Distribution Agreement

In presenting this thesis as a partial fulfillment of the requirements for a degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis in whole or in part in all forms of media, now or hereafter now, including display on the World Wide Web. I understand that I may select some access restrictions as part of the online submission of this thesis. I retain all ownership rights to the copyright of the thesis. I also retain the right to use in future works (such as articles or books) all or part of this thesis.

Griffin Davis April 12, 2022

Design of a Collagen-Mimetic Peptide for Dynamic Template-Directed Polymerization

by

Griffin Davis

Dr. David Lynn
Adviser

Department of Chemistry

Dr. David Lynn
Adviser

Dr. Frank McDonald
Committee Member

Dr. James Kindt
Committee Member

2022

Design of a Collagen-Mimetic Peptide for Dynamic Template-Directed Polymerization

By

Griffin Davis

Dr. David Lynn
Adviser

An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Science with Honors

Department of Chemistry

2022

Abstract

Design of a Collagen-Mimetic Peptide for Dynamic Template-Directed Polymerization

By Griffin Davis

Dynamic chemical networks have been integral in developing the remarkable complexity of life, with the resulting bottom-up process of chemical evolution giving way to the selection, propagation, and diversification of modern biopolymers. Throughout this process, template-directed polymerization has allowed the accurate storage and propagation of molecular information with high fidelity. Combining dynamic polymerization with cross-β peptide architectures, templates have been shown to direct amplification of chain-length-specific oligomers (which are limited in length by the β-sheet template). Here, we set out to adapt a dynamic chemical network that would direct propagation and amplification of a collagen-mimetic peptide that is far more extended than those produced in the established cross-β system. Self-assembly of the C-terminal peptide aldehyde NPG has been characterized to a certain degree, but much remains unknown about its supramolecular structure. In this work, we develop a reliable protocol for the synthesis of H_2N-NPG-CHO monomers and examine ways to tune self-assembly via external templating. The template peptide sequence Ac-$(EOGPOG)_3$EOG-NH_2 was synthesized and purified, and its templating ability was evaluated using computational modeling of resulting assemblies. This template is shown to promote triple helix character when self-assembled and form favorable salt bridges with oligomers of NPG, but further analysis is necessary to determine whether the resulting assembly is stable. Further adaptation of this workflow will allow for preliminary analysis of proposed peptide sequences such that promising templates may be investigated experimentally. In this way, external templating of NPG self-assembly builds on pathways for production of chain-length specific oligomers and models Nature's ability to direct chemical evolution through template selection.

Design of a Collagen-Mimetic Peptide for Dynamic Template-Directed Polymerization

By

Griffin Davis

Dr. David Lynn
Adviser

A thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Science with Honors

Department of Chemistry

2022

Acknowledgements

First, I would like to thank Dr. Lynn for being a supportive, kind, and thoughtful mentor to me over the past couple years. I joined the Lynn lab at the end of my sophomore year and just as the pandemic was starting, and Dr. Lynn quickly became a household name thanks to our weekly lab meetings on Zoom over the next year. Thank you for allowing me to learn from you, especially throughout such an exceptional period of time. Your patience, understanding, and mentorship has meant a lot to me.

I would also like to thank the other members of the Lynn lab for all they've taught me and the genuine friendship I've shared with some of them. I'm truly grateful for the learning experiences and memories from my time in the lab, I would not have made this far without our shared spirit of collaboration.

Next, I'd like to extend my gratitude to Dr. McDonald for taking the time to evaluate my thesis and encouraging my growth as both a chemist and a peer mentor. Because of him, I've learned to think about chemistry through critical and creative lenses, which has opened my mind to fascinating new possibilities. Through serving as a Learning Assistant for his CHEM 203Z class, I discovered my love of mentorship and made many great connections that have fundamentally changed my time at Emory for the better.

I'd also like to thank Dr. Kindt for taking the time to evaluate my thesis and providing guidance many times along the way. His support has been incredibly encouraging and instrumental in helping me make it this far, and I could not be more grateful for his advice (especially because I definitely had a lot of concerns). Ever since taking CHEM 205 with him, I always enjoy seeing him around Atwood and being able to catch up.

Next, I want to thank my family for all their support, especially through such a tumultuous year. Mom, Dad, and Chloe, I would not have made it this far without your help. I feel like you've each been a part of this process ever since sitting in on my first Zoom lab meetings during the pandemic. I love you all endlessly.

Thank you to every single one of my friends who has supported me along the way, whether you let me practice my presentation to you or listened to me talk about the whole process. There are almost too many of you to name, but I'll try anyway. Thank you to Kylie, Esteban, Sally, Erin, Nestor, Sri, Haley, Fiona, Olivia, Rohini, Sean, and Kenny. I love and appreciate you all.

Finally, I'd also like to thank Joni Mitchell for her extensive discography, to which most of this thesis was written. It was an invaluable source of emotional support. Credit is also due to Adrianne Lenker's *instrumentals*, which probably inspired most of my productivity.

Table of Contents

1. Introduction

The tremendous diversity and complexity of molecular information, structure, and function has emerged through open-ended evolution of dynamic chemical networks (DCNs) over time. Specifically, in these DCNs, simple network components assemble into more complex structures that develop an added dimension of functionality, and the following Darwinian selection process determines the network's fitness over time.[1-3] Creating DCNs similar to those involved in prebiotic chemistry can help provide insight into nature's selection processes in chemical evolution and also evaluate alternative chemistries of life by which new functional possibilities emerge. Combining a peptide-based DCN with the progression of phase transitions, self-selection of internal cross-β templates has been shown to direct production of monodisperse oligomers.[4] Moving beyond the use of cross-β architectures as templates, which are intrinsically limited by their length, analogous DCNs should be compatible with other morphologies and test the templating efficiency of the extended triple helix, as found in collagen. By exploiting the triple helix, another DCN may be able to direct propagation of peptides that are much longer than those achieved using cross-β templates.

Collagen — a family of proteins with at least 28 known variants — is the most abundant class of proteins in mammals, contributing roughly 30% of the total protein mass in the human body.[5,6] Often considered the prototypical collagen fibril, type I collagen plays an integral structural role in the extracellular matrix (ECM), blood vessel walls, skin, and nerve sheaths.[7,8] This natural collagen adopts a triple-helical supramolecular structure and may be homo- or heterotrimeric in composition depending on the peptide sequences of its constituent α chains.[9] More specifically, three parallel α chains coil around a central axis in a staggered fashion to adopt a left-handed polyproline type II conformation, forming an overall triple helix that is right-

handed. These tight helical turns in the secondary structure dictate that every third residue in the peptide sequence be glycine, which imparts a great deal of flexibility due to its hydrogen side chain. This tendency results in a recurring Xaa-Yaa-Gly triplet in the chain sequences of collagen strands, wherein the Xaa position and Yaa position are most frequently proline (Pro, P) and hydroxyproline (Hyp, O), respectively.[8] Chains of Pro-Hyp-Gly repeats are capable of self-assembly into this triple-helical structure at a critical length, which is held together and stabilized by one interstrand hydrogen bond per triplet, between the amide nitrogen in glycine and carbonyl oxygen in proline.[10] These triple helices associate with each other to form fibers through the entropically-driven process of fibrillogenesis, in which non-covalent interactions drive packing. The resulting collagen fibers also form hydrogel networks, which are formed as non-covalent associations drive assembly formation, which can then be stabilized by specific covalent cross-linking.[11,12] Collagen fibers themselves can be rigid and inflexible, but transient movement between neighboring fibers is responsible for the dynamic tensile strength of skin and resistance to traction in ligaments. Beyond this long-range order and contribution to mechanical properties, collagen also plays a role in the extracellular matrix where a network of tangled collagen fibers provide critical structure. Some of these dynamic assemblies have been shown to regulate cell growth, differentiation, and migration.[5,7]

As a result of collagen's versatile and invaluable role in the human body, there have been many attempts to design synthetic analogs to gain insight into Nature's rules for self-assembly and chemical evolution, so as to create new therapeutic scaffolds. Previously-developed collagen-mimetic peptides (CMPs) have successfully achieved the characteristic long-range order and mechanical strength of collagen by directly synthesizing peptide sequences of intermediate length (18 – 36 residues) with "sticky ends" that form higher-order assemblies

through association of electrostatically-charged residues.[9, 12-14] However, these approaches have fallen short of capturing the dynamic, flexible nature of collagen's triple helical structure.

