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Targeted Drug Delivery using Peptoid Based Nanospheres

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Chapter 1: Introduction

Eighty – eight years ago (in 1928) penicillin, “the miracle drug”, was discovered. It was until 1942 that penicillin was used to successfully treat a civilian patient. [1] Since then modern medicine has given us vaccines for the flu, pneumonia, and meningitis; allowed us to transplant hearts, kidneys, and lungs; and enabled us to sequence the human genome. [2] Even with all of these incredible advances in technology, currently most delivery systems are still non-selective, meaning the drug harms healthy cells as well as disease cells. The damage to healthy cells is what leads to the undesirable side effects caused by many medicines today. By designing a selective delivery system such as peptoid nanospheres, drug side effects could be drastically reduced and possibly eliminated.

Chapter 2: Background

Section 2.1: Peptoids

Peptoids are poly-N-substituted glycines as shown in Figure 1. [3] By using peptoids instead of peptides, protease degradation can be avoided. [4] Protease degradation is particularly concerning for this application because the goal is to deliver medicine to diseased cells. If protease degradation were to occur, the medicine would be released before reaching the desired destination rendering the targeting mechanism useless.

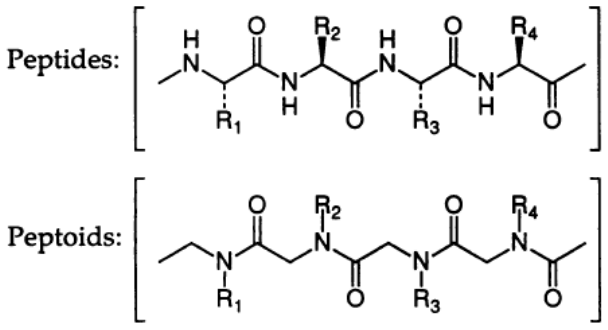


Figure 1. Peptoids vs. Peptides [4]

Another benefit of peptoids is that peptoid alpha helixes have a three pitch turn meaning every third side chain stacks precisely on top of the previous one. [5] This allows the charges to align on one side which is believed to aid in nanosphere formation.

Section 2.2: Peptoid Selection

Having the side chain bonded to the nitrogen makes the backbone achiral but chirality is needed for peptoid formation. To induce chirality, large chiral side chains are used. Peptoids were chosen such that two aromatic rings are followed by a charged side chain. Two positively charged side chains followed by two negatively charged side chains were used because previous research showed this sequence was most likely to induce nanosphere formation. An example structure is shown in Figure Figure 2: Peptoid Sequence2.

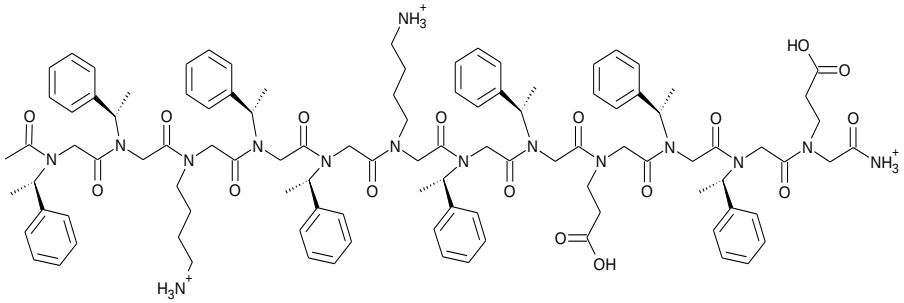


Figure 2: Peptoid Sequence

Section 2.3: Mechanism Theory

The peptoids will be used to form nanospheres then the nanospheres will be attached to peptoids known to bind to specific proteins found at much higher concentrations or exclusively on diseased cells. The peptoid nanospheres will form tertiary structures, as shown in Figure 3, which will allow them to form spheres. The aromatic rings will stack on two sides and the charges align on the third.

