CATCH Peptides Coassemble into Structurally Heterogeneous β-sheet Nanofibers with Little Preference to β-strand Alignment

*Kong M. Wong,[1] Qing Shao,[2] Yiming Wang,[3] Dillon T. Seroski,[4] Renjie Liu,[4] Annabelle H. Lint,[1] Gregory A. Hudalla,[4] Carol K. Hall,[3] Anant K. Paravastu[1]**

1. School of Chemical and Biomolecular Engineering, Georgia Institute of Technology, Atlanta, GA 30332, United States

2. Department of Chemical and Materials Engineering, University of Kentucky, Lexington, Kentucky 40506, United States

3. Department of Chemical and Biomolecular Engineering, North Carolina State University, Raleigh, NC 27695-7905, United States

4. J. Crayton Pruitt Family Department of Biomedical Engineering, University of Florida, 1275 Center Drive, Biomedical Sciences J293, P.O. BOX 116131, Gainesville, FL 32611, United States

Abstract

Coassembling peptides offer an additional degree of freedom in the design of nanostructured biomaterials when compared to analogous self-assembling peptides. Yet, our understanding of how amino acid sequences encodes coassembled nanofiber structure is limited. Prior work on a charge-complementary pair, CATCH+ and CATCH- peptides, detected like-peptide nearest-neighbors (CATCH+:CATCH+ and CATCH-:CATCH-) within coassembled β-sheet nanofibers; these self-associated peptide pairs marked a departure from an "ideal" coassembled structure. In this work, we employ solid-state NMR, isotope-edited FTIR, and coarse-grained molecular dynamics simulations to evaluate the alignment of β-strands within CATCH peptide nanofibers. Both experimental and computational results suggest that CATCH molecules coassemble into structurally heterogeneous nanofibers, which is consistent with our observations in another coassembling system, the King-Webb peptides. Within β-sheet nanofibers, β-strands can have nearest neighbors aligned in-register parallel, in-register antiparallel, and out-of-register. In comparison to the King-Webb peptides, CATCH nanofibers exhibit a greater degree of structural heterogeneity. By comparing the amino acid sequences of CATCH and King-Webb peptides, we can begin to unravel the sequence-to-structure relationships, which may encode more precise coassembled β-sheet nanostructures.

Introduction

Coassembling β-sheet peptides broaden the architectural complexity accessible during the fabrication of synthetic biomaterials. Functional molecules or groups can be covalently attached to the peptide termini to impart biological function and produce functional peptide hydrogels for biotechnological applications, such as enzyme immobilization,[1-2] drug delivery,[3-5] and immunoengineering.[6-8] In theory, peptide coassembly allows finer organizational control of immobilized functional motifs along β-sheet

nanofibers than better-studied peptide self-assembly. One common approach to designing peptides that coassemble utilizes charge complementarity; β-sheet-forming sequences are patterned with charged amino acids to produce a negatively charged peptide and a positively charged partner peptide. Electrostatic repulsion between like-charged peptides disfavors self-association while electrostatic attraction between complementary peptides promotes cooperative coassembly behavior. Charge-complementary peptides were hypothesized to coassemble into a uniform β-sheet structure with perfect alternation of positively and negatively charged β-strands. However, our recent investigation of the King-Webb peptides (KW+: KKFEWEFEKK and KW-: EEFKWKFKEE), a charge-complementary pair from King et al., revealed measurable deviations from an ideal nanofiber structure; like-peptides self-associated to form clustered pairs, and β-strands aligned in both parallel and antiparallel orientations.[9-10] This structural heterogeneity likely exists in other charge-complementary designs, such as the CATCH peptides (CATCH+: Ac-QQKFKFKFKQQ-Am and CATCH-: Ac-EQEFEFEFEQE-Am; Ac-:acetylated C-terminus; -Am: amidated N-terminus) by Seroski et al., but has not been thoroughly evaluated.[1] In this paper, we combine experimental and computational approaches to characterize the molecular organization of coassembled CATCH nanofibers.

