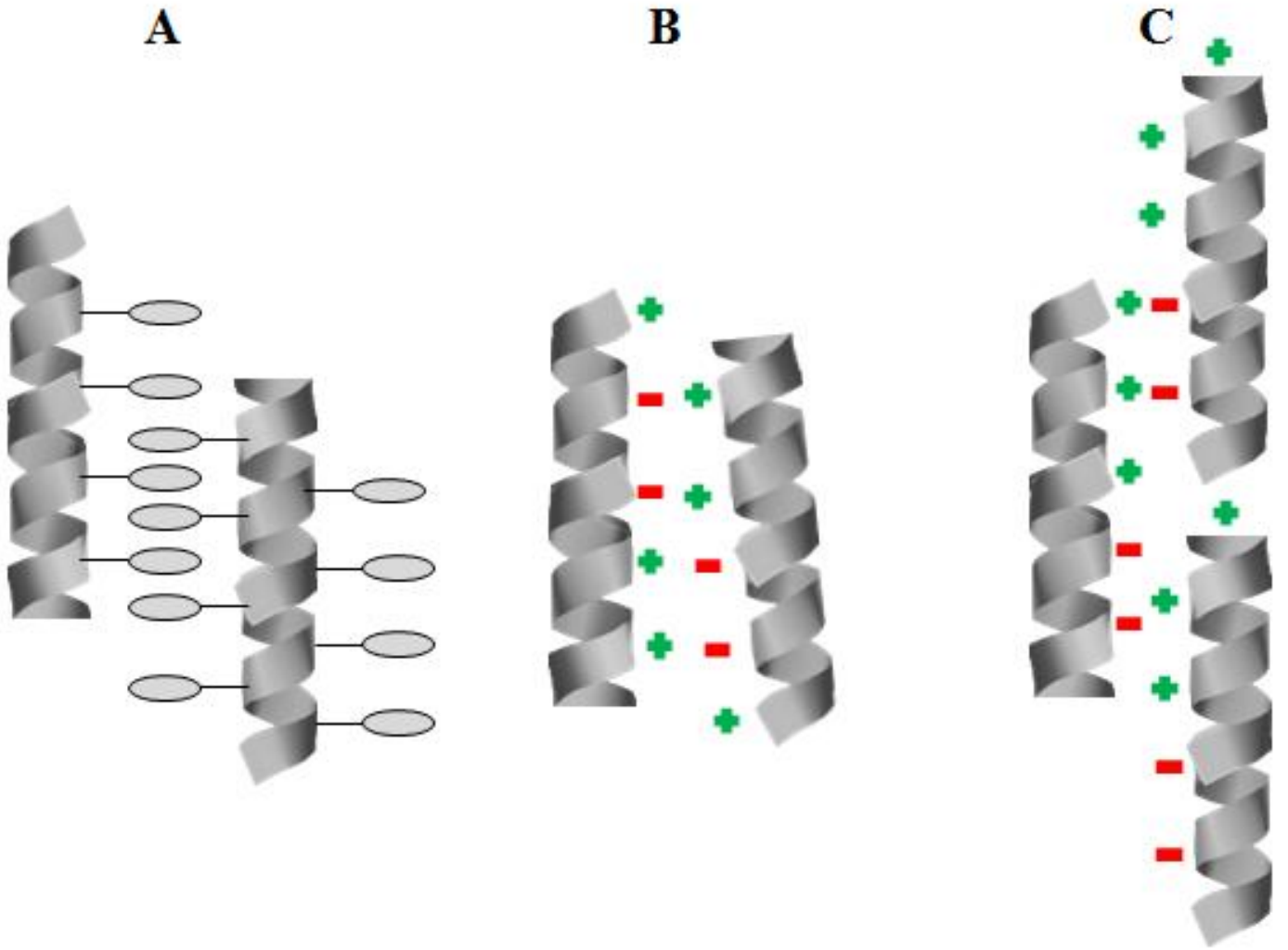


Figure 10. Schematic representation of aromatic stacking mechanisms that induced by hydrophobic interactions within the molecules (A) and charge interactions of P1 (B) where alternation of charged functional groups causes repelling of peptoids in solution and P2 (C) where organization of charged functional groups allows for alignment and grouping of peptoids in solution.

P2 also contained hydroxyl containing functional groups, but they were located on the opposite end of the molecule. This organization of like charges allowed the peptoids in solution to line-up or stack against one another without repelling forces causing disorganization (Figure 10C).

Secondary helical structure also plays an important role in microsphere formation by creating a structure that is conductive to aromatic stacking that aids with stabilization. This characteristic was determined through the comparison of P3 and P4 that only differ by chirality of their functional groups. The chiral, aromatic side-chains included in P3 allow it to adopt a polyproline type 1-like secondary helical structure, while the lack of chiral side chains in P4 leads to a random coil structure. We found the helical peptoid to consistently form self-assembled microspheres (Figure 9B) while the non-helical (random coil) peptoid did not show any evidence of uniform microsphere formation (Figure 8D). Although P4 possessed the hydrophobic characteristics necessary to group together in solution, it did not have any stable aromatic faces to induce the stacking mechanisms to aid in self-assembly.[21, 48, 49]

6. Peptoid Application Process Study

Peptoids 2 and 3 were chosen to complete an in depth application process study, as they had already been shown to self-assemble into microspheres in organic/water solutions due to their partial water solubility and secondary helical structures. Their partial water solubility caused the peptoids to group together in the aqueous/organic solutions due to their hydrophobicity. When the peptoids were in close proximity in solution and had secondary helical structure, we believe the aromatic faces of the helices aligned and induced aromatic stacking mechanisms that have been shown to help stabilize the spherical structures formed by the grouped peptoids.[21, 48, 4921] Although in the previous study both peptoids 2 and 3 consistently formed microspheres, the surface coatings created by each peptoid were not consistently uniform.

6.1 Results and Discussion

6.1.1 Experimental Application Methods

The coating methods consisted of the following: (1) administering a small sample volume (~5 µL) to the center of a silicon wafer chip using a pipette, (2) fully coating the top of the silicon chip with a sample volume between 20 and 50 µL using a pipette, and (3) fully submerging the silicon chip in a holding container with 500 µL of sample and immediately removing it in a vertical orientation. These coating methods will be referred to from now on as pipette spot, full coverage, and dip, respectively. The drying methods consist of (1) an open air environment where the wafers were placed in an open Petri dish and then left to dry without any added

airflow, (2) a humidity chamber that was held at a constant 60% humidity, and (3) a vacuum chamber. These drying methods will be referred to as open air, humidity chamber, and vacuum chamber, respectively.

6.1.2 Application Process Experimental Array

Uniform surface coatings are necessary for reliable detection coatings. ELISA microarray substrates with spotty or non-uniform coatings could lead to unreliable data for disease detection. Although some of the preliminary peptoid microsphere coatings were uniform (Figure 9), the application process characteristics need to be studied for their individual effects on microsphere formation. This will allow for the creation of a process protocol that will lead to consistenly uniform surface coatings.

The application process experimental parameters were chosen to create a matrix in which we could analyze each step of the application process from the sample solvent type, to the way in which the sample was administered onto the substrate, and finally the fashion in which the coating was allowed to dry and form its final state. The three solvents, coating, and drying techniques were chosen to investigate the effect of sample volume and solvent evaporation rate on microsphere formation. Two peptoids were analyzed to help achieve a broader analysis of self-assembled peptoid microsphere coatings as a whole rather than the characteristics of one molecular design. Although a combined average is displayed below for the effect each parameter has on the microsphere formation, individual results were analyzed to determine if any coupling effects were arising between parameters. We determined that the combination of parameters

chosen for any particular experiment did not greatly affect the average scores for the individual parameters and we believe the data displayed in Table 4 is a complete representation of the application process effects on peptoid microsphere formation.

