

Synthesis and Evaluation of Polyaspartate Peptoid Polymers against *Pseudomonas aeruginosa*

by
Rand O. Hasan

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Thesis Committee:

Dr. Kevin Bicker, Thesis Director

Dr. Souvik Banerjee, Second Reader

Dr. Richard Nagorski, Thesis Committee Chair

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APPROVED:

Dr. Kevin Bicker, Thesis Director
Professor, Department of Chemistry

Dr. Souvik Banerjee, Second Reader
Associate Professor, Department of Chemistry

Dr. Richard Nagorski, Thesis Committee Chair
Chair, Department of Chemistry

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Abstract

Polyaspartic acid and its derivatives, such as peptoid polymers, have garnered attention for their potential as antimicrobial agents due to their biocompatibility and structural versatility. This research project delves into the synthesis and evaluation of novel polyaspartate peptoid polymers, aiming to unveil their antimicrobial potential and shed light on their structure-activity relationships. The primary objective is to explore the impact of these novel polyaspartate peptoid polymers on *Pseudomonas aeruginosa* biofilms, investigating their potential as disruptors or inhibitors of biofilm formation. Furthermore, an examination of their susceptibility to proteases aims to determine their stability and potential application as protease substrates. Central to this investigation is the comparison between peptide versions of polyaspartic acid and the peptoid versions being synthesized. Elucidating the differences in their structures and properties will provide valuable insights into the tailored design of antimicrobial materials. The study's methodology involves synthesizing polyaspartate peptoid polymers of varying lengths to evaluate their efficacy against microbial growth and biofilm formation. By systematically varying the length of these polymers, the aim is to discern the relationship between polymer structure and antimicrobial activity, thereby contributing to the understanding of structure-function correlations in these materials.

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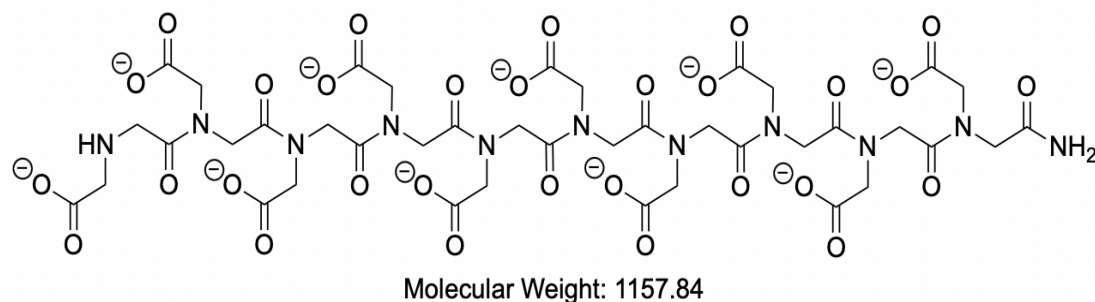
Introduction

Peptoid polymers are a class of synthetic polymers that mimic the structure and properties of peptides and proteins. Due to their unique properties and design flexibility, peptoid polymers have found applications in drug discovery, materials science, biotechnology, and other fields.¹ Peptoid polymers are being explored for drug development due to their ability to mimic biological molecules. They can be designed to interact with specific biological targets, potentially leading to the development of novel therapeutic agents.² Peptoid polymers can also be employed in biological assays and diagnostics due to their ability to bind to target molecules selectively.³ This makes them useful in the development of biosensors and diagnostic tools. Researchers continue to explore and develop new peptoid-based materials for various applications, continuously expanding the potential of this versatile polymer class.

Polyaspartate peptoid polymers are a yet unexplored class of peptoid polymers derived from polyaspartic acid. Polyaspartic acid, a biodegradable polymer composed of repeating aspartic acid units, finds diverse applications in various industries including coatings, adhesives, and pharmaceutical formulations. It serves as a key ingredient due to its excellent corrosion resistance and water solubility, making it suitable for protective coatings in infrastructure projects.⁴ Additionally, its biocompatibility and ability to enhance drug solubility have led to its utilization in pharmaceutical formulations as drug carriers and excipients.⁵ In cosmetics, polyaspartic acid is gaining attention for its potential as an anti-aging agent and moisturizer.⁶ When modified into a peptoid form, it transforms into a polyaspartate peptoid polymer, further expanding its applicability in materials science and biotechnology. Polyaspartate peptides have demonstrated significant antimicrobial

properties, particularly in the inhibition of bacterial biofilms. This antimicrobial capability is particularly valuable in combating persistent bacterial infections⁷, which is why the research focuses on developing the peptoid version of polyaspartate. By creating polyaspartate peptoid polymers, we aim to enhance these antimicrobial properties, specifically targeting the prevention and disruption of *Pseudomonas aeruginosa* biofilms.

