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The Synthesis, Purification, and Characterization of the P3 Peptoid

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Introduction

Peptoids are small molecules physically analogous to peptides.[1] The benefits of using peptoids are vast as they are highly customizable and can be made into foldamers. Foldamers are artificial systems that self-organize into stable secondary structures usually characterized by having a molecular surface area larger than their organic counterparts. These foldamers are tunable in terms of orientation of side-chain functional groups.[2] Both peptoids and foldamers have uses in a plethora of industries - medicine, material science, chemical processing, energy, etc. Investigating the characteristics of different peptoids in different conformations is necessary for the growth of the field of nano- and micro-systems.

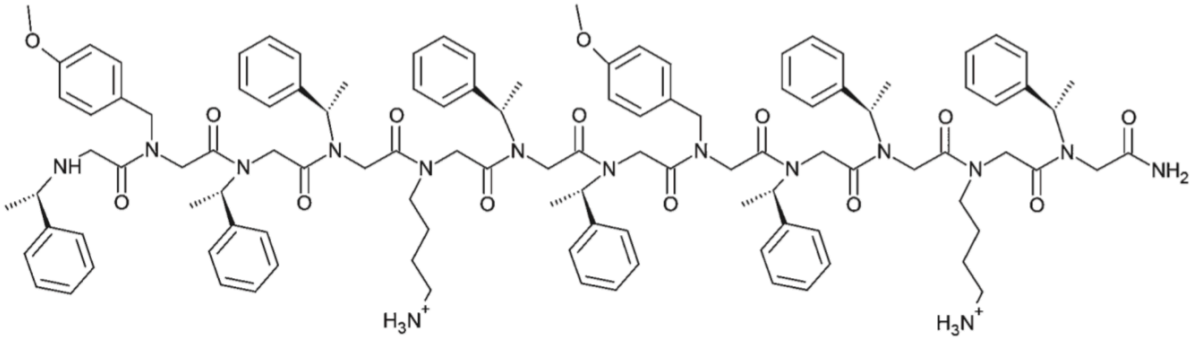

Figure 1 - Molecular Structure of the P3 Peptoid [3]

P3 is the peptoid investigated throughout this paper. Its composition is essentially a poly-N-substituted glycine backbone consisting of three different side chains: L (-)-alpha-methylbenzylamine (F), tert-butyl N-(4-aminobutyl) carbamate (K), and 4-methoxybenzylamine (M). The sequence of these twelve side chains is F M F F K F F M F F K F. A molecular diagram

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[1] Orwell. (1995). The 'peptoid' approach.

[2] Mandity et al. (2015). An overview of peptide and peptoid foldamers.

[3] Herbert et al. (2013). Tunable peptoid microspheres.

for the peptoid can be seen above in Figure 1. The established molecular weight of P3 is 1,919 daltons. These peptoids have an achiral backbone and can form stable helices. These helices are able to interacting with neighboring helices to form microspheres presumably due to stacking of the aromatic groups. A diagram of this self-assembly process can be seen in Figure 2.

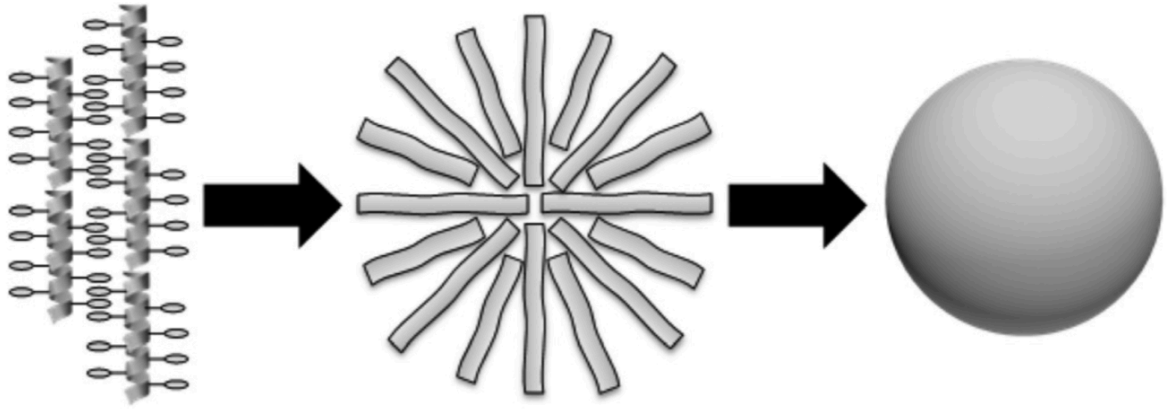

Figure 2 - Sphere Formation From Helices [4]