To this end, previous research in our lab has demonstrated that selective production of monodisperse oligomers is possible through the design of peptide-based DCNs wherein emergent physical phases can select for a specific template and propagate with high fidelity.[4] Using the dipeptide H_2N-Asn-Phe-CHO with a reduced aldehyde at the C-terminus as the chosen monomer, the N-terminus of one monomer may condense with the aldehyde C-terminus of another, yielding an imine intermediate from this condensation reaction. This imine intermediate is subsequently trapped by cyclization of the neighboring N-terminal asparagine's side chain to form an *N*, *N*-acetal (4-pyrimidinone) linkage between the two monomers (Figure 1). The 4-pyrimidinone linkage was also found exist in its protonated and positively charged state at physiological pH, contributing both stability and dynamics in typical cellular and extracellular matrices.[15]

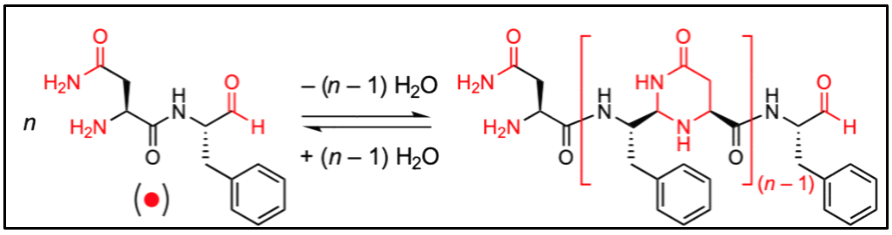

Figure 1. Condensation polymerization scheme of H_2N-NF-CHO from Chen et al.[4,15]

Exploiting this dynamic polymerization, a tripeptide monomer was constructed that undergoes liquid-liquid phase separation (LLPS), self-polymerization, self-selection for a stable internal template, and propagation from the initial particle phase to form nascent peptide chains that self-assemble into a higher-order assembly.[4,15] We realized that the cyclic nature of the 4-pyrimidinone linkage and its hydrogen-bonding capability might allow for comparison to the

characteristic hydroxyproline of collagen strands, leading to the design of the peptide H_2N-Asn-Pro-Gly-CHO. The polymerizing linkage of this tripeptide was designed to imitate the Pro-Hyp-Gly triplet repeat that is ever-present in collagen. The 4-pyrimidinone linkage is also hydrolytically reversible, which allows for additional conformational dynamics along the peptide backbone and creates a more dynamic assembled structure.[16] In this way, the described dynamic chemical linkages enabled the design of a dynamic chemical network that would produce a collagen-mimetic peptide through template-directed polymerization (Figure 2).

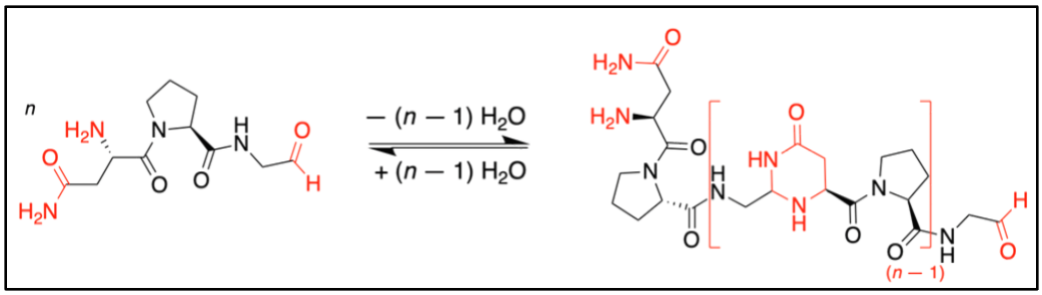

Figure 2. Condensation polymerization scheme of H_2N-NPG-CHO.

This collagen-mimetic peptide sequence could potentially play a role in the survival of cultured cells lacking a circulatory system as well. Cellular organoids, derived from pluripotent stem cells, can capture dynamic features of the human brain at the structural, molecular, and cellular level. However, these cellular organoids lack a centralized vascular system, compromising their viability particularly due to difficulty with nutrient uptake.[17] With its dynamic polymerizing linkage and triple-helical structure, self-assemblies of H_2N-NPG-CHO may lower viscosity in the cellular matrix of the brain organoids and improve their viability for disease modeling by increasing their ability to acquire necessary nutrients.

While H_2N-NPG-CHO self-assembles into homotrimer repeat polymers, structural motifs from collagen heterotrimer assemblies may be translatable to confer greater stability to the overall triple helix. Taking inspiration from nature's ability to facilitate chain association through

disulfide bridges between C-propeptide domains, some collagen-mimetic peptide models have incorporated selective interstrand electrostatic interactions.[9] Considering the positive charge of the 4-pyrimidinone linkage, I set out to design an external template that would pre-order collagen-mimetic peptide strands for supramolecular assembly and stabilize the overall triple helix. I wanted the template to feature negatively charged residues dispersed throughout the sequence to allow for charge complementarity with the 4-pyrimidinone linkage while maintaining the characteristic triplet repeat character of natural collagen where possible. It is known that missense mutations that replace glycine are devastating to triple helix stability and responsible for the collagen disorder known as osteogenesis imperfecta,[18] whereas the proline and hydroxyproline residues can be substituted and still form a triple helix.[19] Additionally, analysis of the propensity for amino acid substitutions in the collagen triple helix has found that proline to glutamic acid (Glu, E) mutations are the most stable.[19] With this information, I sought to investigate the 21-mer sequence Ac-(EOGPOG)$_3$EOG-NH$_2$, where O represents hydroxyproline, as an external template that could potentially further stabilize the triple helix due to added Glu – 4-pyrimidinone salt bridges and accelerate the growth of collagen-like fibers.

In the following sections, I will discuss the path to optimizing the synthesis of H$_2$N-NPG-CHO, building upon the work previously completed in this area by Seth Young. I will then discuss the use of an external template to facilitate self-assembly of a more stable triple-helix and select for chain-length specific oligomers. I will present molecular modeling that details rudimentary non-covalent interactions between strands during the assembly process, as well as outline proposals for extensions of this newly developed peptide aldehyde chemistry.

2. Methods

2.1 Peptide Aldehyde Synthesis

i) Boc-N(trt)PG synthesis on resin and subsequent cleavage

Solid-phase peptide synthesis (SPPS) was performed on a Liberty Blue synthesizer that utilizes a DIC/Oxyma-based coupling scheme. Boc-N(trt)PG was synthesized at a 0.1 mmol scale on a 2-Cl(trt) resin that was preloaded with glycine (with a loading number of 0.787 mmol/g). Upon completion of the synthesis, the resin was rinsed out of the reaction vessel with dichloromethane (DCM) and poured into a vial. The resin was then poured into a Büchner funnel fitted with filter paper, rinsed with DCM to remove residual dimethylformamide (DMF), and then allowed to dry for 15 minutes to ensure complete removal of both solvents from the resin. Meanwhile, a 10 mL cleavage cocktail was prepared using 3 mL hexafluoroisopropanol (HFIP)[20] and 7 mL of DCM, which was then poured into a glass scintillation vial. The dried resin was then added to the vial with the cleavage cocktail and allowed to rock for 2 hours. The contents of the vial were then poured through a filter and into a round-bottom flask. The resin in the filter was then washed with DCM several times, resulting in a glittery red to dull brown color change. The resulting solution was then evaporated using Büchi rotavapor R-200, alternately rinsing with hexanes and DMF to encourage the peptide to precipitate out of solution. The final cleavage product was a fluffy white powder.

ii) Reduction of Boc-N(trt)PG-COOH to Boc-N(trt)PG-CHO

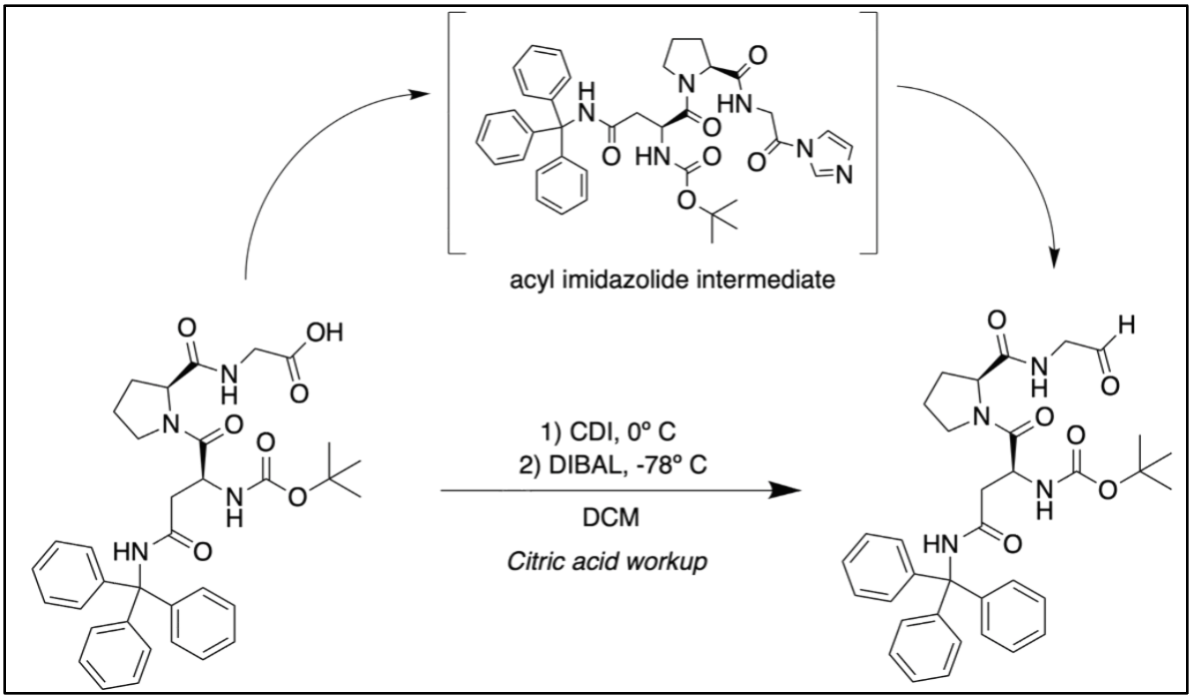

Figure 3. Synthetic scheme for reduction of Boc-N(trt)PG-COOH to Boc-N(trt)PG-CHO. Special thanks to Alexis Blake and Alexis Roberson for their hard work in developing and optimizing the process.[21]