Multiple SEM images were collected of surface coatings for each parameter of the experimental array. These SEM images were then qualitatively analyzed for microsphere coating uniformity and then given a quantitative rating based on a scoring system of 0.1 (no microspheres), 0.5 (spotty microspheres), and 1.0 (uniform microspheres). The data from P2 and P3 were combined and the average score of each experimental parameter was calculated and displayed in Table 4 where a consistent formation score is indicated by the dotted line at 0.4. The average score was found by utilizing all scores from the individual SEM images where a specific parameter was used.

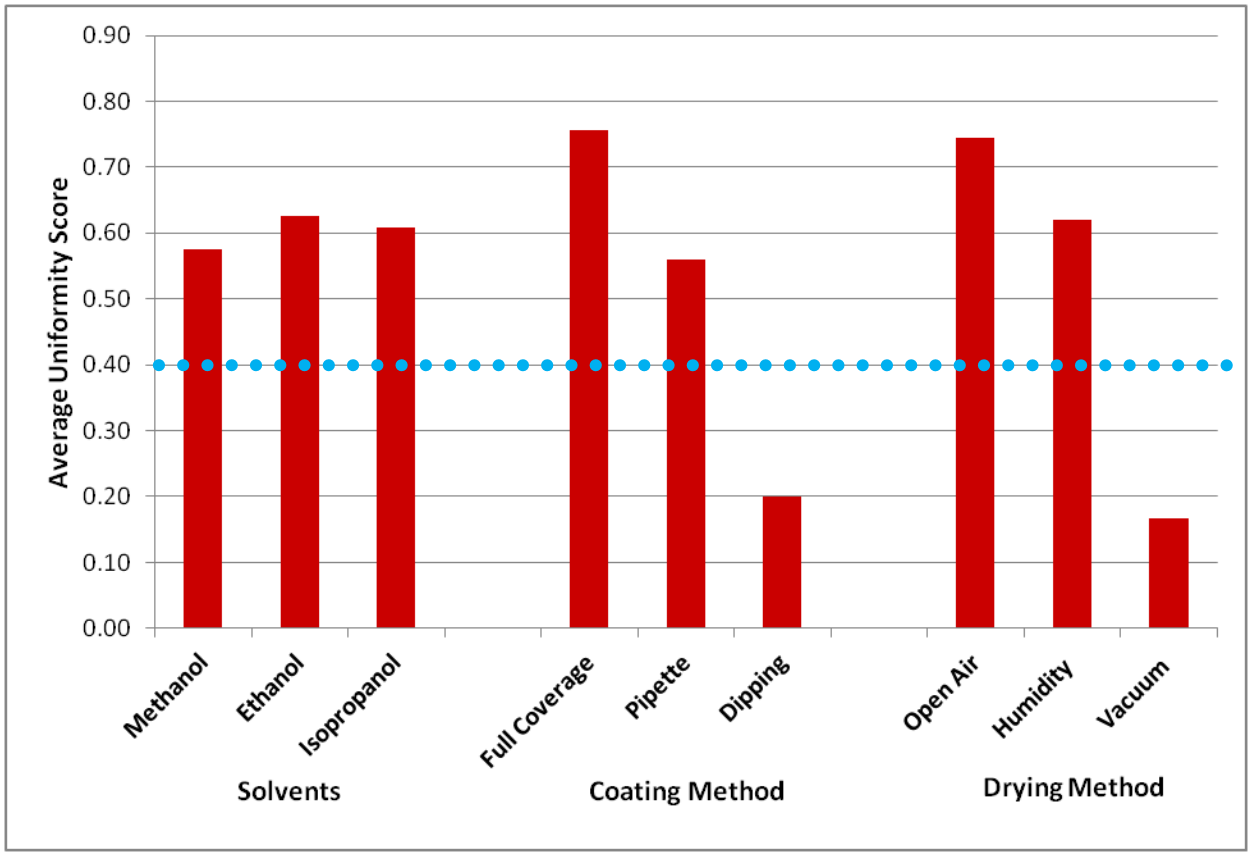
The effect of sample solvent choice was determined through analysis of three 4:1 alcohol:water solutions, as depicted in Table 3. The solvents were all chosen because of their protic characteristics, as these types of solvents have been shown to help stabilize secondary helical structure through hydrogen-bonding between the solvent and the functional groups.[59] Table 4 shows that for both P2 and P3, all three alcohol-based solvent solutions consistently allowed for uniform formation of microspheres. We hypothesize that as long as the solvent chosen is protic and the peptoid is soluble in the solution, microspheres should readily form and sufficiently adhere to the surface of the coated substrate.

Three coating techniques were utilized to investigate how much sample volume would be necessary to evenly and uniformly coat ELISA microarray detection slides. Small, medium, and large sample volumes were analyzed through pipette spot, full coverage, and dip coatings, respectively (Table 4 and Figure 11A-C). The volume of peptoid sample solution allowed to evaporate from the slide during the drying process was found to have great effect on the uniformity of the microsphere surface coatings. This is evident through the analysis of the image for the dip method surface coating displayed in Figure 11C. Although a large volume was used to dip the substrate and completely cover the surface, the substrate was quickly removed and allowed to dry separate from the large sample reservoir. Analysis of the surface showed very spotty to no microsphere formation. This indicates that the peptoid microspheres do not adhere to the substrate until the solvent begins to evaporate from the surface. This conclusion was further confirmed by an extended dip method experiment where a small silicon wafer chip submerged in a peptoid sample solution (P2 at a concentration of 3 mg/ml and a solvent solution of 4:1 ethanol:water) for five minutes. The silicon chip was then removed from the sample solution in a vertical orientation, and allowed to dry in an open-air environment. As with the original dip method experiments, there was no uniform formation of microspheres observed on the substrate for the extended submersion time (data not shown but comparable to Figure 11C).

Table 4. Experimental parameters array used for analysis of peptoid microsphere application process

Drying	Humidity (60%)	Open Air	Vacuum
Solvent	Methanol:Water	Ethanol:Water	Isopropanol:Water
Coating	Pipette Spot	Full Coverage	Dip

Table 5. Analysis of effect of sample application process on peptoid microsphere formation based on experimental parameter array (solvent, coating and drying methods) and visual scoring (0.1 = no uniform formation, 0.5 = spotty formation, 1.0 = uniform formation



Three drying techniques, as described in section 6.1.1, were utilized to analyze how the solvent evaporation rate would affect the peptoid microsphere formation. Slow, average, and quick solvent evaporation rates were evaluated through the use of humidity, open-air, and vacuum chambers, respectively (Table 4). A humidity chamber was tested to create a stable environment for the peptoid coating to form. Humidity samples that were allowed to dry for a long period of time were observed to dry starting around the outer edges and finishing in the middle. This led to different microsphere formations throughout the sample and changing densities over the area of the substrate. According to Table 5, the humidity chamber still allowed for consistent formation of microspheres (Figure 11D). On the other end of the spectrum, samples were dried under vacuum to speed up the drying process to potentially remove inconsistencies observed throughout the sample coatings. The vacuum chamber drying method led to no or spotty microsphere coatings, as indicated in Table 4 and shown in Figures 11D-F. The microspheres imaged after vacuum drying were miniscule compared to those coatings formed in the other two drying environments, as if the peptoids were not given sufficient time to adhere to the substrate and were removed from the surface along with the solvent. A constant, "natural" drying process allows for the most uniform coating of self-assembled microspheres based on the data displayed in Table 5 and shown in Figure 11E. These application process results are exciting as they show ELISA coating processes already used in practice can be applied to peptoid microsphere coatings without adaptation to equipment to cater to special application environments.