This paper will examine the synthesis, purification, and characterization of the P3 peptoid. The characterization will focus on the robustness of P3 microspheres. High robustness of a peptoid is essential to its viability for drug delivery. After the peptoids have been synthesized and conformed into microspheres, the peptoids can be taken out of solution. The peptoids can then be brought back into solutions containing the desired drug. This allows the drug to be encapsulated inside the microspheres.

[4] Herbert et al. (2013). Tunable peptoid microspheres.

Methodology

Synthesis

The synthesis process begins with the equipment set-up. The main piece of synthesis equipment is the reaction vessel. This vessel has a drain that can be opened into a beaker. A nitrogen inlet stream allows for mixing in the reaction vessel. An attached vacuum system allows for the reaction vessel to drain effectively. After the equipment is arranged, the solutions of side chains need to be prepared. As there are eight F side-chains, the F solution is 1M 35 mL in dimethylformamide (DMF). As there are two K side-chains, the K solution is 1M 10 mL in DMF. As there are two M side-chains, the M solution is 0.2M 10 mL in DMF.

The rink amide resin is then prepared at a ratio dependent on the millimoles per milligram of the resin. Resin is essentially the base for the peptoid to be built upon. The resin used had a substitution ratio 0.79 mmol/mg. Using the sample equation for a 0.25 mol peptide synthesis (Equation 1), the milligrams of resin required for a desired amount of synthesized peptoid can be calculated. The resin is then added to the reaction vessel and swelled via mixing and suspension with DMF.

$$\frac{0.25\ mmol\ of\ peptoid}{0.79\ \frac{mmol}{mg}} = 316.5\ mg\ of\ resin$$

Equation 1 - Sample Resin Calculation

The next step is Fmoc deprotection which is necessary for the upcoming additions of side-chains. 0.8 milliliters of piperidine in 3.2 milliners of DMF is suspended in resin. This step is then repeated with an extended suspension period. Several DMF washes follow to ensure the

piperidine has been removed from the reaction vessel. The piperidine effectively renders the resin able to attach side-chains to its surface; however, left-over piperidine would destroy the incoming side-chains. Bromoacetic acid (BAA) is then added to the vessel to prepare the resin for the upcoming addition reactions.

The addition of a side-chain is a combination of both bromoacetylation and coupling steps. The bromoacetylation is achieved by combining N,N'-Diisopropylcarbodiimide (DIC) and BAA. A DMF wash follows the bromoacetylation before the addition can take place. The coupling begins by adding the side-chain solution to the vessel. A DMF wash follows the coupling step. This two step cycle is repeated for each side-chain in the desired order. After the addition of the final side chain, a dichloromethane (DCM) wash effectively ends the reaction process by blocking off the amine group in the final F group. The resin is then dried and stored in a vial at -20°C.

A test cleavage is then undergone to provide a sample for matrix-assisted laser deprotonation/ionization (MALDI) and analytical high-performance liquid chromatography (HPLC) characterization. These tests ensure the synthesis was performed correctly. The cleavage process is described in-full below.

Cleavage

The cleavage process begins by measuring a third of the freshly synthesized peptoid-resin complex and placing it into a vial. A mixture of triisopropylilane (TIS) and water is created in a separate vial. Trifluoroacetic acid (TFA) is then added to this mixture. This multicomponent mixture is then combined with the resin-peptoid complex, which is subsequently placed on a belly dancer for vigorous mixing. This step allows for the peptoid to be separated from the resin.

Then, the resin is filtered from the solution via a filter column. The solution is filtered into a rotovap glass so that it can be transformed into its oil state. An equimolar solution of water and acetonitrile is added to the peptoid. The amount of solution added is dependent upon the initial resin weight, as a solution concentration of three milligrams per milliliter is desired. The peptoid solution is then refrigerated.