A 1000 mL two-neck round-bottom flask with a Schlenk adapter, magnetic stirring bar, and a glass stopper was heated, dried *in vacuo* and sparged with N_2. The cleavage product was added and dissolved in DCM. The solution was then placed in an ice bath and allowed to cool to 0° C before adding 1.1 equivalents of 1,1'-carbonyldiimidazole (CDI). The glass stopper was replaced with a gas bubbler to relieve pressure in the flask. After letting the peptide stir with the CDI for 1 hour, the gas bubbler was removed, and the flask was cooled to -78° C in a bath of dry ice and acetone for approximately 15 minutes. The glass stopper was replaced with a septum and a very gentle flow of N_2 was re-established. 2.1 equivalents of DIBAL (1.0 M in toluene) was added to the flask dropwise over the course of 11 minutes, using a syringe through the septum. The reaction was stirred and allowed to proceed in the acetone/dry ice bath for 30-60 minutes (until TLC indicated reactant conversion). The reaction was quenched via addition of ethyl acetate, the

acetone bath was removed, and tartaric acid solution was added to the flask while stirring with the gas bubbler attached. The flask was then placed in a room temperature water bath and stirred vigorously for 15 minutes. At this point, the layers were separated through liquid-liquid extraction with ethyl acetate, and the combined organic extracts were washed with 0.2 M HCl, 0.8 M $NaHCO_3$, and brine before being dried with Na_2SO_4 and filtered. The resulting product was frozen with liquid nitrogen and placed under a high vacuum, and this process was repeated two more times.

2.2 Tuning H_2N-NPG-CHO Assembly

i) Template synthesis and cleavage

Solid-phase peptide synthesis (SPPS) was performed on a Liberty Blue synthesizer that utilizes a DIC/Oxyma-based coupling scheme. The peptide sequence $(EOGPOG)_3EOG$ was synthesized at a 0.1 mmol scale on Rink Amide (RAM) resin (with a substitution number of 0.848 mmol/g) with an acetylated N-terminus using Fmoc-protected L-amino acid monomers. The resin was collected and dried on a Büchner funnel, subsequently rinsing and drying with DCM a few times. A TFA cleavage cocktail consisting of 9 mL TFA, 0.5 mL thioanisole, 0.3 mL ethane dithiol, and 0.2 mL anisole was prepared in a glass scintillation vial. The resin was added to the cleavage cocktail and allowed to shake for 3 hours. The contents of the vial were then poured into a conical tube fitted with a filter, separating the colored solution from the resin. 25 mL of cold ether (stored at -20º C) was poured into the conical tube, and peptide precipitation was observed. The tube was then centrifuged at 4000 rpm for 20 min at 4º C. The supernatant was poured off, and the peptide pellet was washed with ether, vortexed, and centrifuged again. This step was repeated 2 more times, the ether was poured off, and the conical tube with the peptide was covered with a Kimwipe and placed in the desiccator to dry overnight.

ii) Template purification

Ac-(EOGPOG)$_3$EOG-NH$_2$ was dissolved in 7 mL acetonitrile and 1.5 mL triethylamine for purification by New Waters Reverse-Phase High-Performance Liquid Chromatography (HPLC). Solvents A (neat MeCN) and B (100 mM triethylammonium acetate, TEAA, in water) were allowed to equilibrate the semipreparative C18 column for 10 minutes. The peptide solution was injected into the HPLC, and a linear gradient of 5-15% solvent B was run with a steepness of 0.22%/min and a flow rate of 20 mL/min. The elution of peptides was detected by monitoring UV absorption at 222 nm versus peptide retention time. Peptide peaks with absorbance units greater than 0.02 were collected for analysis via electrospray ionization (ESI) mass spectrometry. The peak containing the peptide sample was then frozen in liquid nitrogen, lyophilized, and placed in a desiccator for storage.

iii) Computational Modeling

Template sequence, Ac-(EOGPOG)$_3$EOG-NH$_2$, and 21-mer H$_2$N-NPG-CHO oligomers were built manually in Maestro Version 13.0.137 (software by Schrödinger) and their structures consequently underwent a simple energetic minimization. Different triple helices were then modeled: one homotrimeric assembly consisting only of NPG oligomers, a heterotrimeric assembly differing only by inclusion of one template strand, and a homotrimeric assembly consisting only of the template sequence (to roughly verify that it could adopt a triple-helical conformation). These rudimentary models were prepared by placing component strands near one another, selecting all three, and performing another energy minimization. Admittedly, modeling supramolecular assemblies in Maestro has its limitations. Specifically, the minimized assembly is highly-dependent on starting geometry (and the equilibrium geometry must be relatively accessible from this starting point). As a result, many starting geometries were tested and a

simple reality check was performed on each minimized assembly to make sure it was feasible, especially keeping an eye on excessive steric clashes. Non-covalent hydrogen bonds were visualized and salt bridge distances were measured with the in-application tool.

3. Results

3.1. H_2N-NPG-CHO Preparation

After Seth's apparent success in obtaining H_2N-NPG-CHO, I worked with Youngsun to purify the sample via HPLC, only to find that the ESI results of our collected peak were not consistent with the expected mass. I then set out to test Seth's protocol and try to replicate his results, to no avail. After multiple failures to produce an aldehyde using the existing protocol, I worked closely with Alexis Blake and we tested many variations of the procedure. We tried different reagents (DIBAL, $LiAlH_4$), various molar quantities of reagents, and several different reaction conditions (0º C, -78º C, room temperature). Discouraging results followed, with most protocols yielding products that could not clearly be characterized by spectroscopy, or no product at all. We began to investigate using different resins, eventually pursuing the 2-Cl(trt) resin for solid-phase peptide synthesis. We considered two different routes: cleaving the peptide off the (glycine-preloaded) resin as an acid and reducing it to the corresponding aldehyde or cleaving the peptide off the (glycinol-preloaded) resin as an alcohol and oxidizing it to the corresponding aldehyde. At this point, Alexis found literature[21] for cleavage off the resin and reduction using CDI/DIBAL that led to a breakthrough, and she and Alexis Roberson were able to optimize the procedure. At this point, we can now reliably produce H_2N-NPG-CHO in good yield and purity.

3.2. Template Synthesis

The outlined method for solid-phase peptide synthesis was followed, and Ac-$(EOGPOG)_3$EOG-CHO was synthesized on Rink Amide Resin via the Liberty Blue Synthesizer.

The outlined cleavage procedure was followed as well, and the cleaved template sequence was washed with ether and centrifuged several times before being allowed to dry in the desiccator prior to purification via reverse-phase HPLC.

3.3. Template Purification

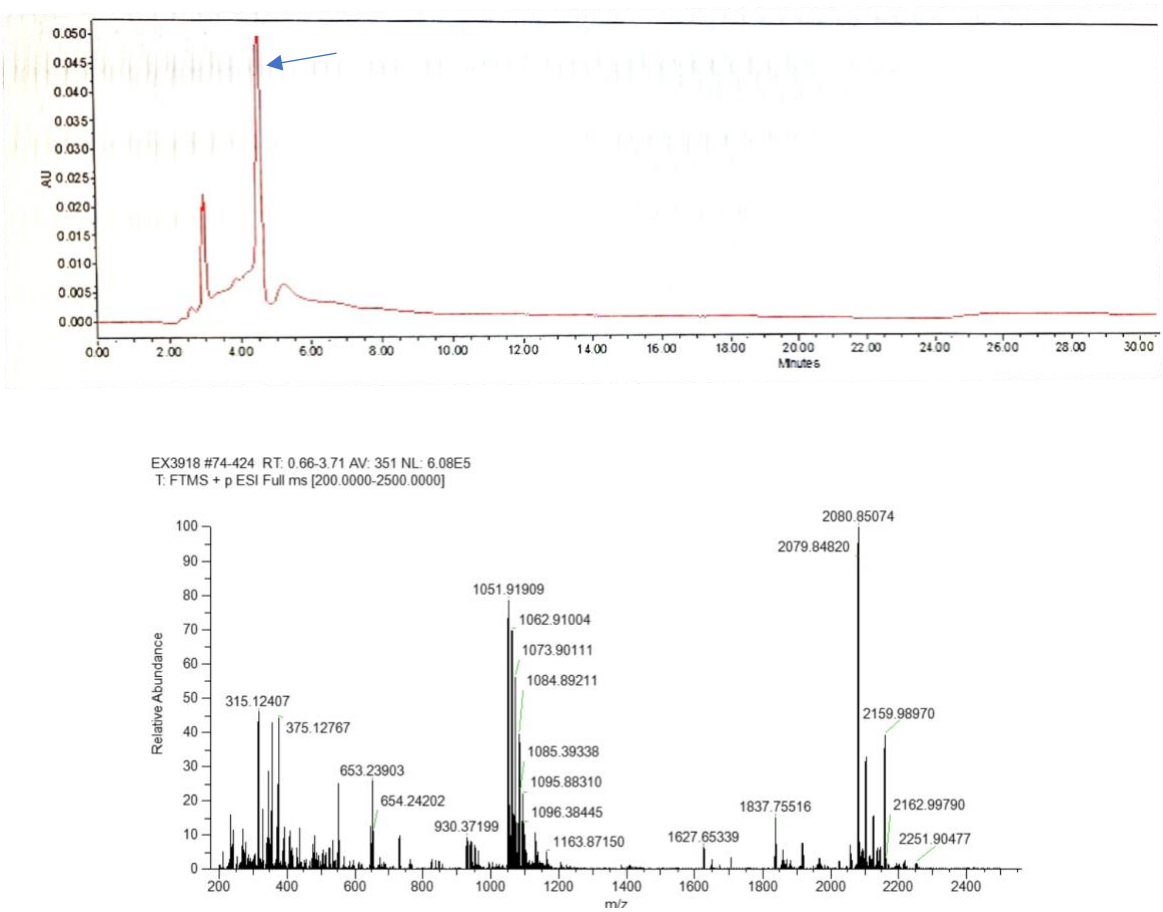

Figure 4. HPLC output (top) and ESI spectrum for indicated sample peak (bottom).