The structure of polyaspartate peptoid polymers is distinctive and underpins their remarkable stability and functionality. These polymers retain the backbone structure of polyaspartic acid but incorporate side chains that confer additional stability and resistance to proteolytic degradation, making them more robust than their peptide counterparts⁸. As depicted in the 10-mer in **Figure 1**, the polyaspartate peptoid polymer features a well-defined architecture that supports its application in challenging environments where stability is paramount. This structural integrity, coupled with its water solubility, positions polyaspartate peptoid polymers as highly suitable for applications in drug delivery systems,



biomaterials, and environmentally friendly coatings.

Figure 1. Structure of Polyaspartate peptoid polymer.

One of the key features of polyaspartate peptoid polymers is their stability, particularly in combating bacterial biofilms, our main focus being *Pseudomonas aeruginosa* biofilms.

Pseudomonas aeruginosa biofilms are tough communities of bacteria that can cause persistent infections and are particularly challenging to treat. *Pseudomonas aeruginosa* biofilms present a significant challenge across various sectors due to their inherent antibiotic resistance and propensity to cause chronic infections.⁹ These biofilms form on medical devices, such as catheters and implants, exacerbating the difficulty of treating infections and increasing the risk of systemic illness, particularly in immunocompromised individuals. Moreover, in industrial and environmental settings, *Pseudomonas aeruginosa* biofilms can contaminate water systems and surfaces, posing a threat to public health. Their resilience to conventional antimicrobial agents and disinfectants further complicates treatment efforts, necessitating ongoing research into innovative strategies for prevention and eradication.¹⁰

Unlike some synthetic polymers that degrade easily, peptoids are known for their resilience and resistance to breakdown. This stability is crucial for mitigating the formation of bacterial biofilms, which is, again, a primary focus of this project. By remaining intact over time, polyaspartate peptoid polymers can effectively reduce the environmental impact associated with biofilm formation. Polyaspartate peptoid polymers are often water-soluble, making them suitable for various applications where solubility in aqueous solutions is essential.¹¹ This characteristic is particularly valuable in fields such as biomedicine, where water solubility is often a critical factor. Polyaspartate peptoid polymers exhibit structural similarity to polyaspartate peptides but offer improved stability against proteases. This

enhanced stability makes them advantageous in various applications. With their unique combination of properties, polyaspartate peptoid polymers find utility across diverse fields. They are explored for use in drug delivery systems, biomaterials, and environmentally friendly coatings.¹²

To synthesize peptoids larger than 20 monomers, N-carboxyanhydride or NCAs are utilized. NCA serves as the monomeric precursor from which the polyaspartate backbone of the peptoid polymers is synthesized. Through controlled ring-opening polymerization (ROP) of NCA, facilitated by appropriate initiators and catalysts, the backbone structure of the polymer is established. This method allows for precise control over the polymerization process, yielding polymers with tailored molecular weights and architectures.¹³ Furthermore, the unique properties of NCA, such as its stability and reactivity, make it a versatile building block for the synthesis of a diverse range of peptoid polymers. By harnessing the reactivity of NCA, a high degree of structural control can be achieved, enabling the design and development of peptoid polymers with desired functionalities and applications in various fields, including biomedicine, materials science, and nanotechnology.¹⁴

NCA-based synthesis is compatible with automated synthesis platforms and scalable, allowing for quick and efficient high-throughput screening of peptoid libraries for a variety of bioactive characteristics. All things considered, the ability to create peptoids using NCAs is a significant step toward creating a variety of molecular structures with adjustable characteristics, making them attractive options for tackling a wide range of biological and materials-related problems.¹⁴

In the pioneering work conducted by Luxenhofer and colleagues, they started a thorough study to create peptoids using NCAs and explained their structural and functional characteristics. Their approach involved meticulously designing and synthesizing a series of NCA monomers, followed by polymerization to construct peptoid chains with tailored sequences and side chain functionalities. Through a combination of spectroscopic, chromatographic, and microscopic techniques, they characterized the resulting peptoids, probing into their conformational behavior, solubility profiles, and interactions with biological molecules.¹⁵

The overarching goal of Luxenhofer *et al.*'s research was to explore the versatility of peptoids in addressing both biological and materials challenges.¹⁵ They aimed to decipher the fundamental principles governing peptoid structure-property relationships and to leverage these insights for the design of novel biomaterials and therapeutic agents. In this regard, their work can be considered a resounding success. By meticulously studying the physicochemical properties and biological activities of peptoids, they not only achieved their goal but also laid down a solid foundation for further advancements in peptoid-based research.