The CATCH peptide sequences may encode a more homogeneous β-strand alignment than the King-Webb peptide sequences and provide insight into amino acid sequence motifs that promote homogeneous nanofiber structure. Both peptide systems exhibit hydrophobic/hydrophilic amino acid sequence patterning, such that β-strand secondary structure orients even and odd residues onto different faces of the molecule. The patterning of charged amino acids on the hydrophilic faces of CATCH peptides is more uniform than the patterning of charged amino acids on the hydrophilic faces of King-Webb peptides. Each peptide in the King-Webb system, KW+ (KKFEWEFEKK) and KW- (EEFKWKFKEE), combines domains of positively and negatively charged residues that result in a ±1 overall molecular charge per peptide at neutral pH.[10] On the other hand, CATCH+ (Ac-QQKFKFKFKQQ-

Am) is patterned with positive K residues along its hydrophilic face, and CATCH- (Ac-EQEFEFEFEQE-Am) is patterned with negative E residues along its hydrophilic face.[1] Each CATCH peptide has a higher overall molecular charge than the corresponding KW peptide. The high overall charge was previously hypothesized to effectively discourage self-association during coassembly. However, solid-state nuclear magnetic resonance (NMR) and discontinuous molecular dynamics (DMD) simulations observed some self-associated CATCH+ and CATCH- pairs within coassembled nanofibers in our prior study.[11] Solid-state NMR experiments in this previous study were designed to be insensitive to β-strand alignment. Thus, the study did not report on whether β-strands aligned parallel or antiparallel to one another, and it remains unclear how the charge patterning of the CATCH pair versus the King-Webb pair might influence such β-strand alignment. We have also investigated the role of net molecular charge in selectively biasing charge-matched CATCH variant pairs towards coassembly rather than self-assembly.[12] However, structural analysis of the charge-matched pairs was limited to β-sheet content and nanofiber composition. Another sequence design difference between CATCH peptides and King-Webb peptides that may influence β-strand alignment is seen in the peptide termini. CATCH peptides have acetylated and amidated termini while King-Webb peptides have unmodified termini. Acetylation and amidation produce charge-neutral end groups and increase the number of hydrogen bond acceptors and donors, which may bias β-strands to align parallel or antiparallel to one another. Understanding how peptide sequence design affects resulting nanofiber structure will be important in the future design of precise coassembled peptide nanostructures.

Our understanding of structural heterogeneity in coassembled peptide nanofibers draws from studies by Dr. Ruth Nussinov regarding polymorphism in self-assembling peptides. Her work on Aβ(17-42) (p3) oligomers has shown that oligomer conformations play an important role in polymorphic behavior in assembled fibers.[13] Nussinov's computational work on structural polymorphism in peptide nanostructures has also shed light on mechanisms of aggregation as they relate to toxicity in amyloids.[14]

With the de novo self-assembling peptide MAX1, Miller et al. showed in simulations that the total energies of different packing of β-hairpins along the fiber axis were similar within statistical error.[15] However, MAX1 has been conclusively demonstrated to exhibit monomorphism in experiments by Nagy-Smith et al.[16] This behavior falls in line with previous experimental studies of self-assembling peptides, where a single dominant structure is observed for a given set of assembly conditions.[17-19] The discrepancy between computational and experimental results highlights a gap in our knowledge of peptide assembly processes. In this work, we take a combined computational and experimental approach, which allows us to compare computational predictions against experimental measurements and gain a better understanding of heterogeneity in peptide systems.