Purification

The peptoid is purified using a preparative HPLC. Once the equipment has been turned on, the feed is sparged to removed all air from the lines. A blank is sent into the system to calibrate the machine. After the blank has been run, the purification can begin. The HPLC is able to separate the peptoid from the other components of the solution. The purified HPLC is sent to the fraction collector where it is placed in tubes. The fraction collector is set to move to another tube every ten seconds. The limit considered pure for this research was 0.2 Absorbance Units (AU). Sample data for an HPLC run for P3 is found in the Data section (Table 1).

Characterization

The initial MALDI and analytical HPLC synthesis checks proved a proper synthesis over the course of eight different syntheses. Results from a MALDI P3 presence check can be found in the Data section (Figure 3). This data shows that a molecule of 1,917 daltons is present in the sample. This measurement has a two dalton discrepancy from the 1,919 dalton molecular weight of P3. As the two amine groups in the K side-chains can be deprotonated during the process, this two dalton difference can be justified.

To measure the robustness of the microspheres, dried P3 is diluted in a mixture of ethanol and water. This suspension allows for the P3 peptoids to take on the secondary structure of

helices. This solution is then spotted on a glass slide which is placed inside a humid chamber to allow for the helices to come together as microspheres. After the solution is spotted on a glass slide, the remaining peptoid mixture is lyophilized to return the peptoid to its dry state. This process is repeated several times to examine the changes in microsphere morphology after each round of lyophilization. The slides of microspheres were imaged using a scanning electron microscope (SEM).

Results & Discussion

The main results from this study are the SEM images of the microspheres at each stage of the process from pre-lyophilization to after the second lyophilization. The images can be seen in the Data section in Figure 4. The distinct spherical morphology is retained after two rounds of lyophilization. It should be noted that the spheres and peptoids were both absent after a third round of lyophilization. This is most likely due to some unnoticed error in the lab that caused the spheres not to form. If the spheres were able to form again after two rounds of lyophilization, they can be considered robust. More of this type of testing should be performed; however, the primary results make P3 look promising as a robust peptoid option.

After many purifications of P3, it became clear that the majority of the pure component came out between the first and third peaks of AU. The samples around the second peak tended to have the highest overall purity and behaved more as expected. A program could be created to decrease the run time for the preparatory HPLC without losing volume of P3 by focusing on this more purified range. The peaks before the first major peak were often other components that had not been washed away.

Future research should investigate how the spheres are able to form in aqueous solutions, as well as, the degradation rates of the P3 microspheres. This first objective would be accomplished easily enough by seeing if the spheres self-assemble in various aqueous solutions such as phosphate-buffered saline. This second objective could be accomplished by encapsulating a colored solution, leaving the peptoids in homeopathic conditions, and measuring the absorbance of samples from the environmental solution over time.

Conclusion

As peptoids and foldamers become increasingly characterized, their utility will continue to increase in a wide variety of fields. P3 itself shows much potential in many different biomedical applications based on its folder ability. The extensive synthesis and purification methods of P3 are feasible in any laboratory with the proper equipment; therefore, it can continue to be synthesized and characterized for any number of studies involving nano-systems. The characterization of robustness for P3 increases its academic value even further. Overall, P3 is a peptoidic foldamer that shows future potential in many fields, in part because of its ability to form microspheres both consistently and robustly.