By integrating the findings from Luxenhofer *et al.*'s innovative work into our own project, we aim to build upon their discoveries and explore new avenues for the application of peptoids in our research endeavors.¹⁵ Through careful experimentation and analysis, we aspire to contribute to the growing body of knowledge surrounding peptoid chemistry and its implications for various scientific disciplines.

Thesis Statement:

The proposed research aims to investigate the potential of novel polyaspartate peptoid polymers in combating *Pseudomonas aeruginosa* biofilms. Our primary goal is to synthesize these polymers with carboxylic acid side chains, a feat not previously achieved. This achievement will serve as a foundational step towards understanding their effectiveness against biofilms. Subsequently, we will explore their ability to disrupt or prevent biofilm formation and their impact on existing biofilm structures. By comparing the resilience of these polymers to traditional peptides against protease breakdown, we aim to develop antimicrobial materials with enhanced durability. These insights are crucial for addressing biofilm-related infections and advancing healthcare technologies. Additionally, biological characterization of these polymers will further elucidate their potential applications.

Methodology

Phase I: (Nasp)10 by solid-phase synthesis

In the synthesis of peptoids via the submonomer method, the procedure began with the deprotection of the PS-Rinkamide AM resin, liberating amine functionalities for subsequent reactions. **Figure 2** provides a schematic overview of the submonomer method used, illustrating the sequential steps of bromoacylation and amination, which ultimately result in the synthesis of the peptoid structure.

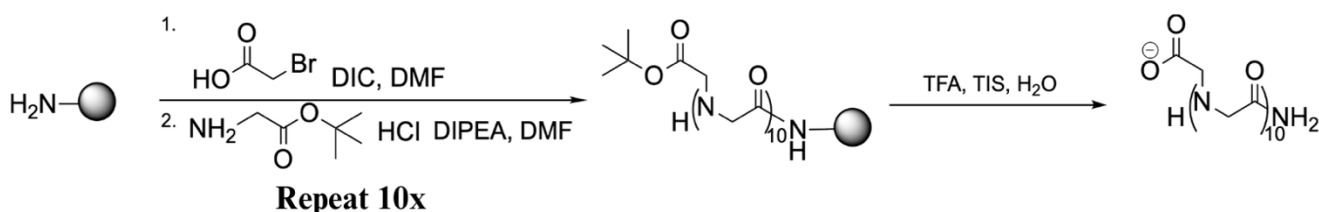


Figure 2. Solid-phase synthetic scheme of (Nasp)10.

Following resin deprotection, the bromoacylation step was initiated by the addition of a solution containing 1.5 mL of 3.2 M N,N'-diisopropylcarbodiimide (DIC) and dimethylformamide (DMF), along with 1.5 mL of 2 M bromoacetic acid in DMF, to the resin. This reaction introduces bromoacetyl groups onto the resin-bound amines. The mixture was then microwaved at 10% power for 30 seconds and rocked for 15 minutes. Subsequently, the resin was washed three times with DMF to remove excess reagents.¹⁶

The amination step followed, involving the addition of a solution with a total volume of 3 mL containing 0.5 M of O-t-butyl glycine HCl, the desired amine, and N,N-

diisopropylethylamine (DIPEA) in DMF to the resin-bound bromoacetyl groups. This step introduces the desired N-substituted glycine functional group onto the peptoid chain. The reaction was again microwaved, rocked, and then washed three times with DMF. The bromoacylation and amination processes were carried out 10 times repeatedly. Once the desired length was achieved, the peptoid was cleaved from the resin by treatment with a cleavage cocktail composed of trifluoroacetic acid (TFA; 95%), triisopropylsilane (TIS; 2.5%), and water (2.5%). This cleavage cocktail facilitates the detachment of the peptoid from the solid support while concurrently removing t-butyl protecting groups from side chains. After cleavage was performed, the product was purified by ether precipitation and mass spectrometry was conducted to confirm the identity and purity of the product, providing evidence that the resulting peptoid matches the desired product. The submonomer method works successfully in synthesizing relatively short peptoids (<20 monomers).¹⁵

To test how effective the (Nasp)₁₀ polymer is against *P. aeruginosa*, we performed Minimum Inhibitory Concentration (MIC) test which was performed following a standardized procedure.¹⁷ Initially, Lysogeny Broth (LB) plates were streaked with the pathogen and left to incubate overnight at 37°C. Subsequently, compound concentrations were prepared at 10x strength and subjected to 2-fold serial dilution in water. Colonies were selected and transferred to Tryptic Soy Broth (TSB) to achieve an OD₆₀₀ of 0.107. This inoculant was further diluted 1:200 in Cation Adjusted Muller-Hinton broth (CAMHB) to yield a final concentration of approximately 5x10⁵ CFU/mL. Ninety microliters of the 1:200 cell inoculant were then added to each well of a 96-well plate, with 10 microliters of media only added to designated controls. Subsequently, 10 microliters of the 10x

compound stocks were added to appropriate wells, resulting in a final concentration of 1x in each well. Additionally, a negative control (vehicle) and a positive control (20 µg/mL