Here, we present an experimental and computational investigation of CATCH+ and CATCH- peptide alignments within coassembled β-sheets. Solid-state NMR measurements on ^{13}C- and ^{15}N-labeled nanofiber samples detected in-register antiparallel and in-register parallel β-sheet structures. DMD/PRIME20 simulations of a 96-peptide system of CATCH+ and CATCH- molecules predicted the formation of structurally heterogeneous β-sheet nanofibers and did not indicate a strong organizational preference during the addition of CATCH peptides to the fiber ends. Nanofibers formed in DMD/PRIME20 simulations contained in-register parallel, in-register antiparallel, and out-of-register β-strand neighbors. Spectral analysis of isotope-edited Fourier transform infrared (FTIR) measurements on a series of isotopically labeled nanofiber samples suggest CATCH+ and CATCH- peptides coassemble into heterogeneous β-sheets. Quantitative analysis of dipolar recoupling NMR measurements also agreed with a structurally heterogeneous β-sheet model. The percentage of β-strands out-of-register with nearest neighbors was estimated to be 30.1%. The coexistence of multiple β-strand arrangements in coassembled CATCH nanofibers illustrates the challenge in designing amino acid sequences to create precise charge-complementary nanostructures.

Materials and Methods

Peptide Synthesis and Purification

Peptides were synthesized using standard Fmoc solid-phase peptide synthesis on a CS336X automated peptide synthesizer (CS Bio) using standard amino acids, a ^{13}C-enriched phenylalanine at position F4, or a ^{15}N-enriched phenylalanine at position F8. Peptides were acetylated at their N-termini using 10% acetic anhydride (Sigma), 80% dimethylformamide (Fisher) and 10% *N,N*-Diisopropylethylamine (Fisher). Peptides on resin were collected and washed with acetone ten times and placed *in vacuo* overnight. Peptides were deprotected and cleaved from resin using 95% trifluoracetic acid (TFA) (Fisher Scientific), 2.5% triisopropylsilane (Sigma-Aldrich), and 2.5% ultrapure water. Resin was separated from the soluble peptide by using disposable polypropylene columns with 0.2 µM filters followed by precipitation with diethyl ether (Fisher Scientific) on ice for five minutes. The precipitate was then pelleted *via* centrifugation and washed with fresh diethyl ether three times to remove remaining TFA, and then dried in vacuo overnight. CATCH+ peptide was dissolved in water and CATCH- peptide was dissolved in 200 mM ammonium bicarbonate, then both were frozen, and freeze-dried using a FreeZone 1 lyophilizer (Labconco).

Peptides were purified to greater than 90% purity using a DionexTM Utlimate 3000 System (Thermo Scientific) equipped with a C-18 column (Thermo Fisher) for CATCH-, or a PFP column (Thermo Fisher) for CATCH+. The mobile phase consisted of water with 0.1% TFA and the elution phase was acetonitrile with 0.1% TFA. Peptides were detected via absorbance at 215 nm. Molecular weights were verified using matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry by mixing 1:1 with 10 mg/ml α-Cyano-4-hydroxycinnamic acid matrix in 30:70 water:acetonitrile with 0.1% TFA.

Nanofiber Preparation and Formulation

Peptides were dissolved in either water or 200 mM ammonium bicarbonate with their concentrations determined using phenylalanine absorbance (λ=258 nm). Peptide samples contained equimolar ratio of CATCH+ and CATCH- and are coassembled in 1X PBS (137 mM NaCl, 2.7 mM KCL, 10 mM Na_2HPO_4, pH 7.4).

Solid-state NMR Measurements

Peptide hydrogels were prepared at 10 mM in 1X PBS and then incubated for 24 hours. Nanofibers were sedimented via centrifugation at 12,100 x *g* for 5 minutes and then re-suspended in ultrapure water three times. Samples were then frozen and freeze-dried with a FreeZone 1 lyophilizer. Lyophilized CATCH peptide nanofibers were packed into Bruker 3.2 mm NMR rotors. All measurements were performed on an 11.75 T Bruker Avance III spectrometer with a 3.2 mm Bruker MAS probe. ^{1}H-^{13}C CPMAS measurements were run at 22 kHz magic angle spinning (MAS). Proton decoupling at 100 kHz was applied during acquisition, and the contact time for cross-polarization set to 2 ms. ^{13}C chemical shifts are referenced to tetramethyl silane by using adamantane to calibrate before each experiment.