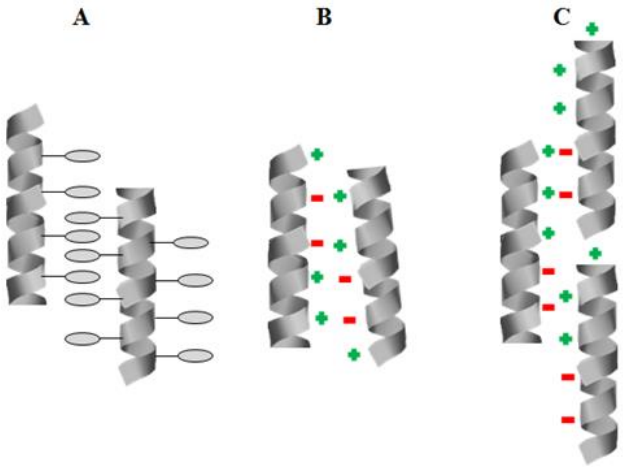


Figure 3: Peptoid Sphere Formation [5]

The peptoid nanospheres will then be attached to affinity peptoids which will target the diseased cells as shown in Figure 4. Upon binding, the medicine will be released at the disease site. The medicine could be released in the cells due to endocytosis. If endocytosis does not occur, the medicine could be released using an external source such as heat or ultrasound.

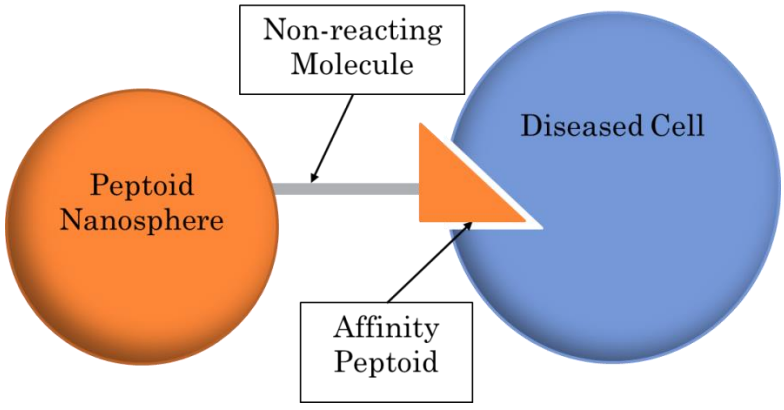


Figure 4: Targeting Mechanism

Chapter 3: Methods

Before conducting experiments, the peptoids were synthesized then purified to greater than 98% using high performance liquid chromatography. Matrix assisted laser desorption ionization was used to confirm the peptoids synthesized properly by verifying the molecular weight. Circular dichroism was used to determine the helicity before proceeding with nanosphere testing. Scanning electron microscopy showed if peptoids formed on a solid substrate. Finally, dynamic light scattering in conjunction with transmission electron microscopy was used to determine if spheres formed in solution.

Section 3.1: Peptoid Synthesis

Peptoid synthesis is a submonomer process that begins with an amine group attached to a rink amide resin. [6] The resin is swelled using 40 mL of dimethylformamide (DMF) per 1 mmol of resin determined by weight, then drained. The Fmoc group is then deprotected using 20% piperdine in DMF at 40 mL/mmol concentration. This step is repeated to ensure the Fmoc group is completely deprotected. The resin is then rinsed using DMF at 40 mL/mmol five times. To begin the submonomer process, 1.2 molar bromoacetic acid at a concentration of 17 mL/mmol resin is used to acylate the amine. This is done in the presence of diisopropylcarbodiimide (DIC) at a concentration of 3.2 mL/mmol resin. The resin is rinsed three times with 40 mL/mmol DMF before 17 mL/mmol of a 1 molar solution of the desired side chain, typically in DMF, is added to the solution. The bromine is displaced by the desired sidechain using an S_N2 mechanism. This process is repeated until the desired peptoid is synthesized.

Before the peptoid can be used it must be cleaved from the resin. To begin this process the peptoid on resin is dissolved in 10 mL of a 95% trifluoroacetic acid (TFA), 2.5% triisopropylsilane, and 2.5% water solution then swirled for ten minutes. Immediately the peptoid solution is filtered to remove the resin beads then rotary evaporated. Once the sample

has reached an oily state in the rotary evaporator, it is dissolved in just enough 50/50 acetonitrile and water mixture to have a concentration of 3 mg/mL based off the original sample weight.