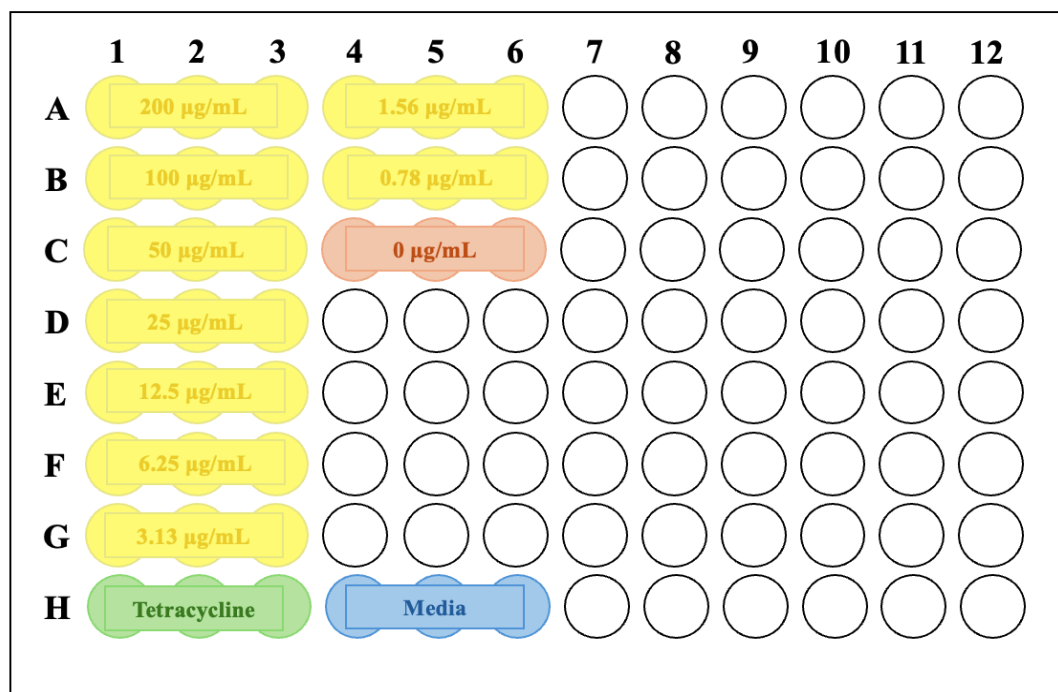


Figure 3. 96-well MIC₉₀ plate for *Pseudomonas aeruginosa*.

tetracycline) were included. The plates were then incubated at 37°C overnight (**Figure 3**). Following overnight incubation, Presto Blue (10 µL) was added to each well and the plates were further incubated at 37°C for 1-2 hours.¹⁸

Phase II: N-n-propylglycine-N-carboxyanhydride NCA

Using N-carboxyanhydrides(NCAs), which are reactive peptoid monomers containing the desired polymer side chain, peptoids larger than 20 monomers are able to be synthesized. N-carboxyanhydride (NCA) polymerization was attempted following the methodology detailed by Luxenhofer *et al.*¹⁵ This method involved the synthesis of NCA

building blocks that would have then been polymerized into the desired length of polyaspartate peptoid polymer. The NCA that we attempted to synthesize was N-propylglycine.

The synthesis of N-propylglycine N-carboxyanhydride (NCA) followed a three-step process, illustrated in **Figure 4**. In the first step, 5 g (54.32 mmols) of glyoxylic acid was combined with 1.86 mL propylamine in 106 mL of dichloromethane, and the solution

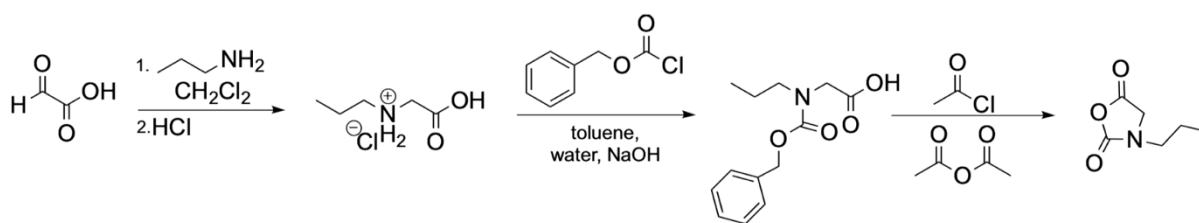


Figure 4. N-propylglycine N-carboxyanhydride NCA Synthetic scheme.

was stirred at room temperature for 24 hours. The reaction mixture was then concentrated using a rotary evaporator to remove the solvent, followed by the addition of 17.67 mL of 6 M hydrochloric acid in 88.33 mL of water to the solution. The resulting mixture was heated under reflux for 24 hours, after which the solvent was removed under vacuum yielding a yellow solid. The crude product was purified by recrystallization in a 1:5 methanol/diethyl ether mixture, yielding 1.781 g of product as white crystals.