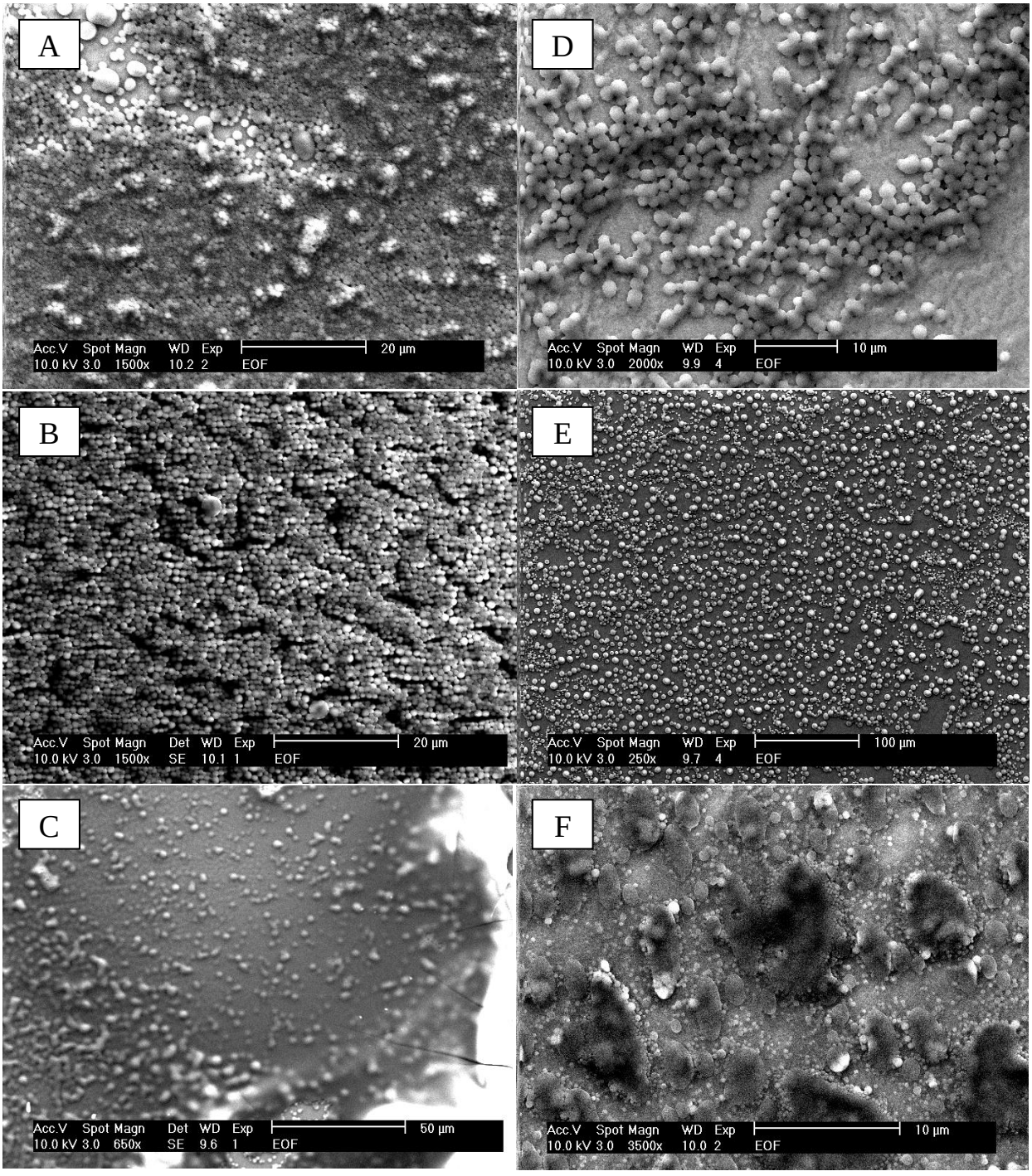


Figure 11. SEM images of self-assembled microsphere coatings administered using pipette spot (A, scale bar = 20 µm), full coverage (B, scale bar = 20 µm), and dip (C, scale bar = 50 µm) coating techniques, and humidity (D, scale bar = 10 µm), open-air (E, scale bar = 100 µm), and vacuum (F, scale bar = 10 µm) drying techniques

6.2 Conclusions

We have shown that both P2 and P3 consistently form microspheres due to their partial water solubility and secondary helical structure (Figure 9). All three solvents (methanol, ethanol, and isopropanol) allowed for the formation of microspheres, with the outcome only being affected by the coating and drying techniques used in conjunction with the individual solvents. It has been shown that the use of protic solvents are helix inducing, allowing for increased stability of the secondary structures.[59] We hypothesize that as long as the chosen solvent possesses protic characteristics, the specific solvent chosen will not greatly affect the self-assembly of the peptoid microspheres.

The substrate coating method has a large effect on the uniformity of the self-assembled microsphere coating. The pipette spot and full coverage coating techniques consistently allow a sufficiently large volume of peptoid sample to evaporate from the slide and therefore adhere to form a uniform robust coating (Figure 11A & B). A dip coating technique does not allow for efficient formation as the microspheres do not adhere to the slide until the solvent begins to evaporate from the surface of the substrate. The dip technique only allows for a small volume of sample solution to evaporate from the surface, causing a very sparse microsphere coating (Figure 11C).

The drying method utilized for the peptoid application process also greatly affects the uniformity of microsphere formation. We have shown through use of both an open air drying environment and a humidity chamber that a slow or “natural” drying environment yields the most uniform

surface coatings (Figure 11D &E). A very quick solvent evaporation rate does not allow the peptoid microspheres to adhere on the surface causing a sparse array of partially formed microspheres (Figure 11F).

We believe these peptoid-based microsphere coatings will help us reach theoretical levels of ELISA microarray detection as they have the structure necessary to achieve the critical properties of specific binding sites, high surface area, retention of antibody structure, long shelf-life, and low non-specific protein bindings.[7, 18]

7. Additional Work Completed

7.1 AFM Coating Analysis

Preliminary images of the coating topography were acquired using atomic force microscopy (AFM). These images were collected to help determine whether or not the spherical objects observed using SEM were full spheres or hemisphere-like formations on the surface and to determine the uniformity of the coatings. Coatings created by P1 and P3 were measured and compared for structural characteristics. We chose to compare these two coatings as P1 was shown to not successfully form uniform microspheres and seemed less 3-dimensional in nature, while P3 consistently formed coatings that contained spherical uniform structures.