PITHIRDS-CT measurements were performed at a spinning frequency of 12.5 kHz. The length of π-pulses during ^{13}C dipolar recoupling was set to 26.7 μs. The parameters were set as follows k1 = 4 and k2 + k3 = 16, which gives a total recoupling time of 61.44 ms.[20] During the PITHIRDS-CT pulse sequence and data acquisition, 100 kHz of continuous wave proton decoupling was applied.

REDOR measurements were performed at a MAS frequency of 10 kHz and at a temperature of 293 K. xy8 phase cycling was used to compensate for pulse imperfections,[21] and EXORCYCLE phase cycling used during the final ^{13}C Hahn-echo refocusing pulse with 95 kHz Spinal64 proton decoupling.[22-24] ^{13}C and ^{15}N π pulses were both set to 10 μs. REDOR data points were calculated from the integrals of the central peaks.

Nuclear spin simulations of dipolar recoupling NMR experiments

To understand the effects of structural heterogeneity on dipolar recoupling NMR measurements, we performed simulations of coassembled β-sheets with varying degrees of in-register parallel β-sheet content and in-register antiparallel β-sheet content. For the homogeneous nanofiber model fits, PITHIRDS-CT and $^{15}N\{^{13}C\}$REDOR curves were simulated using SpinEvolution NMR simulation software.[25] Due to computational limitations of spin simulations, an ideal in-register parallel β-sheet nanofiber was represented by an 8-unit long segment using distances from an all-atom in-register parallel β-sheet model (Supplemental Figure 6A). Similarly, an 8-unit long segment taken from an all-atom model of in-register antiparallel β-sheets (Supplemental Figure 6B) was used to represent an ideal in-register antiparallel β-sheet nanofiber. Linear combinations of a PITHIRDS-CT curve representing an ideal in-register parallel β-sheet and another one representing an ideal in-register antiparallel β-sheet were used to fit experimental PITHIRDS-CT datapoints to the homogeneous nanofiber model. A similar approach was used to fit $^{15}N\{^{13}C\}$REDOR measurements.

To fit datapoints to the heterogeneous nanofiber model, Monte Carlo simulations were used to produce a β-sheet pattern consisting of string sequence of A's and P's. A's represented in-register antiparallel β-strand neighbors, and P's used to represent in-register parallel β-strand neighbors. The addition of a peptide to the end of a β-sheet sequence in the Monte Carlo simulations was determined by the probability of an in-register antiparallel or an in-register parallel β-strand neighbor. The β-sheet length was set to 2000 peptide units to approximate steady state. Then, the β-sheet sequence patterns were used to generate spin simulations to examine heterogeneity effects on PITHIRDS-CT and $^{15}N\{^{13}C\}$REDOR measurements. The Monte Carlo predicted β-sheet sequence patterns were simulated in 8-unit long segments, and interstrand distances were randomized from a distribution of distances calculated from all-atom models simulated 10 times. Each Monte Carlo sequence was also simulated 10 times to eliminate sampling artifacts and produce an averaged simulated curve. Finally, simulated curves

of PITHIRDS-CT and $^{15}N\{^{13}C\}$REDOR measurements were produced by averaging over 10 Monte Carlo sequences at each simulated percentage of in-register parallel and in-register antiparallel β-strand neighbor content to again reduce sampling bias. Similar simulations were conducted to understand the effect of like-peptide nearest neighbors on PITHIRDS-CT and $^{15}N\{^{13}C\}$REDOR measurements; instead of A's and P's, A's and B's were used to represent positive and negative peptides, respectively.