In the second step, N-benzyloxycarbonyl-N-propylglycine was synthesized by suspending N-propylglycine hydrochloride (1.781 g, 11.63 mmol) in 16.4 mL of toluene and cooling the mixture in an ice bath for 10 minutes. A solution of 6.01 mL of 6 M sodium hydroxide in 10.39 mL of water was slowly added, followed by the addition of 1.64 mL of benzyl chloroformate. The solution was stirred for 4 hours and then allowed to phase-

separate. After 4 hours, the aqueous phase was reacidified with concentrated HCl, adjusting the pH to 1-2, and then 30 mL of ethyl acetate was added to the mixture. The solution was then transferred to a separatory funnel, where the organic layer was carefully extracted. This step was crucial for isolating the desired compound. The organic layer was subsequently dried over calcium chloride (CaCl_2) to remove any residual water, and the solvent was evaporated under reduced pressure, yielding 2.78 g of a yellowish oil.

In the final step, N-benzyloxycarbonyl-N-propylglycine (11.03 mmols) was reacted with 1.6 mL of acetyl chloride and 2.10 mL of acetic anhydride under a dry argon atmosphere. The reaction mixture was heated under reflux at 70 °C for 6 hours. Afterward, excess acetyl chloride and acetic anhydride were removed under reduced pressure, yielding a crude brownish oil. The crude product was then distilled under reduced pressure (120 °C, bath temperature), affording the final product as a colorless oil which was not successfully extracted.

Phase III: (Nasp)25 by solid-phase synthesis

After encountering significant challenges in the synthesis of a 25-monomer polyaspartate peptoid using N-carboxyanhydrides (NCAs), we opted to revert to the submonomer method for solid-phase synthesis of this larger polyaspartate peptoid polymer (**Figure 5**).

The synthesis commenced with the deprotection of the PS-Rinkamide AM resin, similar to the process used for the (Nasp)10 peptoid. This deprotection exposed the amine functionalities necessary for subsequent reactions. The synthesis followed a sequence of bromoacylation and amination steps as described above, carried out 25 times to achieve the

desired chain length. Given the increased length of the peptoid, we implemented more stringent control over reaction conditions, ensuring the reactions were complete and minimizing side reactions or incomplete coupling through careful monitoring and additional washes.

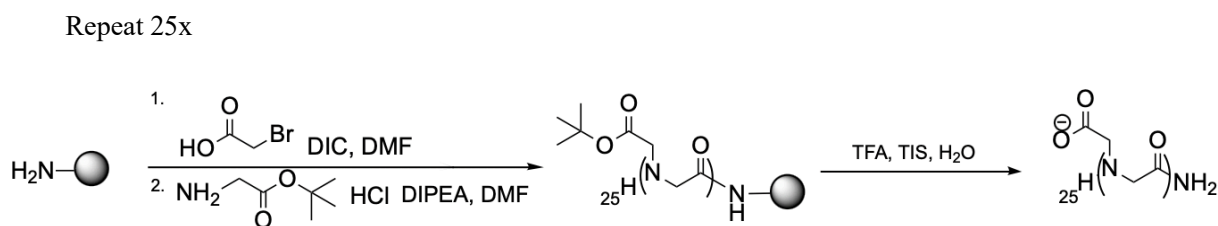


Figure 5. Solid-phase synthetic scheme of (Nasp)10.

Upon completing the 25 cycles of bromoacylation and amination, the peptoid was cleaved from the resin using a cleavage cocktail of trifluoroacetic acid (TFA), triisopropylsilane (TIS), and water. The peptoid was then purified using ether precipitation.

Final analysis will be confirmed by mass spectrometry and additional antimicrobial tests will be performed.

Results and Discussion:

I. (Nasp)₁₀ Peptoid Synthesis Confirmation

Our efforts to synthesize a 10-mer polyaspartate peptoid polymer using the submonomer method were successful. The synthesis proceeded smoothly for this well-established technique. However, purification proved challenging due to the highly hydrophilic nature of the polymer. Initially, we attempted purification using high-performance liquid chromatography (HPLC), but this approach was ineffective. To overcome this, we opted for ether precipitation purification, which successfully removed impurities. This allowed us to obtain a pure 10-mer polyaspartate peptoid polymer with a molecular weight of 1168 g/mol, as confirmed by mass spectrometry (**figure 6**).