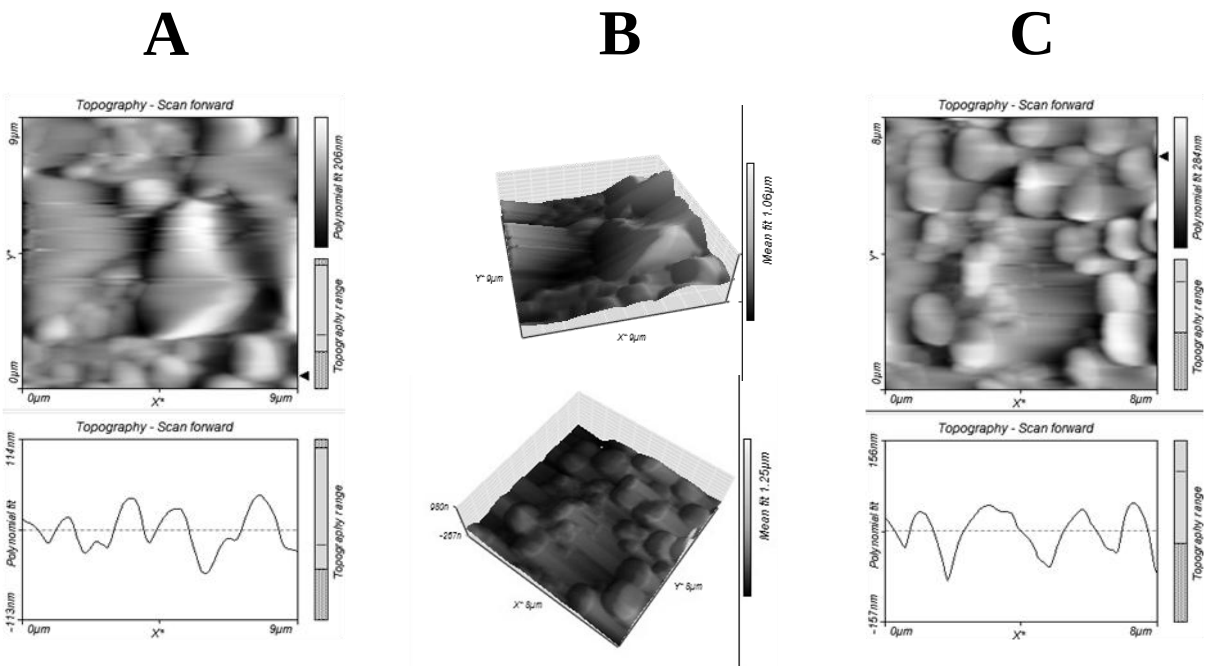


Figure 12. AFM images of P1 (A) and P3 (B) coatings depicting a topographical image (top) and a vertical distance measurement (bottom) of the microspheres followed by three dimensional topography images (C) of both P1(top) and P3 (bottom).

Through analysis of the preliminary AFM images, we can initially conclude that the non-uniform microspheres of P1 are much flatter in a 3-dimensional sense than those formed by P3, having a height to diameter ratios of ~0.015 and 0.08 respectively (Figure 12). These initial findings could lead to the conclusion that the microspheres we have been observing are actually more hemispherical in shape possibly caused by polar interactions between the microsphere and silicon surfaces. Further analysis using AFM, or possibly low angle x-ray diffraction would need to be completed before a final conclusion could be made.

7.2 Glass Substrate Coating

Although we have been able to find ample proof of peptoid microsphere self-assembly on silicon wafer chips, ELISA microarray analysis is completed on glass substrates. In order for the peptoid microspheres to be able to have an increasing effect on the antibody attachment density, they must be able to efficiently and reliably form on glass substrate surfaces. To be able to adequately

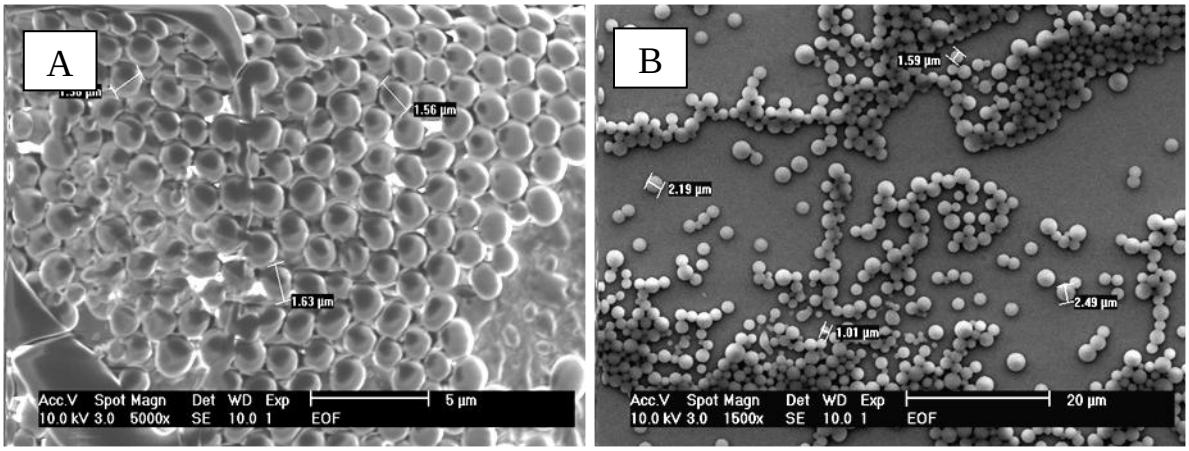


Figure 13. SEM images of glass (A, scale bar = 5 µm) and silicon (B, scale bar = 20 µm) substrates coated side-by-side with a 3 mg/ml peptoid 3 sample in a 4:1 ethanol:water solvent solution. Samples were applied using full coverage coating and open-air drying techniques.

compare the peptoid microsphere formation ability on these glass substrate with that of a silicon wafer, we coated a sample substrate of each material side-by-side from the same peptoid sample solution reservoir (P2 at 3 mg/ml concentration, 4:1 ethanol:water solvent solution). The substrates were coated using the full coverage coating and open-air drying techniques as described in section 6.1.1. SEM images of both sample coatings were analyzed and are displayed in Figure 11. Although the images show some slight differences in microsphere uniformity and density, there is ample formation of microspheres on the glass substrate. Through further analysis of the glass and silicon wafer coating images, it is observed that the self-assembled microspheres created are in the same size range (1 – 3 μm, Figure 13). These observations lead us to believe that the application process conditions investigated thus far will transfer from the silicon wafer substrates to use on the ELISA microarray glass substrates needed for disease detection.

7.3 Robust Microsphere Coating

Once the peptoid microspheres were found to successfully form on both the silicon wafer and glass substrates, the question arose of whether or not the peptoids would be able to withstand the extensive wash steps during ELISA microarray analysis. Sandwich ELISA microarray analysis consists of four steps including capture antibody attachment, antigen bathing, detection antibody attachment, and fluorescence magnification.[16] Each of these processing steps contains numerous intermediate washing steps that pose a danger to the peptoid microspheres that have been coated on the slide as the detection foundation. In order to determine the level of robustness that these peptoid microspheres would exhibit during microarray processing, they were exposed to the

entire array of consecutive microarray washing steps. We re-imaged a peptoid coated silicon chip, which had already shown to have microspheres attached, after completing the washing protocol described below.

7.3.1 Microarray Washing Protocol

The sandwich ELISA microarray washing protocol, used to test robustness of the coating samples, consists of a combination of incubation and washing cycles using PBS-T and deionized water. The samples are first dipped in a solution of PBS-T and then rinsed with deionized water and spun dry. The samples are then exposed to 6 cycles of PBS-T incubation and chip aspirations with an incubation time between 2 and 10 minutes for each cycle. The samples are then rinsed one final time with deionized water and centrifuged till dry.

7.3.2 Results

We found that after the extensive washing process with water and phosphate buffered saline with 10% tween (PBS-T), the majority of the peptoid microspheres were still present on the surface of the slide (Figure 14B). ImageJ analysis of the pre-wash microsphere coating showed that the peptoid structures covered 19.6% of the total slide area, and analysis of the same coating post-wash showed only a slight decrease to 18% of total slide area coverage. Magnified images that depict the individual microspheres left on the surface after the washing process was completed showed that the spheres seemed to have changed in individual appearance. The spheres seemed to lose some of their total volume without much of a change in diameter (Figure 14D). We