The second peak registered an absorbance of ~0.050 AU at 222 nm, eluting at a concentration of 6.5% solvent B (TEAA with water). This peak was collected for analysis by ESI, which yielded a spectrum in which all major peaks past 700 m/z could be accounted for by considering different protonation/charge states and formation of adducts with sodium and triethylamine. The observed noise, mostly between 300 and 400 m/z, is likely due to remaining impurities which can be corrected by further tuning the purification process.

[Peptide + Na] = 2081 m/z, [Peptide + NEt_3] = 2160 m/z]

3.4. Computational Modeling

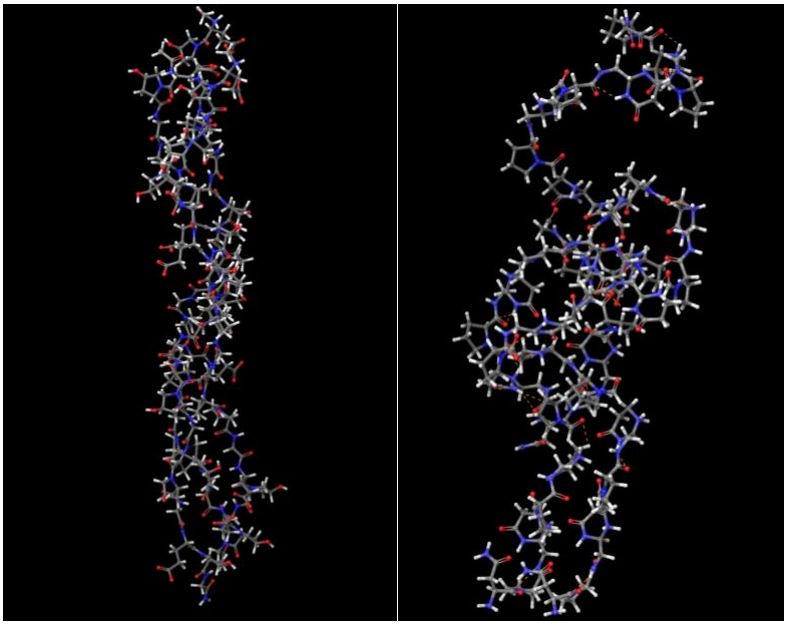

Figure 5. Minimized "triple-helical" assemblies of Ac-(EOGPOG)$_3$EOG-NH_2 alone (left) and NPG oligomers alone (right).

For each of the following assemblies, NPG oligomer strands and the template sequence were built in Maestro and underwent a basic energy minimization. The components of each assembly were then positioned in contact with one another and aligned before the entire assembly was minimized. The assembly of the template sequence alone seems to be the most triple-helical out of the four models, with a uniform width and steep pitch. Glutamate residues extend outward from the assembly, with negative charges decorating the outside of the triple helix. The assembly of NPG oligomers alone seems to be generally triple-helical but less well-defined: individual strands coil upward and around each other about a central axis, but the middle of the helix seems to be more crowded. The assembly of the template sequence with NPG oligomers (in a 1:2 ratio) is intriguing, as it retains recognizable elements of a triple helix, but constituent strands seem to be distorted away from one another. However, closer analysis of the structure did reveal that the assembly places glutamate residues close to the 4-pyrimidinone

linkages, allowing for electrostatic interaction between oppositely charged glutamic oxygens and positively charged nitrogens in the aminal rings. Finally, the assembly of the template sequence with NPG oligomers in a 2:1 ratio yielded a morphology that didn't really resemble a triple helix, nor were there any observed glutamate – 4-pyrimidinone salt bridges despite increased glutamate presence.

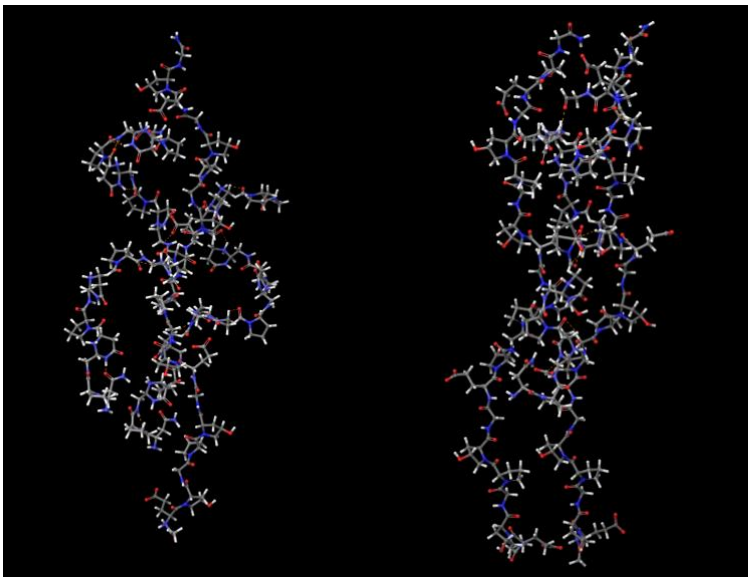

Figure 6. Minimized "triple-helical" assemblies of Ac-$(EOGPOG)_3EOG\text{-}NH_2$ and NPG oligomers in a 1:2 ratio (left) and 2:1 ratio (right).

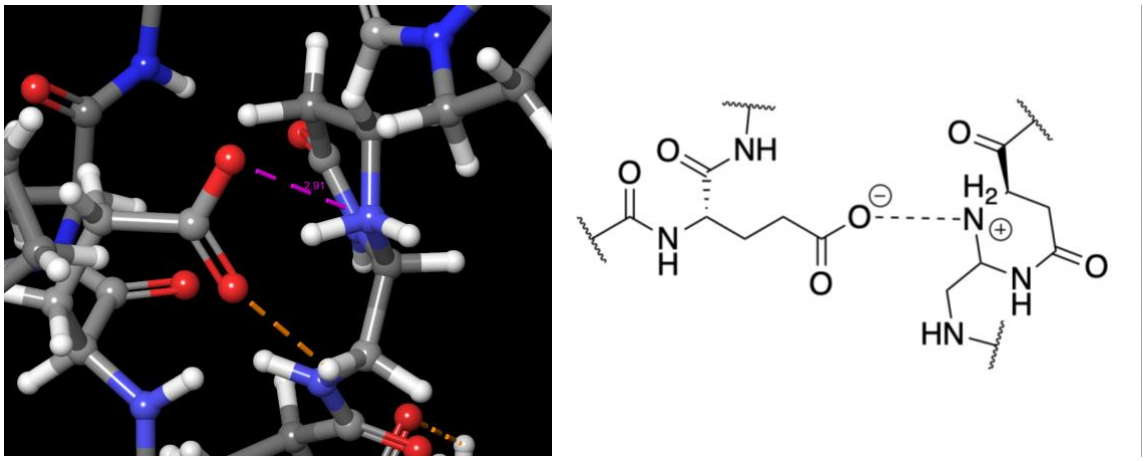

Figure 7. Detailed non-covalent interactions between glutamate residue and 4-pyrimidinone linkage from the structure to the left in figure 6. Salt bridge formation between negatively charged oxygen and positively-charged nitrogen (2.91Å apart) is observed. 2D representation of salt bridge formation (made in ChemDraw) presented for additional clarity.