Section 3.2: High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) was used to purify the sample then analytical HPLC was analyzed to determine the purity of the sample. Before using the HPLC, all solvents were sparged with hydrogen for 30 min to ensure no air entered the column. Once sparging was complete, solvent A (94.5% water, 5% acetonitrile, and 0.5% (TFA) was run for 10 minutes followed by 10 minutes of solvent B (94.5% acetonitrile, 5% water, and 0.5% TFA) then the column was equilibrated with starting conditions for 10 minutes. Three milliliters of 3 mg/mL peptoid dissolved in 50/50 acetonitrile and water was injected into the HPLC then a 1% gradient per minute from 30 to 95% acetonitrile was run. Ultraviolet light was used to determine when peptoid was leaving the column and these samples were collected. The collected samples were analyzed using analytical HPLC and matrix assisted laser desorption ionization.

Section 3.3: Matrix Assisted Laser Desorption Ionization

Matrix assisted laser desorption ionization (MALDI) was used to confirm the correct peptoid was synthesized. To perform MALDI, 1 µl of peptoid sample was mixed with 1 µl of DHB matrix then dried on a metal plate. Standards were ran before testing the samples. The molecular weight estimated by MALDI was then compared to the predicted molecular weight to determine if the peptoid synthesized properly.

Section 3.4: Circular Dichroism

Circular dichroism determines the relative helicity of the peptoids by comparing the difference in light absorption between left and right circularly polarized light. The circular

dichroism value was calculated by subtracting the right circularly polarized light from the left circularly polarized light. [7] This experimental value was then compared to known values to determine the peptoid structure.

Section 3.5: Scanning Electron Microscopy

Scanning electron microscopy (SEM) uses an electron beam to form an image of a surface. [8] The anode directs the electron beam down the column towards the specimen as shown in Figure 5. [9] The beam then proceeds through a series of magnetic lenses. The condenser lens controls the spot size and an objective lens controls where the beam comes into focus. The stagnator makes the electron beam round before it contacts the sample. [10] When the electrons hit the sample, electrons and x-rays are emitted. [9] Detectors collect data from each of these emissions and convert it to a signal which is used to construct an image of the surface. [8]

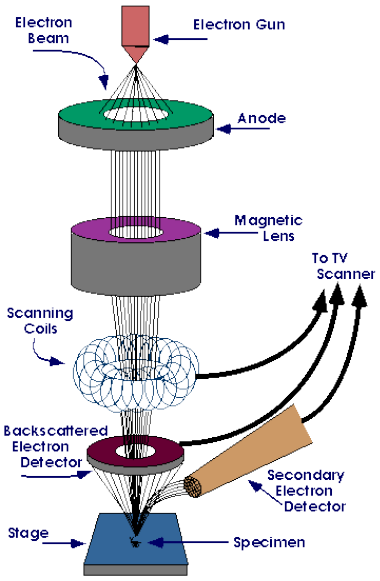


Figure 5: Scanning Electron Microscope [8]

For SEM, samples must be conductive and completely dry. SEM is performed under a vacuum to prevent air molecules from interfering with the electron beam. If water is present in

the sample, it will vaporize and disrupt the equipment. Non-metal samples must be sputter coated with a conductive material. [8]

The peptoid samples were dried on silicon chips at room temperature for 30 minutes then sputter coated with gold before being imaged. These images were used to determine if spheres formed on the surface of a dry substrate.

Section 3.6: Dynamic Light Scattering

Dynamic light scattering (DLS) predicts the size of spherical particles in solution. The instrument sends a laser beam through the sample as shown in Figure 6. It then measures the transmitted light shown as "PD" in the figure and scattered light at either 90° or 173° depending on the concentration of the particles. This data is used to determine the size of the particles in solution. [11]