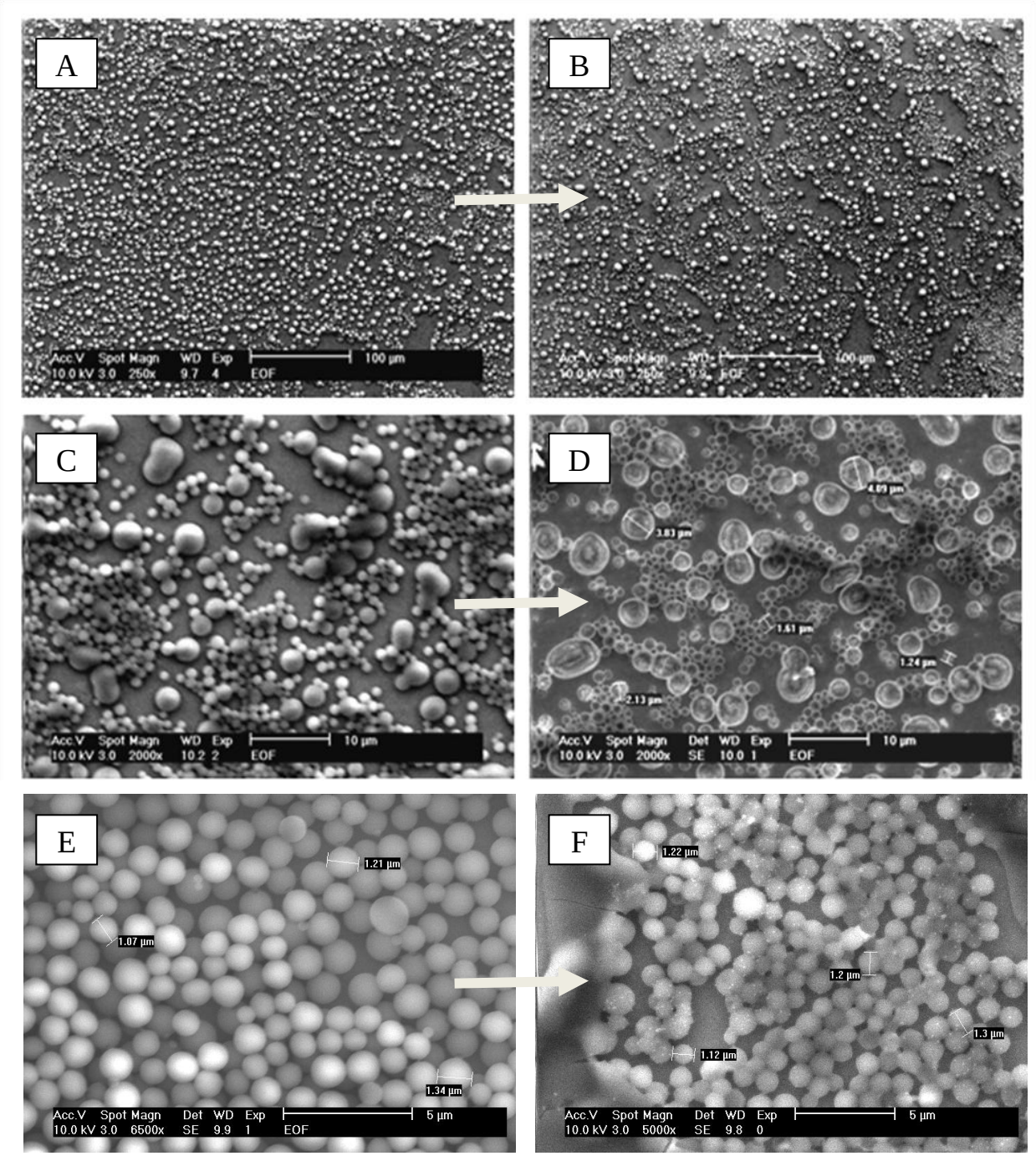


Figure 14. SEM images of coatings on silicon wafer substrates (3 mg/ml peptoid 3 in 4:1 ethanol:water solvent solution) before (A & C) and after (B & D) ELISA microarray washing protocols and also before (E) and after (F) casein blocking and washing protocols, where the top images are full coating views with a scale bar of 100 µm and the bottom images are zoomed-in with a scale bar of either 5 or 10 µm.

believe this change in appearance was due to an effect the PBS-T had on the spheres when it came into contact with them on the surface.

We then completed the same washing protocols on another microsphere coated slide after first incubating the chip with a 1 mg/mL solution of casein (milk protein) in PBS for one hour. In ELISA microarray, casein is used to block the slides and prevent non-specific protein attachment to the slide Comparison of Figures 14E and 14F show that the individual spheres were protected from the PBS-T interactions by the casein solution. Although the casein solution remedied the problems caused by the PBS-T, it formed a layer over the individual spheres causing clumping within the surface coating.

7.3.3 Conclusions

Peptoid microspheres have been shown to be robust when coated on a silicon or glass substrate and subjected to multiple microarray washing protocols. We believe stabilization between the microspheres and the silicon/glass slides is a result of the polar characteristics of both substrate surfaces. If we are correct in our hypothesis that the polar faces of the peptoids are oriented on the outside of the spheres, polar interactions between the spheres and the slide surfaces are allowing a robust coating to be formed. This microsphere/substrate interaction hypothesis can be supported by the AFM analysis (Figure 12B & C) that depicts a hemispherical peptoid structure when coated on the substrate.

Optimization of the current coating and protection protocols is still necessary, but no further substrate attachment mechanisms will be needed. Integration of the preliminary coating design into a robust microarray detection platform that can be used to increase the antibody attachment density for early disease detection should be completed easily and efficiently.

8. Continuing Work Recommendations

The work completed towards the project goals has created a solid foundation that can support continuing work leading to a fully functional peptoid ELISA microarray slide coating. Continuing work is needed to optimize and analyze this coating in order to utilize the knowledge gained thus far and further build towards an industrially applicable ELISA microarray coating. The recommended future work includes (1) continuing investigation of the correlations between peptoid sequence characteristics and microsphere self-assembly, (2) optimization of the peptoid slide coating on a fully processed glass ELISA microarray slide, (3) confirmation of the increased antibody attachment density on the 3-dimensional slide coating, and (4) customization of the peptoid sequence to induce directed orientation of the attached antibodies.

8.1 Peptoid Sequence Characteristics

The sequence characteristics studied thus far, have opened the door to any number of functional group combinations that could potentially self-assemble and form robust microsphere coatings. The exact recipe of functional group characteristics that consistently lead to these self-assembled structures still needs to be determined. Some aspects that we did not consider during these studies were the functionalities of the side-chains and how they will ultimately interact with the attached antibodies. We did try to keep these interactions in mind when designing our peptoids, but testing of the assumed interactions will need to be completed before the slides can be used commercially.

Investigation of the design of peptoids that form a specific size microsphere by altering the chain length or individual side-chain chemistries would be recommended. We observed that the addition of bulky or charged functional groups could greatly affect the diameter of the formed spheres.

Studies determining the ionization characteristics of the peptoids in solution would also need to be completed in order to fully understand the interactions occurring between neighboring peptoids. These studies could be completed using electrophoresis techniques to determine the isoelectric point of the peptoids in solution and the individual side-chain charge characteristics under different solvent, structure, and sequence conditions.[60] Another technique that could be used to determine the actual ionization of the peptoids is nanofilitration using membrane rejection. This technique uses a charged membrane that allows neutral molecules to pass through and rejects charged molecules.[54] A study using these membranes across a pH gradient system would allow for the calculation of the isoelectric point. This information would allow more concrete conclusions to be drawn regarding the interactions between the individual peptoids due to charge characteristics and distribution.

Sequences containing chiral aliphatic side chains could be designed to test the effects of aromatic versus non-aromatic structures. Sequences including protease resistant functionality could also be tested for their formation, as protease resistant structures could lead to a number of new applications. There are many opportunities for peptoid sequence modifications that could greatly affect the size, uniformity, and efficiency of these self-assembled microspheres.

A study to investigate the timeframe in which these peptoid microspheres self-assemble would also be recommended. Preliminary findings suggest that the microspheres are forming while in solution and very quickly such that intermediate structures are difficult to decipher (Unpublished results from Dr. Ronald Zuckermann, Berkley University, 2008). Confirmation of these findings would need to be completed so that formation environments could be studied with the solution conditions, or surface conditions if the spheres were found to form during the drying process.