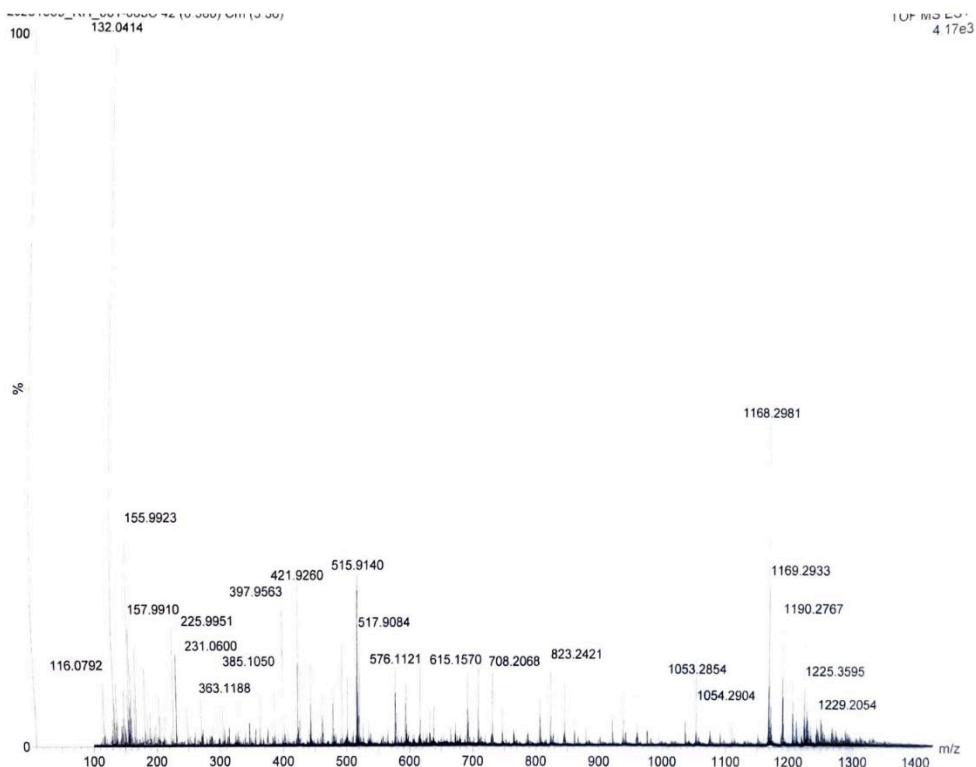


Figure 6. Mass spectra of Polyaspartate peptoid polymer.

Afterward, we tested the 10-mer polyaspartate peptoid polymer against bacterial biofilms, focusing on *P. aeruginosa*. After evaluating its effectiveness over several days, we found that the polyaspartate peptoid did not noticeably affect the *P. aeruginosa* growth in the MIC assay. One possible reason for this outcome is that the polyaspartate peptoid's structure may not have been sufficiently disruptive to the biofilm matrix, which is known to provide significant protection to bacteria within. Additionally, the polymer's hydrophilic nature could have limited its ability to penetrate the biofilm's dense extracellular polymeric substance (EPS), reducing its interaction with bacterial cells. Another contributing factor might be the relatively short length of the 10-mer peptoid, which may not have been long enough to effectively interfere with key biofilm processes such as adhesion, maturation, or structural stability. Longer polymers, such as those seen with thermally synthesized polyaspartate peptides, may have greater potential to disrupt these processes by interacting more robustly with the biofilm matrix.

II. N-n-propylglycine-N-carboxyanhydride NCA Confirmation.

The process of synthesizing the NCA involved the conformation of the two key intermediates:

- N-propylglycine hydrochloride: The successful conversion to N-propylglycine hydrochloride was confirmed using proton nuclear magnetic resonance (^1H NMR) spectroscopy, melting point analysis, and yield determination. The reaction provided a yield of 51%, which, while moderate, indicated that the synthesis was reasonably efficient under the given conditions. The ^1H NMR spectrum, obtained in methanol- D_3 , displayed signals that confirmed the structure of the intermediate: 3H at $\delta = 1.00$ ppm, corresponding to the propyl methyl group ($-\text{CH}_3$); 2H at $\delta = 1.71$ ppm, for the methylene group ($-\text{CH}_2$); 2H at $\delta = 3.00$ ppm, from the $-\text{CH}_2$ adjacent to the amine; and 2H at $\delta = 3.90$ ppm, for the $-\text{CH}_2$ group adjacent to the carboxyl group. A singlet at $\delta = 9.00$ ppm confirmed the presence of the protonated amine, verifying the successful formation of the hydrochloride salt (**Figure 7**). Additionally, the melting point of the product (196°C) was consistent with literature values (195 - 197°C), further validating the identity of N-propylglycine hydrochloride.