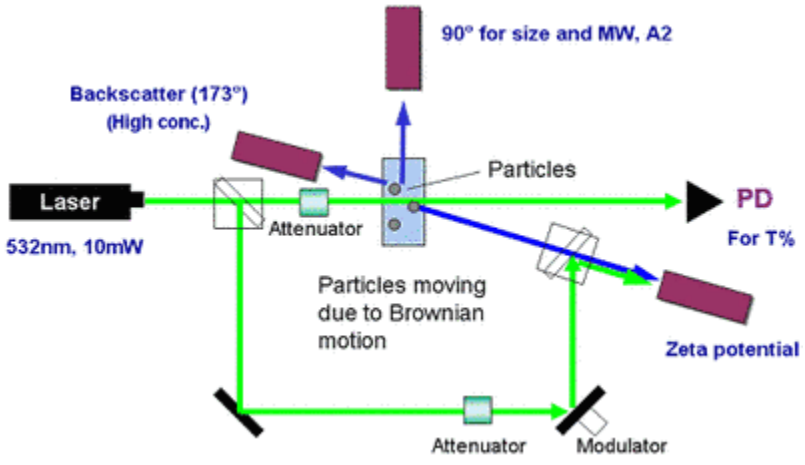


Figure 6: Dynamic Light Scattering Set-up [11]

The particles are assumed to be in Brownian motion therefore the probability density function is known. Using the transmitted and scattered light data, the diffusion constant can be calculated with the probability density function. Stokes-Einstein relation can then be applied based on the assumption that the particles are small. This yields an equation for the diffusion

constant that is based on the particle radius, the Boltzmann constant, temperature, and the viscosity of the solvent. [12]

To conduct this experiment, each peptoid was dissolved in a 4 to 1 ethanol and water solution at 3 mg/mL. The program used the light intensity data to calculate volume and the number of particles for each diameter.

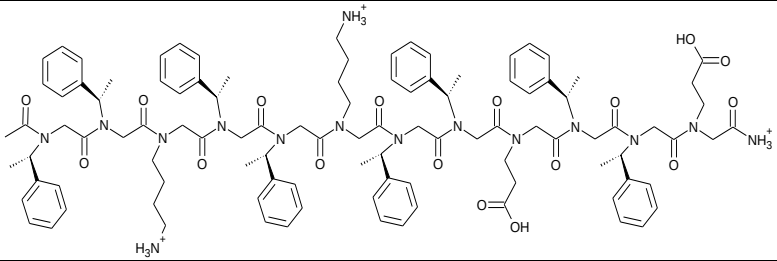
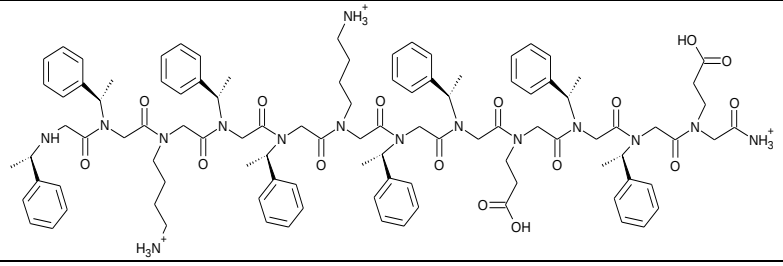
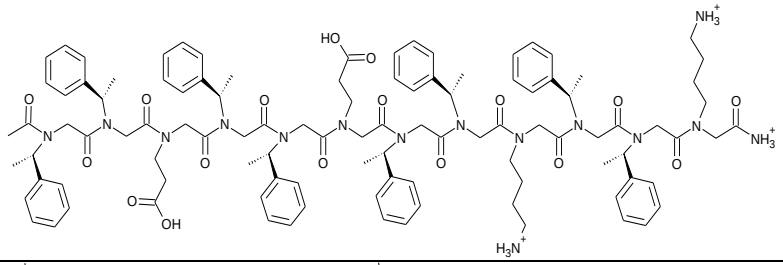
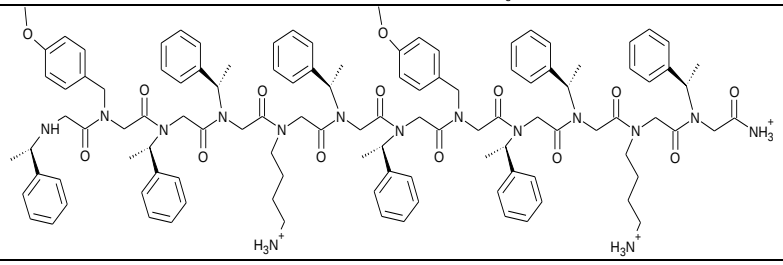
Section 3.7: Transmission Electron Microscopy

To perform transmission electron microscopy (TEM), 1 µL of 3 mg/ mL peptoid dissolved in a 4 to 1 methanol and water solution was mixed with 1 µL 1% uranyl acetate and dried on copper mesh at room temperature for 24 hours. Once dried, the samples were viewed using TEM, which is similar to SEM except the transmitted light is detected instead of the scattered light. [13]

Chapter 4: Results

The peptoids were synthesized then purified using HPLC. MALDI was used to determine if the peptoids were properly synthesized. Analytical HPLC was performed to ensure the peptoids were above 98% pure. The peptoids tested during this study are shown in Table 1 below.