In order to fully understand how these structures are forming, x-ray diffraction (XRD) should be utilized to determine the internal organization of the assemblies as a whole. Zuckermann's group utilized XRD to determine structure thickness and organization characteristics for peptoids self-assemblies.[49, 51] XRD could be used to determine whether or not the peptoids are forming these microspheres in either an amorphous or crystalline manner.[61] A crystalline formation would allow for a greater understanding of the structural organization, and how and why the peptoids are self-assembling into microspheres. Performing XRD with different sequence modifications could help to track the specific effects caused by each individual characteristic, and allow for more definite conclusions to be drawn regarding molecule interactions.

8.2 Coating Optimization for ELISA Microarray Processing

The majority of the uniform coating work done thus far has been on silicon wafer chips so that they could be readily analyzed using an SEM, as it requires the sample to be conductive. The necessary coating characteristics discovered using the silicon wafers need to be tested on glass slides that will be necessary for the ELISA microarray detection process. Preliminary data using

these glass slides was obtained and confirmed peptoid microsphere formation (Figure 13), but extensive testing under microarray processing conditions including those discussed in section 7.3.1 will need to be completed.

Some processing conditions were analyzed during the washing experiments conducted as an extended section of our studies (Figure 14), but a number of steps were omitted to reduce material waste. There are multiple steps during ELISA processing that include extended incubation times in a sample solution. Testing will need to be completed to determine how robust these microsphere coatings will be at this extended soaking times and on the glass substrates. The limits of coating robustness will also need to be tested for the peptoid microspheres on glass substrates. This could be achieved through incubation of the coatings in strong acid and then strong base solutions, as well as introducing them to different thermal levels to determine the environments in which the microspheres remain robust.

Completing the microarray washing steps with the inclusion of salts with increasing concentration could be another way to test the microspheres robustness limits. As the salt concentration increases, and the solvent becomes more polar, that microspheres should be more easily removed from the slide. At some transition point, the polar attraction of the microsphere to the solvent will overcome the original polar interactions with the slide.

The peptoid coatings will also need to be analyzed for their reaction to the attachment of antibodies. We hypothesize that the peptoids will act as merely a solid platform to the antibodies and not transform or interact with other molecules on the surface of the slide. We believe this to

be true due to the casein protection layer that will be applied to the microspheres before the antibodies are introduced to the slides.

The application process study shed light on some potential problems that could arise when attempting to coat an entire microarray processing slide. We observed that the larger the surface that needed to be coated, the more difficult it became to achieve uniform coverage over the entire area. We recommend performing a wax blocking protocol that includes adding waxed squares to the microarray slides for isolation of the individual arrays before administering the coating onto the slides. This wax blocking would reduce the coating area from the entire glass slide to several individual areas of ~0.5cm x 0.5cm.This would require smaller local volumes and allow for more uniform microsphere coverage over each array area.

An optimized, automated coating system will also need to be created so that the peptoid microsphere coatings can be applicable in an industrial environment. The coating application processes discussed thus far are all manually administered. An automated process would reduce human error that can affect the coating uniformity, and allow for a reliably reproducible uniform surface coating. Spin and sputter coatings are both possible automation techniques for our microsphere coatings. For spin coating, the spin speed and sample volume can be controlled so that an even layer of peptoid microspheres can be administered to all sections of the substrate. Sputter coating can also be controlled to give a reliable and repeatable coating through specified sample volumes and sputter patterns. Both of these automation techniques need to be tested for coating uniformity and reproducibility to determine the best avenue for the automation of our peptoid microsphere application process.

8.3 Confirm Increased Attachment Density

The main hypothesis that led to the creation of our microsphere coatings was that if we could create a non-porous, three dimensional, microarray surface, we could increase the attachment density of the antibodies, and ultimately increase the signal intensity without creating an overpowering level of background noise. This hypothesis will need to be evaluated through calculations determining the ratio of surface area between our 3-D coating and a normal 2-D coating, coupled with microarray evaluations depicting ratios of signal intensities between the two styles of coatings. A comparison of these ratios would allow a conclusion to be drawn about whether the 3-D peptoid microsphere coating is allowing a larger number of antibodies to attach to the slide than a currently used 2-D coating. Further microarray analysis would need to be completed to determine the level of background associated with the microsphere coating, and if it could ultimately outperform the slide coatings commercially available.

8.4 Coating Customization

Through customize of the peptoid coating could be used to induce directed orientation of the attached antibodies, as shown in Figure 15. The current random orientation, witnessed in most ELISA microarray applications, has many problems with antibodies being wasted. This occurs by the active sites specified for the disease antigens attaching directly to the glass slide and becoming deactivated.[13] Directed orientation will further increase the range of detection limits and in turn allow us to create a precise detection tool for multiple diseases. Directed orientation of antibodies on the slide could be achieved through the addition of specific binding sites present

on both the slide coating and the capture antibody. Our peptoid microsphere slide coatings are a great candidate for these site additions as the possibilities for functional groups are practically limitless. Most any slide characteristics could be achieved through minor tweaking of our initially tested peptoid sequences. The added slide characteristic of directed orientation would allow a larger number of antibodies to be available for bonding with the antigens found in the sample serum. This would lead to a further decrease in the overall cost of disease detection.

Figure 15. Schematic representation of random and directed orientations of antibodies[27]

9. Conclusion

Through investigation of the charge distribution and secondary structure of designed peptoids, it was concluded that peptoids with secondary helical structure and partial water solubility have the characteristics necessary to self-assemble into tightly packed microspheres when introduced to organic/water solutions. The distribution and specific location of charged functional groups throughout the peptoid sequence has a large effect on the ability of the peptoids to self-assemble. Observations between P1 and P2 concluded that even with the proper secondary structure and hydrophobic characteristics, a peptoid with polar functional groups dispersed throughout its sequence created repelling forces between neighboring peptoids and could not successfully form uniform microspheres (Figure 8A). On the other hand, a peptoid with polar functional groups oriented on opposite ends of the structure greatly increased the bulk organization of the peptoids and allowed for more tightly packed microspheres (Figure 8B).

The secondary structure was also found to play a large part in the ability of the peptoid to successfully self-assemble. Through investigation of P3 (helical) and P4 (non-helical) it was found that a peptoid with partial water solubility but no secondary helical structure was only able to form globules (Figure 8D), and is less likely to achieve the tightly packed microspheres than those peptoids that also possessed helical secondary structures (Figure 8C).

The peptoids analyzed in these studies were shown to self-assemble in an organic/water solution through charged functional group interactions and aromatic stacking mechanisms. These peptoids were shown to have a slight helical spectrum when analyzed in an organic/water solvent

solution and a strong helical spectrum when analyzed in a pure organic solvent. The organic/water solution was causing the hydrophobic interactions between the peptoids and the solution to mask the secondary structure of the individual peptoids.

Through investigation of the application process, it was found that the coating and drying techniques used had a much larger effect on uniformity than the choice of solvent. It was apparent that as long as the solvent had protic characteristics, the specific solvent chosen would not help or hinder self-assembly into microspheres. We believe this is due to polar protic solvents being able to form hydrogen bonds with the peptoids in solution and aid in stabilization of the helical structures formed by the peptoids.[59] The individual coating and drying techniques utilized for each peptoid microsphere surface coating had a great effect on the uniformity and microsphere density observed. It was found that the more complete the coating of the surface area and the larger the volume of solvent evaporated from the coated substrate yielded the most uniform formation of microspheres, both in sphere density (Figure 11B) and size (Table 3). A "natural" drying technique coupled with this full coverage coating process was found to allow for the consistent formation of uniform peptoid microsphere surface coatings.

These uniform coatings were found to be highly robust when exposed to normal ELISA microarray washing protocol conditions. Coatings were imaged using SEM before and after the washing protocol (described in section 7.3.1) to observe changes in attachment density and overall sphere characteristics. It was observed that microsphere attachment density was slightly lower after washing, but not greatly altered. Local microsphere volume characteristics, however, changed dramatically after the washing was complete. We believe this is due to the surfactant in

the PBS-T included during one of the washing steps. This surfactant effect was found to be insignificant when the peptoid coated slides were utilized for the entire ELISA microarray process, which includes an initial slide protection step to reduce such interactions. Since the peptoid coatings tested are highly robust for microarray use, they can be easily integrated into currently used microarray processes without additional protection measures or sequence modifications necessary.