4. Discussion

4.1. Synthesis Optimization

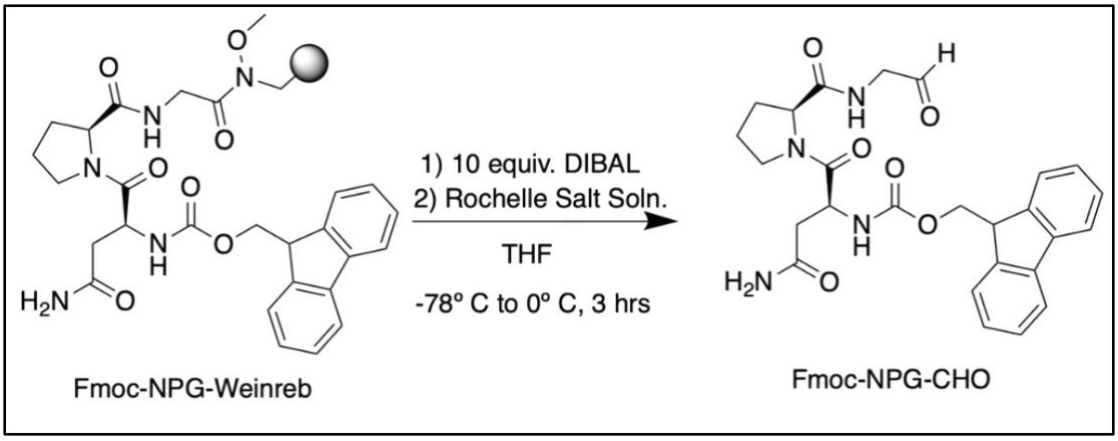

Figure 8. Previously proposed synthetic scheme for reduction to Fmoc-NPG-CHO[16]

The above strategy for synthesizing NPG-CHO monomers, devised by Seth Young, produced the corresponding peptide aldehydes in low yield. Subsequent attempts to synthesize greater quantities of NPG-CHO were unsuccessful, as ^{1}H NMR spectra did not indicate presence of a C-terminal aldehyde and ESI results did not agree with the expected molecular mass of the product. With this setback in synthesis, some issues with the original protocol became clear. First, the Weinreb AM resin used for initial SPPS contains an additional reactive amide group that was not accounted for when tuning the reduction conditions. This extra amide group increases risk of side reactions and ensures that the reduction protocol isn't totally selective for the target amide. Further, the reduction was quenched with Rochelle salt solution (a saturated solution of potassium sodium tartrate), which proved to be a poor quenching agent due to observed complex formation with DIBAL. These aluminum salt emulsions are difficult to break up, and their presence made it difficult to isolate the product in the reaction workup. Finally, the solvent choice of THF became problematic due to its miscibility with water, which complicated the liquid-liquid extraction process in the reaction workup such that product was isolated in low

yield or not collected at all. Moving to the 2-chlorotrityl resin as solid support for SPPS, we opted to first cleave the peptide from the resin to yield a C-terminal carboxylic acid. Notably, the 2-Cl(trt) resin lacks the additional amide bond present in the Weinreb AM resin.

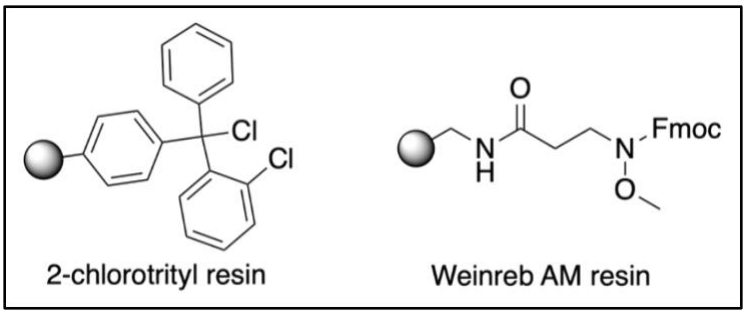

Figure 9. Structures of 2-Cl(trt) and Weinreb AM resins.

Following cleavage from the 2-Cl(trt) resin, the aldehyde is obtained through activation and subsequent reduction of the C-terminal carboxylic acid. Carbonyldiimidazole (CDI) installs an imidazole group on the target amide and activates it for reduction by DIBAL, where the imidazole serves as a better leaving group than the alcohol could have. The structure of the 2-Cl(trt) resin, as seen in the above figure, clearly avoids the issue of non-specific reduction. The choice of DCM as a solvent avoids the issues associated with the miscibility of THF and water, facilitating the extraction process. Further, adding citric acid in the workup helps to break up the aluminum salt emulsions, while using this weak acid sparingly helps decrease the probability of prematurely deprotecting the peptide (which would immediately result in polymerization). Notably, the success of the reduction seems to be highly dependent on the quality of CDI, which must be kept wrapped in parafilm under dry storage conditions.

4.2. Tuning Assembly

Dynamic combinatorial networks, such as those consisting of H_2N-NFF-CHO and H_2N-NPG-CHO, have been shown to self-assemble through two-step nucleation, wherein individual peptides undergo liquid-liquid phase separation and become desolvated in a phase-separated

particle.[6, 15-16] Subsequently, the desolvated peptides undergo a liquid-to-solid phase transition in which solid aggregates form within the particle.[22] In these mixed-phase environments, the system selects for an ordered template over time, allowing for the self-selection and amplification of specific biopolymers. The NFF network utilized cross-β assemblies for template-directed polymerization, but this template was intrinsically limited due to the inherent constraint on strand length in β-sheets.[23] Beyond sequence lengths of 12 residues, the tendency for intrastrand interactions associated with the α-helix seem to outcompete the interstrand interactions associated with β-sheets. This sequence limit then seems to determine that the upper degree of polymerization for H_2N-NFF-CHO be the linear trimer (9 residues in length). Moving away from cross-β assemblies and instead examining the efficiency of a triple-helical strand as an internal template, it should next be possible to direct production of longer oligomers.

Upon self-incubation in aqueous conditions, H_2N-NPG-CHO has been shown to polymerize to high molecular weight polymers, the smallest of which has been identified as the corresponding decamer via ESI.[6] Thanks to extensive EM imaging by Yushi Bai, the resulting collagen-mimetic fibrils have been shown to self-assemble into higher-order bundled structures. These polymers subsequently self-assemble into supra-bundled “balls of yarn” in 50% acetonitrile solution, though the polymerization degree of these fibers remains unknown.[6]

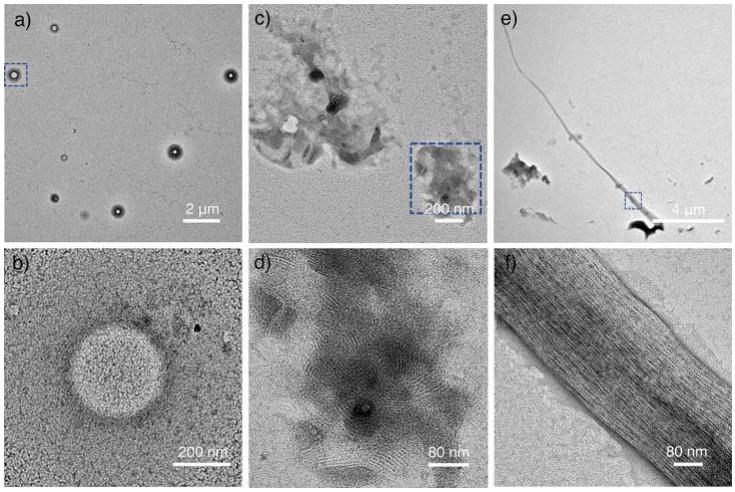

Figure 10. TEM Images of NPG system at a) "first few hours", b) 48 hours, and c) 12 days of assembly in aqueous solution with pH = 4.75 NaOAc/HAc buffer. Data collected by Seth Young and Yushi Bai.[6]

Figure 11. TEM Images of NPG system at 14 days of assembly in 50% acetonitrile solution. Data collected by Seth Young and Yushi Bai.[6]

While the NFF network has inspired the investigation of a dynamic chemical network that generates a different template, it has also inspired the introduction of an external template to the new system. Seeds formed from sonicated assemblies of linear trimers of NFF were introduced to a fresh network of monomers and found to rapidly template growth of the linear trimer assembly, bypassing the previously observed transition stages and emphasizing the importance of template selection for fiber growth.[4] While these seeds consisted of the self-selected internal template, providing an external template with a propensity for triple-helical conformation should

be able to accelerate NPG fiber formation in the same way. Further, it may be possible to tune the self-assembly process to achieve production of monodisperse poly-NPG oligomers through the incorporation of modified monomers or an external template. Ideally, a well-designed external template sequence would alter the nucleation process in self-assembly, accelerating the rate of assembly, and exhibiting chain-length specificity. Further, polymerization would be halted by any modification that interferes with the condensation reaction, which makes N-terminal capping of monomers a promising way to halt the process. Varying concentration ratios of N-acetylated monomers to H_2N-NPG-CHO monomers may therefore be an effective way to select for the length of poly-NPG oligomers.

To this end, the prepared template peptide sequence Ac-(EOGPOG)$_3$EOG-NH_2 may be able to accelerate production of 7-mers of NPG and stabilize the overall assembly.

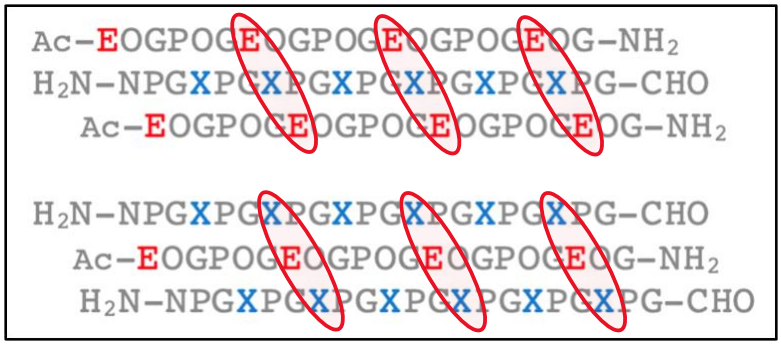

Figure 12. Proposed mechanisms of self-assembly for different template sequence abundance levels. 4-pyrimidinone linkages are represented as X (in blue) for simplicity, while glutamate residues are shown in red.