Table 1: Peptoid Sequences

Peptoid	Sequence	Molecular Weight
P1		1866
wP1		1824
rP1		1866
P3		1919

Section 4.1: Circular Dichroism

Based on previous research conducted in the Servoss lab, in order for nanosphere formation to occur, peptoids need to form a polyproline type 1-like helix. All four peptoids were determined to have this secondary structure based on their minima around 200 and 220 nm. [5] Figure 7 shows typical circular dichroism results from literature and Figure 8 shows the results obtained by another student in Servoss's lab for the peptoids used in this study.

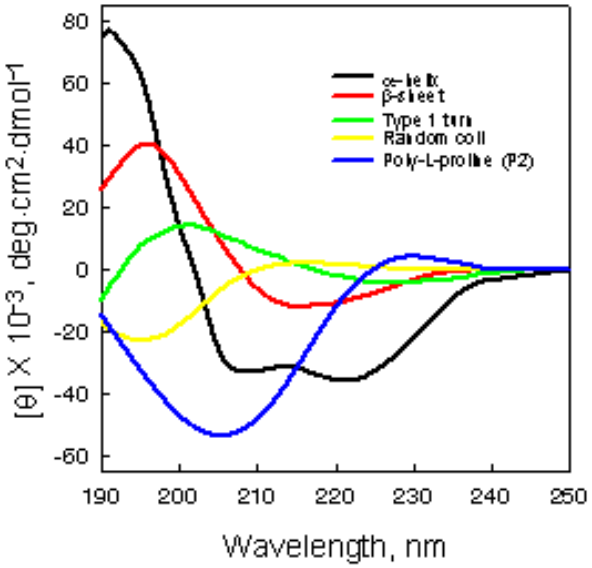


Figure 7: Circular Dichroism Literature Values [14]

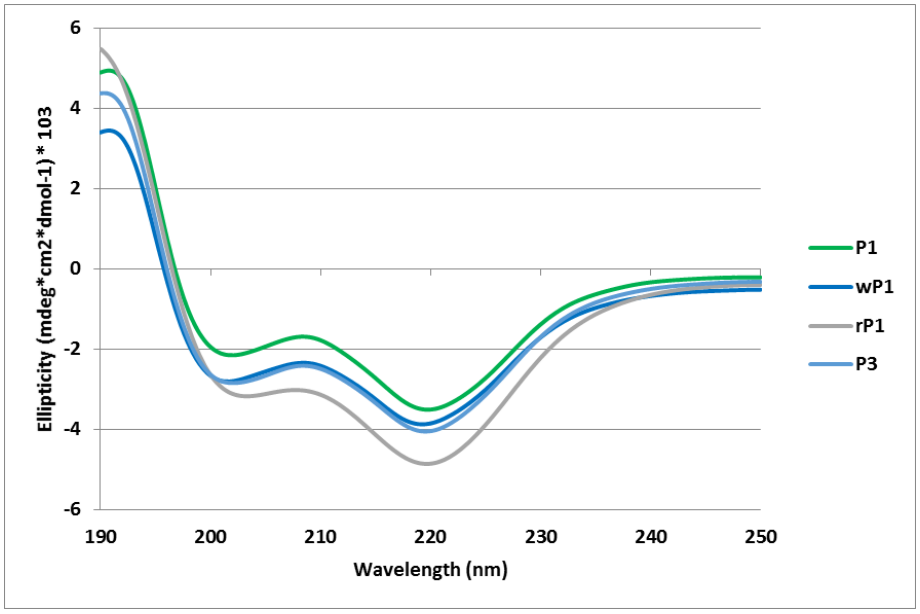


Figure 8: Circular Dichroism Results [5]

Section 4.2: Scanning Electron Microscopy

Scanning electron microscopy was used to determine if peptoid nanospheres formed on a solid substrate. This experiment was performed previously then redone before testing the peptoids further. The figure below shows representative SEM images for the four peptoids tested. P3 was the only peptoid that showed uniform sphere formation when dried on a solid substrate.