Peptoids have great potential for use in disease detection as they are highly tunable,[26, 27] form robust secondary structures,[28-33] and have the ability to self-assemble.[48-51] Peptoid sequences can be designed to include numerous side-chains, as peptoids are synthetic molecules and their sequences do not need to be found in nature.[26, 27] This ability to specify all aspects of the peptoid sequence can lead to customization of these designed surface coatings to functionalize them for any specialization or application.

We have shown that peptoid microspheres form due to a combination of hydrophobic and aromatic stacking interactions when introduced to an aqueous/organic solution. The increased surface area created by microspheres, acting as the base coating for antibody microarray, will lead to a large increase the density of antigen binding, and in turn increase the strength of the microarray detection signal.[27, 62] We believe these peptoid based slide coatings will create the solid foundation needed to build a reliable test for the early detection of diseases leading to overall increased survival rates.

References

1. [Internet]: American Cancer Society Inc.; cBreast Cancer: Early Detection. 2011 [cited 2012 4/8]. Available from: http://www.cancer.org/Cancer/BreastCancer/MoreInformation/BreastCancerEarlyDetection/index.

2. Zangar RC, Daly DS, White AM. ELISA microarray technology as a high-throughput system for cancer biomarker validation. Expert Rev Proteomics 2006 Feb;3(1):37-44.

3. Petricoin EF, Liotta LA. SELDI-TOF-based serum proteomic pattern diagnostics for early detection of cancer. Curr Opin Biotechnol 2004 2;15(1):24-30.

4. Liotta LA, Petricoin EF. Serum peptidome for cancer detection: Spinning biologic trash into diagnostic gold. J Clin Invest 2006 01/04;116(1):26-30.

5. Tang H, Ali-Khan N, Echan L, Levenkova N, Rux J, Speicher D. A novel four-dimensional strategy combining protein and peptide separation methods enables detection of low-abundance proteins in human plasma and serum proteomes. Proteomics 2005;5(13):3329-42.

6. Wulfkuhle JD, Liotta LA, Petricoin EF, Proteomic Applications for the Early Detection of Cancer. Nature Reviews: Cancer 2003;3:267.

7. Seurynck-Servoss SL, White AM, Baird CL, Rodland KD, Zangar RC. Evaluation of surface chemistries for antibody microarrays. Anal Biochem 2007 Dec 1;371(1):105-15.

8. Gonzalez RM, Seurynck-Servoss S, Crowley SA, Brown M, Omenn GS, Hayes DF, Zangar RC. Development and validation of sandwich ELISA microarrays with minimal assay interference. J Proteome Res 2008 06/01; 2012/02;7(6):2406-14.

9. Ekins RP. Ligand assays: From electrophoresis to miniaturized microarrays. Clinical Chemistry 1998 September 01;44(9):2015-30.

10. Kusnezow W, Syagailo YV, Goychuk I, Hoheisel JD, Wild DG, Antiobody microarrays: The crucial impact of mass transport on assay kinetics and sensitivity. Expert Rev Mol Diagn 2006;6:111-24.

11. Nair PR, Alam MA. Performance limits of nanobiosensors. Appl Phys Lett 2006 06/05;88(23):233120-3.

12. Zhao M, Wang X, Nolte D. Mass-transport limitations in spot-based microarrays. Biomed Opt Express 2010 10/01;1(3):983-97.

13. Seurynck-Servoss SL, Baird CL, Rodland KD, Zangar RC. Surface chemistries for antibody microarrays. Front Biosci 2007 May 1;12:3956-64.

14. Haab BB. Methods and applications of antibody microarrays in cancer research. Proteomics 2003 Nov;3(11):2116-22.

15. Huang RP. Detection of multiple proteins in an antibody-based protein microarray system. J Immunol Methods 2001 Sep 1;255(1-2):1-13.

16. Berg JM, Tymoczko JL, Stryer, L. Immunology provides important techniques with which to investigate proteins. Biochemistry. 5th ed. New York: W H Freeman; 2002. .

17. Seurynck-Servoss SL, Baird CL, Miller KD, Pefaur NB, Gonzalez RM, Apiyo DO, Engelmann HE, Srivastava S, Kagan J, Rodland KD, et al. Immobilization strategies for single-chain antibody microarrays. Proteomics 2008 Jun;8(11):2199-210.

18. Angenendt P, Glokler J, Sobek J, Lehrach H, Cahill DJ. Next generation of protein microarray support materials: Evaluation for protein and antibody microarray applications. J Chromatogr A 2003 Aug 15;1009(1-2):97-104.

19. Dong Y, Shannon C. Heterogeneous immunosensing using antigen and antibody monolayers on gold surfaces with electrochemical and scanning probe detection. Anal Chem 2000 06/01; 2012/04;72(11):2371-6.

20. Roberts CJ, Williams PM, Davies J, Dawkes AC, Sefton J, Edwards JC, Haymes AG, Bestwick C, Davies MC, Tendler SJB. Real-space differentiation of IgG and IgM antibodies deposited on microtiter wells by scanning force microscopy. Langmuir 1995 05/01; 2012/04;11(5):1822-6.

21. Gazit E. Self-assembled peptide nanostructures: The design of molecular building blocks and their technological utilization. Chem Soc Rev 2007 Aug;36(8):1263-9.

22. Járai-Szabó F, Aştilean S, Néda Z. Understanding self-assembled nanosphere patterns. Chemical Physics Letters 2005 6/17;408(4–6):241-6.

23. Schatz, G., Self-Assembled Nanospheres: An Exciting Playground. Acta Physica Polonica A 2009;115(2):S. 431-434.

24. Ghadiri MR, Granja JR, Milligan RA, McRee DE, Khazanovich N. Self-assembling organic nanotubes based on a cyclic peptide architecture. Nature 1993 12/25;366(6453):324-7.

25. Plass R, Last JA, Bartelt NC, Kellogg GL. Nanostructures: Self-assembled domain patterns. Nature 2001 08/30;412(6850):875-.

26. Zuckermann RN, Kerr JM, Kent SBH, Moos WH. Efficient method for the preparation of peptoids [oligo(N-substituted glycines)] by submonomer solid-phase synthesis. J Am Chem Soc 1992 12/01; 2012/02;114(26):10646-7.

27. Simon RJ, Kania RS, Zuckermann RN, Huebner VD, Jewell DA, Banville S, Ng S, Wang L, Rosenberg S, Marlowe CK. Peptoids: A modular approach to drug discovery. Proc Natl Acad Sci U S A 1992 Oct 15;89(20):9367-71.

28. Seo J, Barron AE, Zuckermann RN. Novel peptoid building blocks: Synthesis of functionalized aromatic helix-inducing submonomers. Org Lett 2010 Feb 5;12(3):492-5.

29. Wu CW, Sanborn TJ, Zuckermann RN, Barron AE. Peptoid oligomers with alpha-chiral, aromatic side chains: Effects of chain length on secondary structure. J Am Chem Soc 2001 Apr 4;123(13):2958-63.

30. Wu CW, Sanborn TJ, Huang K, Zuckermann RN, Barron AE. Peptoid oligomers with alpha-chiral, aromatic side chains: Sequence requirements for the formation of stable peptoid helices. J Am Chem Soc 2001 Jul 18;123(28):6778-84.

31. Wu CW, Kirshenbaum K, Sanborn TJ, Patch JA, Huang K, Dill KA, Zuckermann RN, Barron AE. Structural and spectroscopic studies of peptoid oligomers with alpha-chiral aliphatic side chains. J Am Chem Soc 2003 Nov 5;125(44):13525-30.