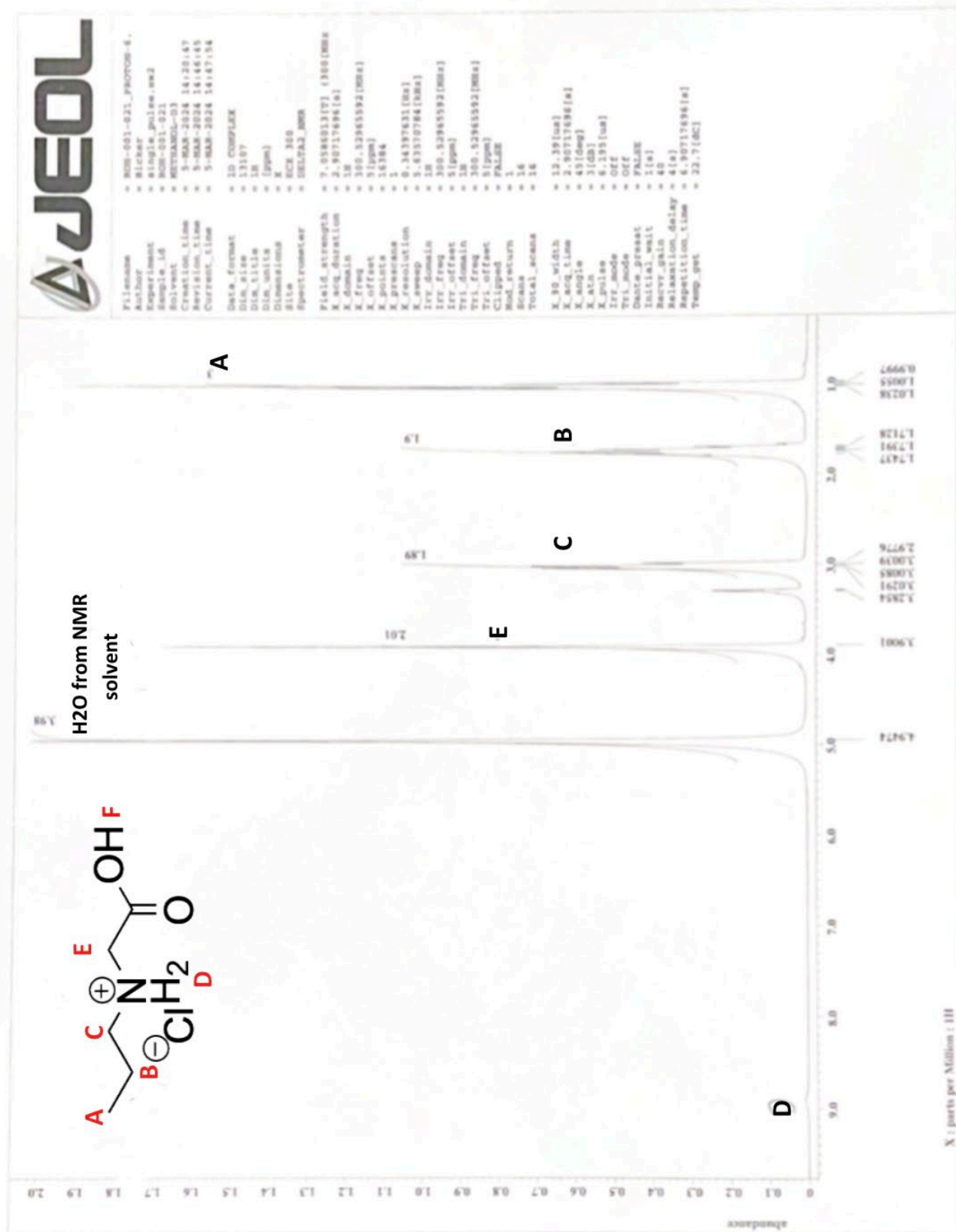


Figure 7. ¹H NMR spectrum of N-propylglycine hydrochloride.

- N-benzyloxycarbonyl-N-propylglycine: The formation of N-benzyloxycarbonyl-N-propylglycine was verified through proton nuclear magnetic resonance (^1H NMR) spectroscopy, which was utilized to confirm the structure of this intermediate (**Figure 8**).