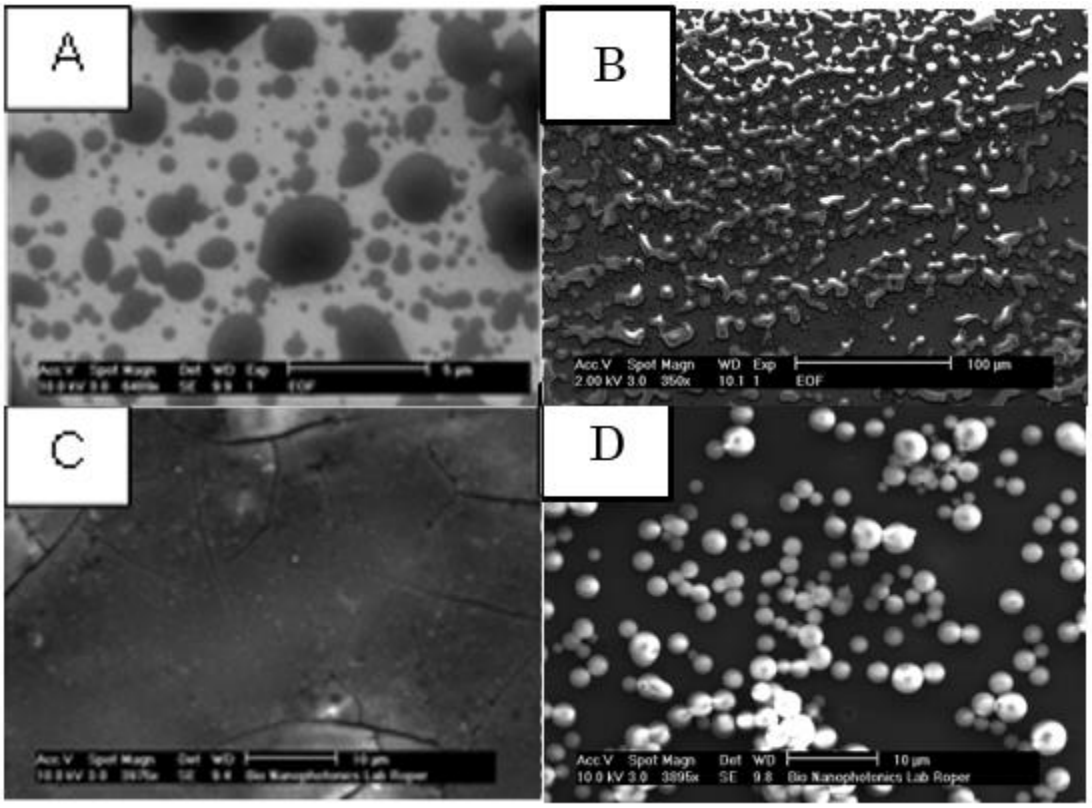


Figure 9: SEM Images A) P1 (scale bar = 5 µm) B) wP1 (scale bar = 10 µm) C) rP1 (scale bar = 5 µm) D) P3 (scale bar = 10 µm)

Section 4.3: Dynamic Light Scattering

Dynamic light scattering was used to determine the size of particles in solution. The graphs with multiple peaks were determined to not form uniform spheres in solution because either they formed spheres of multiple sizes or the particles were different sizes in different directions. The peptoids with a single DLS peak were tested using transmission electron microscopy.