32. Sanborn TJ, Wu CW, Zuckermann RN, Barron AE. Extreme stability of helices formed by water-soluble poly-N-substituted glycines (polypeptoids) with alpha-chiral side chains. Biopolymers 2002 Jan;63(1):12-20.

33. Kirshenbaum K, Barron AE, Goldsmith RA, Armand P, Bradley EK, Truong KT, Dill KA, Cohen FE, Zuckermann RN. Sequence-specific polypeptoids: A diverse family of heteropolymers with stable secondary structure. Proc Natl Acad Sci U S A 1998 Apr 14;95(8):4303-8.

34. Armand P, Kirshenbaum K, Goldsmith RA, Farr-Jones S, Barron AE, Truong KTV, Dill KA, Mierke DF, Cohen FE, Zuckermann RN, et al. NMR determination of the major solution conformation of a peptoid pentamer with chiral side chains. Proceedings of the National Academy of Sciences 1998 04/14;95(8):4309-14.

35. Möhle K, Hofmann H. Secondary structure formation in N-substituted peptides*. The Journal of Peptide Research 1998;51(1):19-28.

36. Armand P, Kirshenbaum K, Falicov A, Dunbrack RL,Jr, Dill KA, Zuckermann RN, Cohen FE. Chiral N-substituted glycines can form stable helical conformations. Fold Des 1997;2(6):369-75.

37. Patch JA, Kirshenbaum K, Seurynck SL, Zuckermann RN, Barron AE. Versatile oligo(N-substituted) glycines: The many roles of peptoids in drug discovery. In: Pseudo-peptides in drug development. Wiley-VCH Verlag GmbH & Co. KGaA; 2004; 2005. .

38. Wu CW, Seurynck SL, Lee KYC, Barron AE. Helical peptoid mimics of lung surfactant protein C. Chem Biol 2003 11;10(11):1057-63.

39. Seurynck-Servoss SL, Dohm MT, Barron AE. Effects of including an N-terminal insertion region and arginine-mimetic side chains in helical peptoid analogues of lung surfactant protein B. Biochemistry 2006 Oct 3;45(39):11809-18.

40. Seurynck SL, Patch JA, Barron AE. Simple, helical peptoid analogs of lung surfactant protein B. Chem Biol 2005 Jan;12(1):77-88.

41. Brown NJ, Wu CW, Seurynck-Servoss SL, Barron AE. Effects of hydrophobic helix length and side chain chemistry on biomimicry in peptoid analogues of SP-C. Biochemistry 2008 Feb 12;47(6):1808-18.

42. Brown NJ, Johansson J, Barron AE. Biomimicry of surfactant protein C. Acc Chem Res 2008 10/21; 2012/04;41(10):1409-17.

43. Dohm MT, Seurynck-Servoss SL, Seo J, Zuckermann RN, Barron AE. Close mimicry of lung surfactant protein B by dimers of helical, cationic peptoids. Peptide Science 2009;92(6):538-53.

44. Chongsiriwatana NP, Patch JA, Czyzewski AM, Dohm MT, Ivankin A, Gidalevitz D, Zuckermann RN, Barron AE. Peptoids that mimic the structure, function, and mechanism of helical antimicrobial peptides. Proc Natl Acad Sci U S A 2008 Feb 26;105(8):2794-9.

45. Miller SM, Simon RJ, Ng S, Zuckermann RN, Kerr JM, Moos WH. Proteolytic studies of homologous peptide and N-substituted glycine peptoid oligomers. Bioorg Med Chem Lett 1994 11;4(22):2657-62.

46. Wu H, Liang T, Yin C, Jin Y, Ke Y, Liang X. Enantiorecognition ability of peptoids with alpha-chiral, aromatic side chains. Analyst 2011 Nov 7;136(21):4409-11.

47. Huang C, Uno T, Murphy JE, Lee S, Hamer JD, Escobedo JA, Cohen FE, Radhakrishnan R, Dwarki V, Zuckermann RN. Lipitoids — novel cationic lipids for cellular delivery of plasmid DNA in vitro. Chem Biol 1998 6;5(6):345-54.

48. Murnen HK, Rosales AM, Jaworski JN, Segalman RA, Zuckermann RN. Hierarchical self-assembly of a biomimetic diblock copolypeptoid into homochiral superhelices. J Am Chem Soc 2010 Nov 17;132(45):16112-9.

49. Nam KT, Shelby SA, Choi PH, Marciel AB, Chen R, Tan L, Chu TK, Mesch RA, Lee BC, Connolly MD, et al. Free-floating ultrathin two-dimensional crystals from sequence-specific peptoid polymers. Nat Mater 2010 May;9(5):454-60.

50. Chen X, Ding K, Ayres N. Investigation into fiber formation in N-alkyl urea peptoid oligomers and the synthesis of a water-soluble PEG/N-alkyl urea peptoid oligomer conjugate. Polym Chem 2011;2(11).

51. Sanii B, Kudirka R, Cho A, Venkateswaran N, Olivier GK, Olson AM, Tran H, Harada RM, Tan L, Zuckermann RN. Shaken, not stirred: Collapsing a peptoid monolayer to produce free-floating, stable nanosheets. J Am Chem Soc 2011 Dec 28;133(51):20808-15.

52. Sato T, Tsuneda T, Hirao K. A density-functional study on pi-aromatic interaction: Benzene dimer and naphthalene dimer. J Chem Phys 2005 09/08;123(10):104307-10.

53. Po HN, Senozan NM. The henderson-hasselbalch equation: Its history and limitations. J Chem Educ 2001 11/01; 2012/05;78(11):1499.

54. Hestekin JA, Smothers CN, Bhattacharyya D. Nanofiltration of charged organic molecules in aqueous and non-aqueous solvents: Separation results and mechanisms. In: Membrane technology in the chemical industry. Wiley-VCH Verlag GmbH; 2001. .

55. Israelachvili J A. 14.14 the debye length. In: Intermolecular and surface forces. 3rd ed. Waltham, MA: Elsevier Inc.; 2011. .

56. Dong HF, Du HF, Qian X. Theoretical prediction of pKa values for methacrylic acid oligomers using combined quantum mechanical and continuum solvation methods. The Journal of Physical Chemistry.A JID - 9890903 EDAT- 2008/12/05 09:00 MHDA- 2008/12/05 09:01 CRDT- 2008/12/05 09:00 AID - 10.1021/jp807315p [Doi] AID - 10.1021/jp807315p [Pii] PST - Ppublish 0326.

57. Dong HF, Du HF, Qian X. Prediction of pKa values for oligo-methacrylic acids using combined classical and quantum approaches. The Journal of Physical Chemistry.B JID - 101157530 0212.

58. Langer R, Tirrell DA. Designing materials for biology and medicine. Nature 2004 04/01;428(6982):487-92.

59. De Santis E, Hjelmgaard T, Caumes C, Faure S, Alexander BD, Holder SJ, Siligardi G, Taillefumier C, Edwards AA. Effect of capping groups at the N- and C-termini on the conformational preference of alpha,beta-peptoids. Org Biomol Chem 2012 Feb 7;10(5):1108-22.

60. Stenesh J, Biochemistry, Biomolecules: 2.2.2 The isoelectric point. Volume 1 ed. New York: Plenum Press; 1998. .

61. Klug HP, Alexander LE. X-ray diffraction procedures for polycrystalline and amorphous materials. 2nd ed. New York: Wiley; 1974. .

62. Shmanai VV, Nikolayeva TA, Vinokurova LG, Litoshka AA. Oriented antibody immobilization to polystyrene macrocarriers for immunoassay modified with hydrazide derivatives of poly(meth)acrylic acid. BMC Biotechnol 2001;1:4.