Data

P3_crude

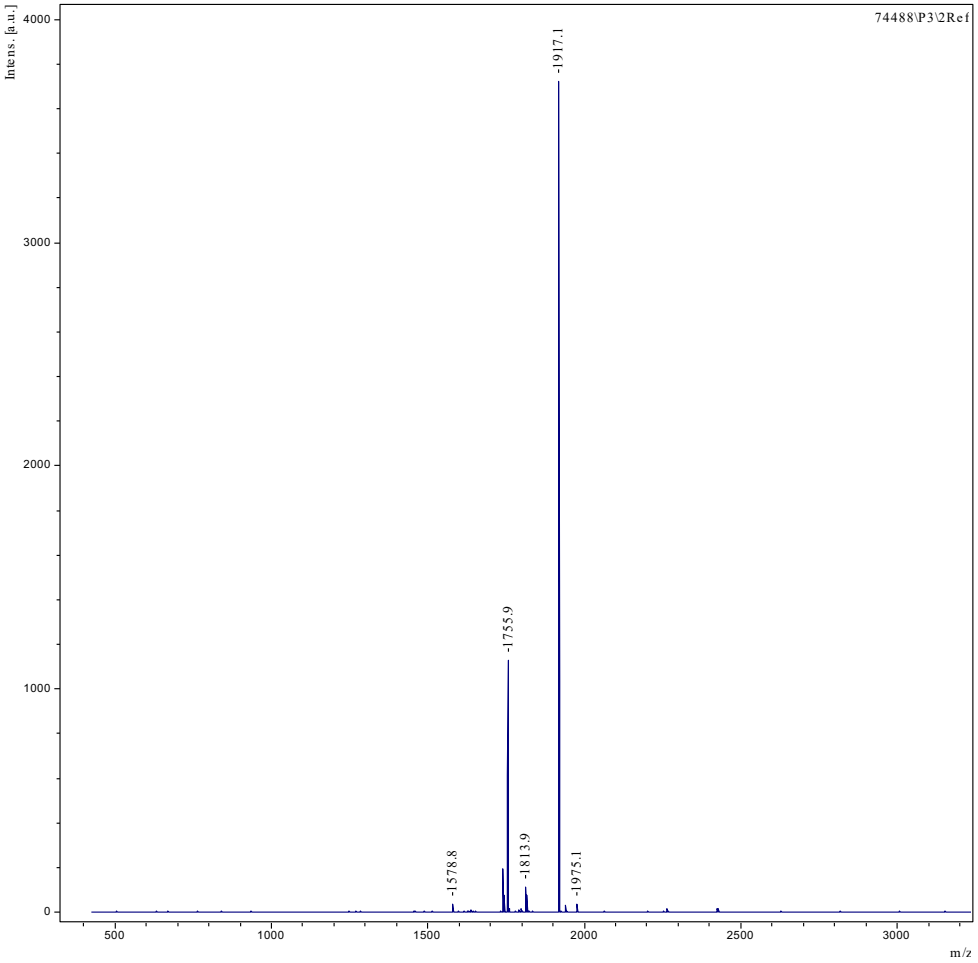

Figure 3 - MALDI Results

Vial Number	Start (AU)	End (AU)	Notes
1	0.2000	0.2000	Peaked at approximately 0.2198 AU
2	0.2000	0.2200	
3	0.2200	0.2345	
4	0.2345	0.2635	
5	0.2635	0.2981	
6	0.2981	0.3552	
7	0.3552	0.4801	
8	0.4801	0.6173	
9	0.6173	0.7445	
10	0.7445	0.9024	Contained the first peak
11	0.9024	0.8922	
12	0.8922	0.8200	
13	0.8200	0.7321	
14	0.7321	0.8289	
15	0.8289	1.2822	
16	1.2822	1.6652	
17	1.6652	2.2215	
18	2.2215	2.2485	
19	2.2485	1.8568	
20	1.8568	1.4058	Contained the second peak
21	1.4058	1.0336	
22	1.0336	0.7434	
23	0.7434	0.5786	
24	0.5786	0.7061	
25	0.7061	1.0273	
26	1.0273	1.3974	
27	1.3974	1.9249	
28	1.9249	1.9103	Contained the third peak
29	1.9103	1.5476	
30	1.5476	1.0566	
31	1.0566	0.6254	
32	0.6254	0.3593	
33	0.3593	0.2000	