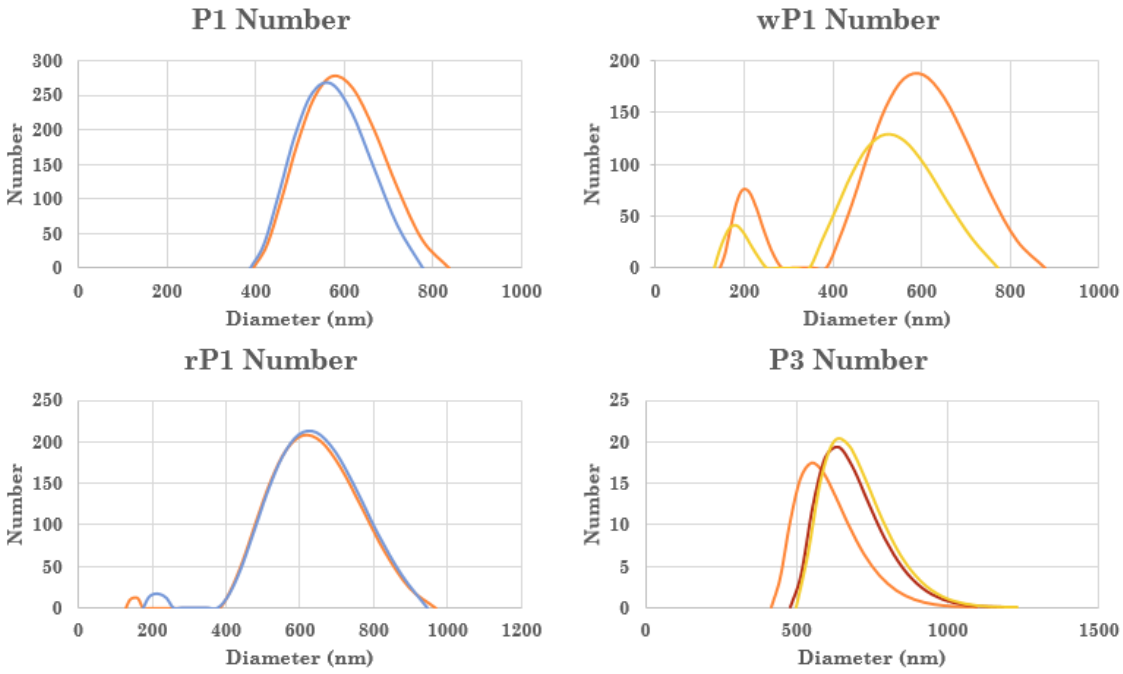


Figure 10: DLS Data

Section 4.4: Transmission Electron Microscopy

Transmission electron microscopy was performed on two of the peptoids. One of the peptoids, P3, formed spheres which were analyzed using Image J. The data from Image J was averaged in excel. P3 was found to have an average diameter of around 500 nm.