Considering an assembly registry that is staggered by just one residue, intrahelical associations can be mapped out to gauge potential stabilizing interactions between constituent peptide strands. Both cases above share a staggered stabilization motif in which there is electrostatic interaction between every glutamate residue and every other 4-pyrimidinone linkage (except for the glutamates near the first repeat unit of the poly-NPG strand where there is not yet

a polymerizing linkage). While it's not initially clear from this rudimentary structural drawing alone whether there is truly significant Glu – 4-pyrimidinone interaction between strands, there is reason to believe that the length and rotational flexibility of glutamate's side chain may allow for leniency in the proximity of the charged residues. Further, considering the case with greater template sequence concentration and noting both the non-associated pyrimidinone linkages and increased glutamate abundance, this scheme may theoretically allow for interhelical association and packing as observed in natural collagen. The precise registry of H_2N-NPG-CHO self-assemblies have yet to be defined, so the choice to model it using this specified registry is an assumption that may be proven wrong through more comprehensive assembly characterization.

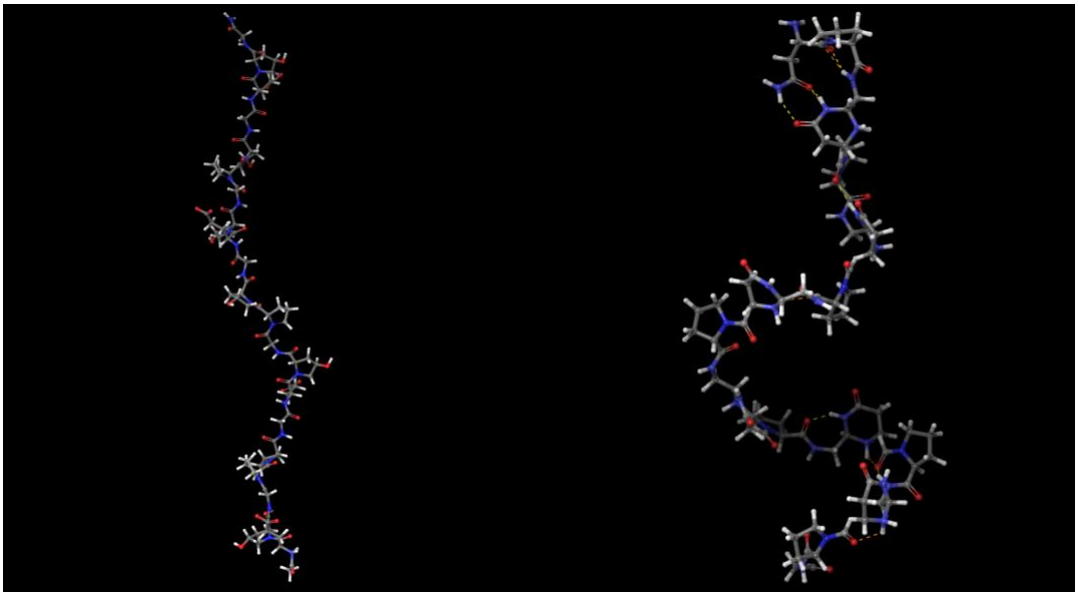

Figure 13. Molecular models of Ac-(EOGPOG)$_3$EOG-NH_2 (left) and a 21-mer of H_2N-NPG-CHO (right) following a simple energetic minimization in Maestro.

Although this developed template sequence couldn't be tested experimentally, it was evaluated computationally to investigate key prerequisites for its templating ability; namely, that it can adopt the very conformation it aims to template. Ac-(EOGPOG)$_3$EOG-NH_2 formed a relatively tight, high-pitch helix with glutamate side chains oriented outwards. This makes sense, as the template sequence alone features no positive charges to allow for charge compensation.

The overall assembly seems to be characterized by hydrogen bonding between carbonyl oxygens and amide nitrogens along the peptide backbone (notably not including hydrogen bond pairs involving the carbonyl oxygen in proline as seen in natural collagen). This difference may be reconciled by considering that minimizing unfavorable interactions between the glutamate side chains may be the dominant factor in the assembly process, potentially altering the orientation of the residues such that the traditional hydrogen bond pairs don't align properly. Still, the assembly seems to successfully form a triple helix.

The assembly of NPG oligomers seems to vaguely resemble a triple helix, though it doesn't appear entirely uniform. This lack of clear triple-helix structure seems to challenge previous findings about NPG assembly structure, but making definitive conclusions about structure feels premature based solely on these minimizations and their inherent limitations. The positively charged nitrogen atoms of the 4-pyrimidinone linkages are oriented outwards and away from the central axis of the assembly (just as the glutamate residues were in the template assembly). Introduction of the 4-pyrimidinone linkage allows for a new array of hydrogen bonding interactions, particularly between aminal hydrogens in the *N*, *N*-acetal and neighboring carbonyl oxygens. It's also notable that a single oligomer adopts a collagen-like helix, though clearly with a much lower pitch than that observed for the template sequences.

Finally, looking at the assembly of NPG oligomers with the template sequence (first with just one template strand), there appears to be divergence from the triple helix structure. Each constituent strand seems to coil around a central axis but the space between strands seems to increase, resulting in a much looser overall assembly. Looking at specific interactions, it's clear that salt bridges do indeed form between the glutamate and 4-pyrimidinone, with observed interatom distances of 2.59, 2.91, and 4.42 Å (the 4th glutamate residue was too distant from the

other strands to associate). These distances are on par with (and, in some cases, even shorter than) reported values for electrostatic interactions in templates that have proven to be experimentally successful,[12] showing that the intended association between templates and oligomers did occur in this sense, at least. These charged residues were oriented away from the assembly when their sequences self-assembled, but salt bridge formation now dictates that they are forced inward to make contact with one another. It then makes sense that this change in orientation of the charged residues may have resulted in distortion of the triple helix, though it cannot be stated conclusively. Conversely, minimization of an NPG oligomer strand with two template strands seemed to form a structure that was also somewhat triple-helical in nature, though none of the expected salt bridges were observed. This was especially surprising since an increased number of glutamate residues from the added template strand should have resulted in a greater number of 4-pyrimidinone linkages being charge-satisfied. It's possible that this increase in charge density was ultimately destabilizing in the interior of the helix, leading the strands to orient the charged residues outwards at the expense of the potential salt bridge interactions.

By virtue of the oligomer and template strands having such different pitches, it's difficult to achieve good overlap for every residue in the sequences, thus ensuring that the starting geometry for minimization is less than ideal. The best way to truly evaluate templating efficiency would be to force the strands into a triple-helical conformation, fix salt bridge distances, and run a molecular dynamics simulation to see whether the assembly falls apart. Preliminary data does suggest that the designed template sequence is capable of electrostatic interaction with NPG oligomers and adopts a triple-helical conformation itself, but the described simulations would be the best way to computationally verify whether these interactions would compromise the integrity of the overall assembly.

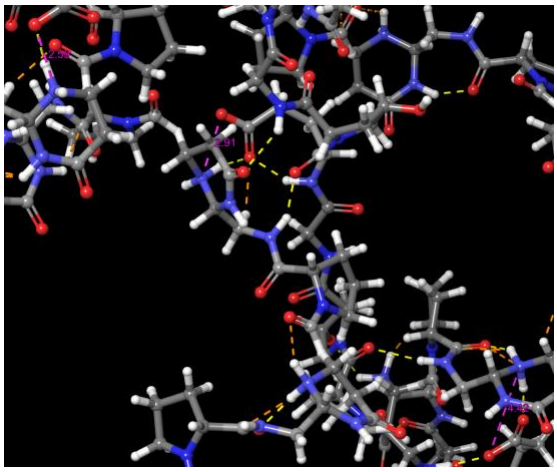

Figure 14. Closer view of salt bridges between glutamate residues and 4-pyrimidinone linkages (represented in pink dashed lines).

4.3. Organoid Viability

Introduction of fresh H_2N-NPG-CHO monomers to growing brain organoid cultures has been shown to increase cellular proliferation and decrease apoptosis rates, though the exact mechanism of this relationship has yet to be discovered.[16] Caspase 3 is an indicator of apoptosis, and its fluorescence indicates active cell-programmed death.[24] SOX2 is a critical transcription factor in maintaining the pluripotency of stem cells, and its continued activity is an indicator of good organoid health.[25] MAP2 stains for microtubule-associated protein 2, which is a neuron-specific cytoskeletal protein that stabilizes neuron morphology during development.[26] DAPI (4',6-diamidino-2-phenylindole) fluoresces upon binding to AT regions of DNA and thus indicates the nuclear region of the organoids.[27] Finally, Ki67 is often used as a marker for cell proliferation and can indicate whether brain organoids are expanding and growing.[28]