Table 1 - HPLC P3 Results

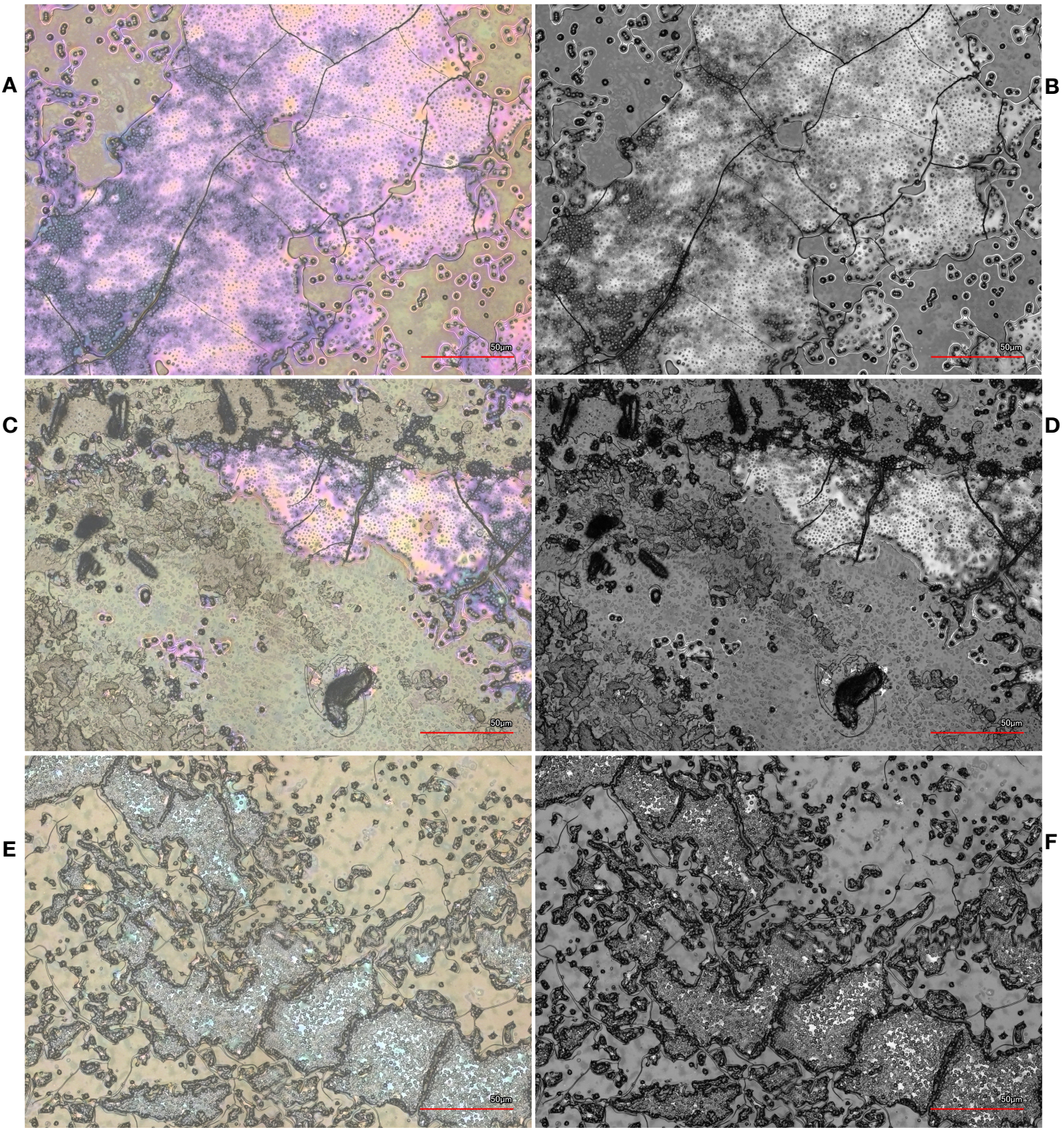

Figure 4 - SEM imaging of the peptoid microspheres pre-lyophilization (A & B), one round of lyophilization (C & D), and two rounds of lyophilization (E & F)

NOMENCLATURE

AU	Absorbance units
BAA	Bromoacetic acid
DMF	Dimethylformamide
DCM	Dichloromethane
DIC	N,N'-diisopropylcarbodiimide
F	L (-)-alpha-methylbenzylamine
HPLC	High-Performance Liquid Chromatography
K	Tert-butyl N-(4-aminobutyl) carbamate
M	4-methoxybenzylamine
MALDI	Matrix-assisted Laser Desorption/Ionization
SEM	Scanning Electron Microscope
TFA	Trifluoroacetic acid
TIS	Triisopropylsilane

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