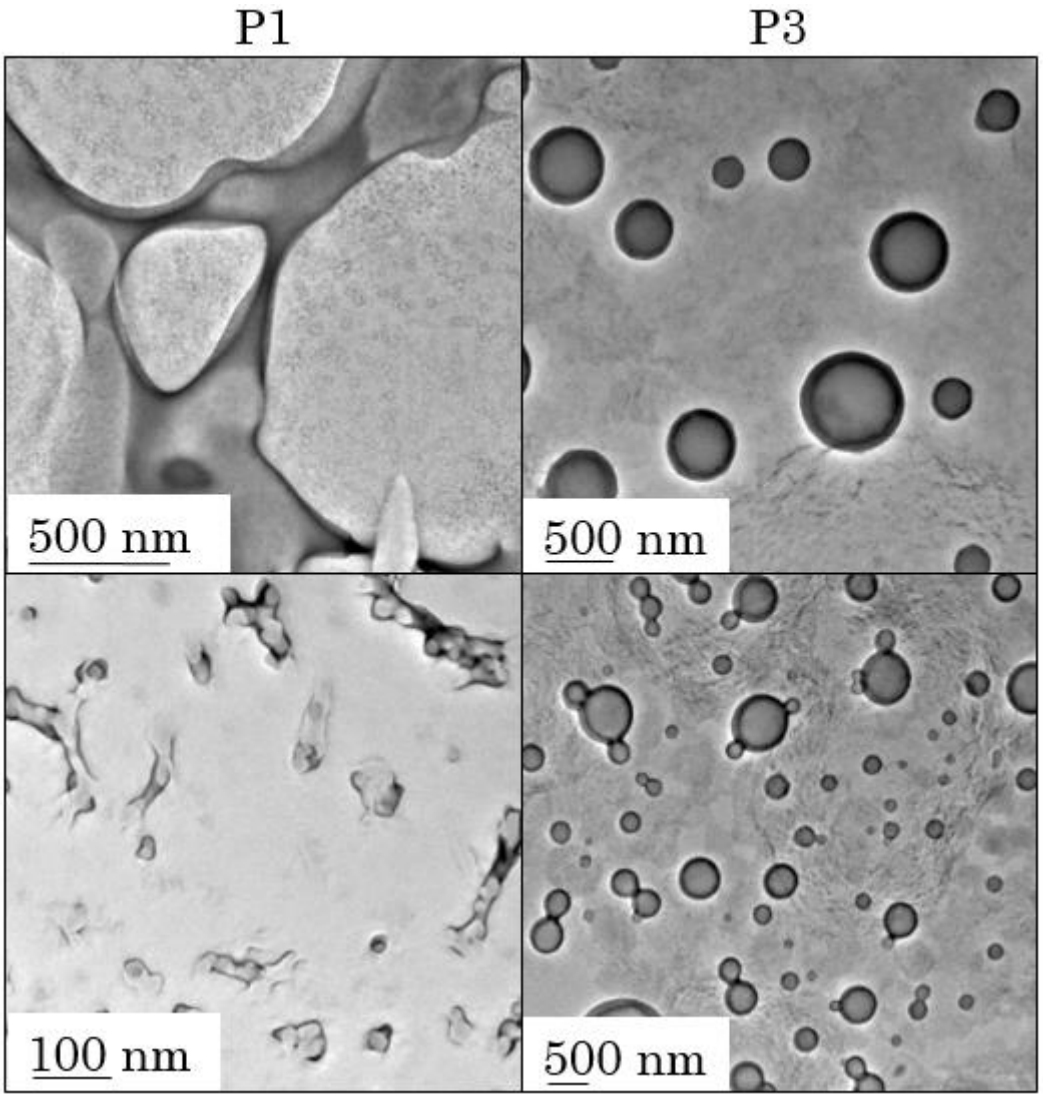


Figure 11: TEM Images

Chapter 5: Discussion

The scanning electron microscopy showed if spheres formed when dried on a solid substrate. To determine if the spheres formed in solution or during the drying process, dynamic light scattering and transmission electron microscopy tests were performed. The TEM images were analyzed and the average diameter was determined. This was compared to the diameters predicted by DLS. These were approximately 500 nm and 600 nm respectively. Because the diameters were less than 20 % different, it can be assumed that the spheres formed in solution. The error could be due to the inaccuracy of using ImageJ to estimate the size of the spheres, but it is more likely due to error in the DLS calculations. DLS accounts for solvent properties when making size predictions. For these tests a pure solvent was not used; however, DLS was set to use the properties of pure methanol for its predictions. This mixture would change the viscosity of the solvent which would change the size predictions.

Chapter 6: Conclusion

While all four peptoids appear to be forming secondary structures, only one of the peptoids formed nanospheres in solution. This indicates that although helicity may be required for nanospheres formation, it is not the only factor. Peptoids are protease resistant and can be manipulated to carry drugs to the disease site. Based on these results peptoids show promise as a drug delivery system.

Chapter 7: Recommendations

To build on this study the peptoids should also be tested in other solvents. Specifically, the peptoids should be tested in a biocompatible solvent and in the presence of salts. Determining if the spheres are stable in the body is key for them to be used for drug delivery. Using the knowledge gained from these experiments, new peptoids can be designed that are

more biocompatible and more stable in the body. The spheres should then be attached to a peptoid that is known to target VGFR. DLS and TEM should be repeated to determine if VGFR interferes with sphere formation.

Chapter 8: Acknowledgments

In addition to thanking my advisor, Dr. Shannon Servoss, I would like to thank the graduate students Dhaval Shah and German Perez who helped train me in the lab as well as Dr. Mohammadmahdi Malmali, Melissa Hebert, Dr. Suresh Kumar, and Dr. Philip Blake. Tests were performed at Arkansas Statewide Mass Spectrometry Facility, Arkansas Nano-Bio Materials Characterization Facility, and Arkansas Biosciences Institute. Funding was provided by SURF, University of Arkansas Honors College, and University of Arkansas Start-up funds.

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