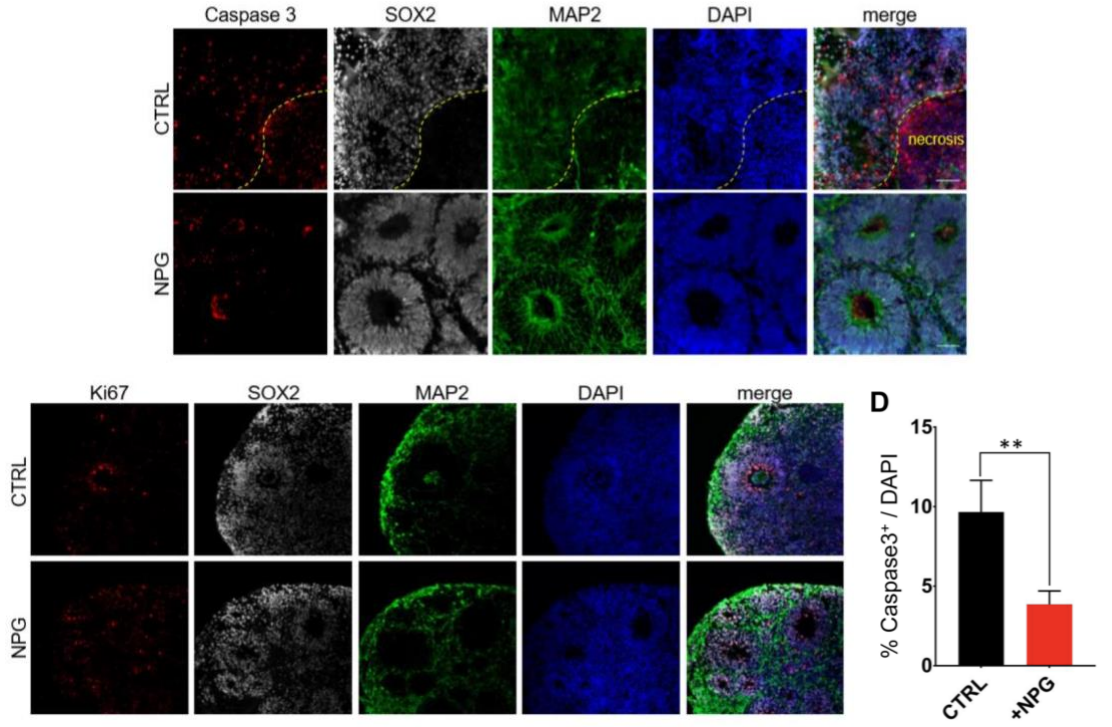

Figure 15. Immunostaining assays for organoids grown in the presence or absence of H_2N-NPG-CHO monomers in PBS buffer with a pH of 7.4. Quantitative comparison of Caspase3/DAPI activity between both treatment groups. All organoid data collected by Dr. Wen and Xianxing.[16]

Decreased fluorescence of Caspase 3 in the samples grown with NPG is consistent with NPG decreasing apoptosis rates and overall necrosis in the brain organoids. Additionally, NPG was found to increase cellular proliferation and promote the formation of cortical units. The critical question resulting from this data is the following: how does NPG extend the lifetime/viability of the brain organoid samples? There exists a possibility that organoid survival increases in the presence of NPG simply because its introduction provides an accessible source of nutrients. To determine whether this is true, unreduced NPG monomers in the form of H_2N-NPG-COOH could be introduced to the organoids and the same immunostains could be used. These monomers with the C-terminal acid will be unable to self-polymerize and self-associate, so monitoring immunostaining under these conditions and comparing to the original case should provide insight into whether survival is due to

triple-helical binding or just monomer uptake. Another possible explanation is that the triple-helical assemblies are able to associate with the extracellular matrix (as natural collagen does), and their dynamic nature reduces matrix viscosity and thus makes nutrients more accessible to cells in the organoids. This hypothesis could be investigated using a fluorophore that binds to the extracellular matrix, and comparison of fluorescence half-life between a control system and the NPG system would determine whether the viscosity of the ECM has been altered. Though the current mechanism of action is unclear, these organoid studies nonetheless demonstrate how dynamic chemical networks, composed of simple abiotic peptides, are capable of self-polymerization and self-assembly with structural specificity that results in emergence of biological function.

4.4. Extending Peptide Aldehyde Chemistry to Disease Pathology

One of the most important relationships in biology is that between the function of a protein and its three-dimensional structure. This three-dimensional structure is a product of a complex folding phenomenon governed by thermodynamics and free energy, frequently represented as a folding funnel with various relative free energy minima associated with different conformations or "native states" the peptide may adopt. In some instances, the free energy minima are extreme and represent thermodynamically trapped (irreversible), fibrillar aggregates.[29] These aggregates are formed via a two-step nucleation process consisting of liquid-liquid phase separation and a liquid-solid phase transition within the phase-separated particle. In Parkinson's Disease, the accumulation of aggregates of the protein α-Synuclein leads to loss of neurological function over time.[30]

α-Synuclein is an intrinsically disordered protein (IDP) and has no single native state; rather, it has multiple accessible conformations separated by relatively low energy barriers. It is especially remarkable, then, that such a dynamic and transient protein has the capability to transition into fibrillar aggregates with essentially no conformational flexibility. This newly developed peptide aldehyde chemistry may help provide insight into whether proteins in the phase-separated particle are conformationally limited prior to aggregation and fibrillization. Using αSyn as a model system, it's possible to distinguish and synthesize separate fragments of the peptide based on the observation that glutamine (Gln, Q) residues with negatively-charged side chains appear to contribute to long-range ordering of the peptide.[31] As with the NPG-CHO system, cyclization (from addition of an asparagine side chain to the imine intermediate) will result in reversible formation of *N*, *N*-acetals. Upon incubating these peptide fragments, one can observe whether a reversible 4-pyrimidinone linkage forms between fragments in order to track favorable associations within the folding space. Formation of these cyclic linkages should allow combined fragments to become trapped in relative low energy minima and allow for simpler detection by mass spectrometry.

To determine the impact of residue mutations on the rate of fibrillization and the stability of these associations, key residues within the α-Syn fragments can be altered. It is known that aggregation of α-Syn is highly dependent on electrostatics.[32] Specifically, negative charges can play a role in preventing fibril formation.[33] Replacing negatively charged residues with neutral or positively-charged residues can have a profound impact on the favorability of associations between fragments, which would provide insight into how common mutations in the familial region of α-Syn impact the degree to which the protein is pre-ordered prior to aggregation. Determining if the protein is pre-ordered may help to explain the biophysical mechanisms of fibril diversity. This should be relatively simple to measure: the formation of the pyrimidinone linkage should indicate that pre-ordering is, in fact, necessary for fibrillization. The reversible nature of the linkage is also key because only stable associations between fragments should be detectable by this design.

Sequence Name	**α-Syn Residues**	**Peptide Sequence**	**Modification from Wild Type**	**Expected Mass (Da)**
Familial	44 – 64	Ac-TKEGVVHGVATVAEKTKEQVG-CHO	N-terminal acetylation; C-terminal aldehyde; T64G	2180.45
NAC Core	65 – 80	H_2N-NVGGAVVTGVTAVAQG-CHO	C-terminal aldehyde; K80G	1399.57
NAC C-term	81 – 101	H_2N-NVEGAGSIAAATGFVKKDQLG-NH_2	C-terminal amide; T81N	2033.27

Figure 16. Proposed fragment sequences, names, modifications, and expected masses. "NAC" refers to the non-amyloid-β component of α-Syn. Adapted from Will McFadden.[31]

The above fragment sequences were designed based on the concept of stickers and spacers and their critical role in driving association in the LLPS process. In this way, the 4-pyrimidinone linkage formed from the condensation reaction functions as a covalent sticker that drives

association. Sequence analysis revealed that glutamine neighboring charged residues were enriched, pointing to this dipeptide's possible role as a sticker motif in α-Syn. Each fragment thus contains one glutamine residue, and the terminal residues of each fragment have been modified to N-terminal asparagine and/or C-terminal glycine with an aldehyde to allow polymerization to occur.

Potential pitfalls with this project involve clean synthesis and purification of the peptide fragments. The current reduction protocol has been optimized for short tripeptides, while the proposed α-Syn fragments are 17 – 22 residues in length and vary in chemical nature. As such, the existing protocol may very well have to be modified to obtain C-terminal aldehydes for the α-Syn fragments. The extension of peptide aldehyde chemistry may be more achievable by first considering a model system in which proposed fragments are more intermediate in length.

5. Conclusion

Collagen, one of the most important and versatile biomolecules in the human body, presents a fantastic opportunity through which to gain further insight into the mechanisms of nature's selection processes and chemical evolution. Using collagen's triple-helical structure as inspiration for an extended template, the H_2N-NPG-CHO network has been shown to undergo LLPS, select for an internal template, and form extended fibers that behave like natural collagen. After an extended roadblock, a robust cleavage and reduction protocol has been developed that reliably produces more NPG monomers for future study. The peptide sequence Ac-$(EOGPOG)_3EOG$-NH_2 was thought to act as an external template capable of accelerating and stabilizing triple-helical assembly, working towards template-directed production of monodisperse NPG oligomers. The template sequence was synthesized and purified via HPLC,

but time constraints dictated that its efficiency be evaluated through computational modeling. Modeling revealed that the template sequence alone was capable of forming assemblies reminiscent of triple helices, as were strands of NPG oligomers (though less uniform). While NPG oligomers and the template sequence exhibited the expected salt bridge formation, the overall assembly resembled a distorted triple helix, so Ac-(EOGPOG)$_3$EOG-CHO may not be the best choice of external template to direct polymerization. Before arriving at this conclusion, however, molecular dynamics simulations should be performed to determine whether the hypothesized assembly is feasible. As NPG monomer production ramps up, these monomers can be co-assembled with the template sequence and the resulting assembly monitored by CD over time to look for a characteristic triple-helix profile. In sum, the use of a dynamic chemical network to create a collagen-mimetic peptide represents a profound extension of established template-directed polymerization, a process which may be further tuned by an external template that directs amplification of chain-length specific oligomers and allows more comprehensive characterization of the resulting peptide assembly.