-

The ^1H NMR spectrum, obtained in chloroform- D (CDCl_3), revealed characteristic signals for each functional group. The peak at $\delta = 0.88$ ppm (3H) corresponds to the methyl protons ($-\text{CH}_3$) of the propyl group. The signal at $\delta = 1.56$ ppm (2H) is assigned to the methylene protons ($-\text{CH}_2$) adjacent to the methyl group. A peak at $\delta = 3.24$ ppm (2H) represents the methylene group attached to the amine ($-\text{CH}_2$), while the signal at $\delta = 4.04$ ppm (2H) corresponds to the methylene group attached to the carboxyl group ($-\text{NCH}_2\text{COOH}$). The signal at $\delta = 5.15$ ppm (2H) confirms the presence of the benzyl group ($-\text{C}_6\text{H}_5\text{CH}_2\text{O}$) linked to the nitrogen, and the broad peak at $\delta = 7.24$ ppm (5H) corresponds to the aromatic protons of the phenyl group ($-\text{C}_6\text{H}_5$).

These distinct signals validate the formation of the N-benzyloxycarbonyl protecting group and its attachment to the N-propylglycine backbone, confirming the successful synthesis of this intermediate. The purity of the compound was further supported by the absence of unexpected peaks, and the integration values were consistent with the expected ratios for each proton environment.

- N-propylglycine NCA: The synthesis of N-propylglycine NCA was performed by following the steps outlined in the literature, all of which were completed except for the part where excess acetic anhydride and acetic chloride were removed. While acetic chloride was successfully evaporated, despite numerous attempts to eliminate the excess acetic anhydride—

including simple distillation and column chromatography with TLC monitoring—NMR analysis consistently revealed signals corresponding to both the propyl NCA and residual acetic anhydride (**Figure 9**). Ultimately, the acetic anhydride could not be separated from the product using the published protocols. Additional methods are being explored to purify this compound.

III. (Nasp)₂₅ Peptoid Synthesis Confirmation

The primary issue arose during the final step of NCA preparation, where the inability to effectively separate acetic anhydride from the NCA compromised the integrity of the synthesis and resulted in poor yields and purity. Consequently, we determined that utilizing the solid-phase synthesis approach would provide a more reliable pathway for constructing the longer peptoids. After the synthesis was completed, the peptoid structure needed to be confirmed by MS; however, due to the MS machine being non-operational, confirmation is still ongoing, followed by an MIC test.

Future Directions

In our ongoing efforts to synthesize functional homopolypeptoids, we have encountered significant challenges during the synthesis of N-carboxyanhydrides (NCAs) that have hindered our progress. Specifically, the synthesis of N-propylglycine-NCA faced difficulties due to the inability to effectively separate residual acetic anhydride from the final product. Despite employing various purification techniques, including distillation and column chromatography, residual acetic anhydride persisted, compromising the purity and yield of the desired NCAs.

This challenge has created a hurdle for subsequent steps, including the polymerization of N-propylglycine-NCA and the synthesis and polymerization of N-(O-*t*-butoxy)carboxymethylglycine-NCA. To address these challenges and move forward, we will explore the refinement of NCA synthesis. We plan to investigate alternative synthetic routes or modifications to existing methods for producing NCAs, optimizing reaction conditions, and exploring different solvents to facilitate better separation of by-products. Additionally, we aim to develop a more rigorous purification process for the NCAs, incorporating extra washing steps and advanced chromatographic techniques to ensure a cleaner starting material for polymerization.

Once high-purity NCAs are obtained, we will re-initiate the controlled ring-opening polymerization (ROP) of both N-propylglycine and N-carboxymethylglycine to synthesize the corresponding homopolypeptoids. This approach should enable us to tailor their properties for specific applications. By addressing the synthesis and purification

challenges, we aim to successfully produce high-quality homopolypeptoids that can be further investigated for their functionality and applications in various fields.

Conclusion

In conclusion, our research endeavors to advance the field of antibacterial polymers by synthesizing novel polyaspartate peptoids and exploring their efficacy against *P. aeruginosa* biofilms. Through a systematic approach involving careful synthesis, testing, and refinement, we aimed to enhance the antibacterial properties of these polymers, ultimately contributing to the development of innovative solutions for combating bacterial infections.

The synthesis of a 10-monomer polyaspartate peptoid was achieved and tested against *P. aeruginosa*, observing no inhibition. To explore whether increased polymer length would affect antibacterial efficacy, we proceeded to synthesize a 25-mer polyaspartate peptoid. The synthesis initially involved the N-carboxyanhydride (NCA) method, which provided valuable learning opportunities despite challenges in achieving a pure NCA product in the absence of acetic anhydride. Ultimately, the 25-mer was synthesized using the solid-phase method, which will or will not then demonstrate inhibition against *P. aeruginosa*. These experiences not only expanded our understanding of polymer synthesis and antibacterial testing but also laid a strong foundation for future work in developing potent antibacterial agents to address antibiotic resistance.

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