6. References

1) Goodwin, J.T.; Lynn, D.G.; Burrows, C. Walker, S.; Amin, S.; Armbrust, E.V. *Alternative Chemistries of Life: Empirical Approaches*, 2014.
2) Frenkel-Pinter, M.; Samanta, M.; Ashkenasy, G.; Leman, L.J. Prebiotic Peptides: Molcular Hubs in the Origin of Life. *Chem. Rev.* **2020**, *120* (11), 4707 – 4765.
3) Childers, W.S.; Anthony, N.R.; Mehta, A.K.; Berland, K.M.; Lynn, D.G. Phase Networks of Cross-β Peptide Assemblies. *Langmuir* **2012**, *28* (15), 6386 – 6395.

4) Chen, C.; Tan, J.; Hsieh, M.-C.; Pan, T.; Goodwin, J.T.; Mehta, A.K.; Grover, M.A.; Lynn, D.G. Design of Multi-Phase Dynamic Chemical Networks. *Nature Chemistry* **2017**, *9*, 799 – 804. DOI: 10.1038/nchem.2737

5) Ricard-Blum, S. The Collagen Family. *Cold Spring Harb. Perspect. Biol*. **2011**, *3*(1), a004978. DOI: 10.1101/cshperspect.a004978

6) Bai, Y.; Young, S.; Lynn, D.G. Alternative Self-Producing Collagen. *Unpublished*.

7) Ottani, V.; Raspanti, M.; Ruggeri, A. Review: Collagen Structure and Functional Implications. *Micron* **2001**, *32*, 251 – 260. DOI: 10.1016/S0968-4328(00)00042-1

8) Shoulders, M.D.; Raines, R.T. Collagen Structure and Stability. *Annu. Rev. Biochem*. **2009**, *78*, 929 – 958. DOI: 10.1146/annurev.biochem.77.032207.120833

9) Hentzen, N.B.; Islami, V.; Köhler, M.; Zenobi, R.; Wennemers, H. A Lateral Salt Bridge for the Specific Assembly of an ABC-Type Collagen Heterotrimer. *J. Am. Chem. Soc.* **2020**, *142*, 2208 – 2212. DOI: 10.1021/jacs.9b13037

10) Egli, J.; Siebler, C.; Köhler, M.; Zenobi, R.; Wennemers, H. Hydrophobic Moieties Bestow Fast-Folding and Hyperstability on Collagen Triple Helices. *J. Am. Chem. Soc.* **2019**, *141*, 5607−5611, DOI: 10.1021/jacs.8b13871

11) Ottani, V.; Martini, D.; Ruggeri, A.; Raspanti, M. Hierarchical Structures in Fibrillar Collagens. *Micron* **2002**, *33*, 587 – 596. DOI: https://doi.org/10.1016/S0968-4328(02)00033-1

12) O'Leary, L.E.R.; Fallas, J.A.; Bakota, E.L.; Kang, M.K.; Hartgerink, J.D. Multi-hierarchical Self Assembly of a Collagen Mimetic Peptide from Triple Helix to Nanofibre and Hydrogel. *Nature Chemistry*, **2011**, *3*, 821 – 828. DOI: 10.1038/nchem.1123

13) Rele, S.; Song, Y.; Apkarian, R.P.; Qu, Z.; Conticello, V.P.; Chaikof, E.L. D-Periodic Collagen-Mimetic Microfibers. *J. Am. Chem. Soc.* **2007**, *129*, 14780 – 14787. DOI: 10.1021/ja0758990

14) Sarkar, B.; O'Leary, L.E.R.; Hartgerink, J.D. Self-Assembly of Fiber-Forming Collagen Mimetic Peptides Controlled by Triple-Helical Nucleation. *J. Am. Chem. Soc.* **2014**, *136*, 14417 – 14424. DOI: 10.1021/ja504377s

15) Chen, C. Metastable Phases Direct Polymer Emergence and Evolution in Dynamic Chemical Networks. *Emory Theses and Dissertations Database* **2015**.

16) Young, S. Controlling Biopolymer Phase Networks with Dynamic Chemical Networks. *Emory Theses and Dissertations Database* **2021**.

17) Chiaradia, I.; Lancaster, M.A. Brain Organoids for the Study of Human Neurobiology at the Interface of in vitro and in vivo. *Nature Neuroscience* **2020**, *23*, 1496 – 1508. DOI: 10.1038/s41593-020-00730-3

18) Clements, K.A.; Acevedo-Jake, A.M.; Walker, D.R.; Hartgerink, J.D. Glycine Substitutions in Collagen Heterotrimers Alter Triple Helical Assembly. *Biomacromolecules* **2017**, *18*, 617 – 624. DOI: 10.1021/acs.biomac.6b01808

19) Persikov, A.V.; Ramshaw, J.A.M.; Kirkpatrick, A.; Brodsky, B. Amino Acid Propensities for the Collagen Triple-Helix. *Biochemistry* **2000**, *39*, 14960 – 14967. DOI: 10.1021/bi001560d

20) Bollhagen, R.; Schmiedberger, M.; Barlos, K.; Grell, E. A New Reagent for the Cleavage of Fully Protected Peptides Synthesised on 2-Chlorotrityl Chloride Resin. *J. Chem. Soc.* **1994**, *22*, 2559 – 2560. DOI: 10.1039/c39940002559

21) Ivkovic, J.; Lembacher-Fadum, C.; Breinbauer, R. A Rapid and Efficient One-Pot Method for the Reduction of N-Protected α-Amino Acids to Chiral α-Amino Aldehydes using CDI/DIBAL-H. *Org. Biomol. Chem.* **2015**, *13*, 10456 – 10460. DOI: 10.1039/C5OB01838B

22) Zbinden, A.; Pérez-Berlanga, M.; De Rossi, P.; Polymenidou, M. Phase Separation and Neurodegenerative Diseases: a Disturbance in the Force. *Dev. Cell* **2020**, *55*, 45 – 68. DOI: 10.1016/j.devcel.2020.09.014

23) Stanger, H.E.; Syud, F.A.; Espinosa, J.F.; Giriat, I.; Muir, T.; Gellman, S.H. Length-Dependent Stability and Strand Length Limits in Antiparallel β-Sheet Secondary Structure. *PNAS* **2001**, *98* (21), 12015 – 12020. DOI: 10.1073/pnas.211536998

24) Bressenot, A.; Marchal, S.; Bezdetnaya, L.; Garrier, J.; Guillemin, F.; Plénat, F. Assessment of Apoptosis by Immunohistochemistry to Active Caspase-3. *J. Histochem. Cytochem.* **2009**, *57* (4), 289 – 300. DOI: 10.1369/jhc.2008.952044

25) Zhang, S.; Cui, W. Sox2, a Key Factor in the Regulation of Pluripotency and Neural Differentiation. *World J. Stem Cells* **2014**, *6* (3), 305 – 311. DOI: 10.4252/wjsc.v6.i3.305

26) Soltani, M.H.; Pichardo, R.; Song, Z.; Sangha, N.; Camacho, F.; Satyamoorthy, K.; Sangueza, O.P.; Setaluri, V. Microtubule-Associated Protein 2, a Marker of Neuronal Differentiation, Induces Mitotic Defects, Inhibits Growth of Melanoma Cells, and Predicts Metastatic Potential of Cutaneous Melanoma. *Am. J. Pathol.* **2005**, *166* (6), 1841 – 1850. DOI: 10.1016/S0002-9440(10)62493-5

27) Tarnowski, B.I.; Spinale, F.G.; Nicholson, J.H. DAPI as a Useful Stain for Nuclear Quantitation. *Biotech Histochem.* **1991**, *66* (6), 297 – 302.

28) Li, L.T.; Jiang, G.; Chen, Q.; Zheng, J.N. Ki67 is a Promising Molecular Target in the Diagnosis of Cancer (Review). *Mol. Med. Rep.* **2015**, *11*, 1566 – 1572. DOI: 10.3892/mmr.2014.2914

29) Uversky, V.N. Intrinsically Disordered Proteins from A to Z. *Int. J. Biochem. Cell Biol.* **2011**, *43* (8), 1090 – 1103. DOI: 10.1016/j.biocel.2011.04.001

30) Flagmeier, P.; Meisl, G.; Vendruscolo, M.; Knowles, T.P.J.; Dobson, C.M.; Buell, A.K.; Galvagnion, C. *PNAS* **2016**, *113* (37), 10328 – 10333. DOI: 10.1073/pnas.1604645113

31) McFadden, Will. Rotation Report: Design of a Modular α-Synuclein to Investigate Biased Conformational Order of Proteins in Undergoing Liquid-Liquid and Liquid-Solid Phase Transitions. *Unpublished*.

32) Wu, K.-P.; Weinstock, D.S.; Narayanan, C.; Levy, R.M.; Baum, J. Structural Reorganization of Alpha-Synuclein at Low pH Observed by NMR and REMD Simulations. *J. Mol. Biol.* **2009**, *391* (4), 784 – 796. DOI: 10.1016/j.jmb.2009.06.063

33) Gallardo, J.; Escalona-Noguero, C.; Sot, B. Role of α-Synuclein Regions in Nucleation and Elongation of Amyloid Fiber Assembly. *ACS Chem. Neurosci.* **2020**, *11* (6), 872 – 879. DOI: 10.1021/acschemneuro.9b00527