Fourier Transformed Infrared Measurements

The FTIR spectra were recorded on a Frontier FTIR spectrophotometer (PerkinElmer) equipped with a universal ATR sampling accessory. For aqueous samples, the FTIR spectrophotometer was blanked with 1X PBS prior to scanning. Samples were prepared at 10 mM and 1X PBS with 5 µl spotted onto the ATR accessory. Aqueous samples were scanned 50 times with the average of the spectra reported.

DMD/PRIME20 Simulations

PDB files of final simulation snapshots from Shao et al. can be found within the data repository associated with our previous publication.[11] Setup conditions for DMD/PRIME20 simulations of 48 CATCH+ and 48 CATCH- peptides are also detailed in our previous publication.[11] VMD (Visual Molecular Dynamics) software was used to visualize coassembled CATCH peptide nanofibers.[26] Intermolecular distances used in structural analysis of coassembled nanofibers were calculated using custom code in Wolfram Mathematica.

All-Atom Models of Ideal β-Sheet Structures

Idealized all-atom models of in-register parallel and in-register antiparallel β-sheet nanofiber structures were built to aid interpretation of DMD/PRIME20 simulations and dipolar recoupling NMR

measurements. All β-sheet models were constructed using NAMD molecular dynamics and VMD software.[26-28] Initial models of CATCH+ and CATCH- peptides were individually created in VMD with the molefacture plugin. CATCH+ and CATCH- monomers were manipulated depending on the β-sheet structure and stacking and repeated along the fiber axis using custom code in Wolfram Mathematica to produce 2 β-sheets with 10 units each (5 CATCH+ and 5 CATCH-) which were stacked to form a hydrophobic core. First, simulations were run for 40 ps to randomize initial sidechain conformations by fixing backbone atom positions. Then, artificial dihedral angle and hydrogen bond constraints were introduced and constraints on backbone atom positions removed. Simulations proceeded with a 20 ps energy minimization step followed by a 10 ps production step. Then, artificial bonds were placed between alpha carbons across the two β-sheets. Following an initial energy minimization run of 10ps, the temperature was raised by 10 K per 10 ps of simulation time until a final temperature of 300 K was achieved. At 300 K, the nanofiber simulation ran for an additional 20 ps of production. Finally, the spring constant for all artificial constraints were decreased from 1 to a value of 0.1, and the constrained bilayer nanofiber structure was run for 40 ps at 300K. Visualization of coassembled CATCH+ and CATCH- peptide nanofibers was performed using VMD software.[26] Custom code in Wolfram Mathematica calculated intermolecular distances for comparison with solid-state NMR measurements.

Results

Dipolar recoupling NMR measurements detected in-register parallel and in-register antiparallel β-strand neighbors

In-register parallel β-strand neighbors were observed in the PITHIRDS-CT measurement on coassembled CATCH nanofiber samples isotopically enriched with ^{13}C. Centrifuged and lyophilized nanofiber samples were prepared from equimolar mixtures of CATCH+ and CATCH- peptides, which were isotopically labeled on the carbonyl carbon of F4 (Figure 1A-B). By labeling both peptides in this

manner, we can evaluate the orientation and alignment of CATCH peptides within coassembled nanofiber samples. PITHIRDS-CT measurements report on distance-dependent ^{13}C-^{13}C dipolar couplings between labeled sites, which allows us to distinguish between in-register parallel β-sheets and in-register antiparallel β-sheets. If CATCH+ and CATCH- peptides coassemble into an ideal in-register parallel β-sheet, the ^{13}C-labeled sites would be placed in close proximity, within 0.5 nm of each other (Figure 1A). A proximity of approximately 0.5 nm would result in a strong ^{13}C-^{13}C dipolar coupling and would appear as a strong ^{13}C signal decay in PITHIRDS-CT measurements (black curve in Figure 1C). In contrast, an ideal in-register antiparallel β-sheet structure places the ^{13}C sites greater than 1.0 nm apart (Figure 1B). With a distance greater than 1.0 nm between labeled sites, ^{13}C-^{13}C dipolar couplings are weaker and produce a minor signal decay in PITHIRDS-CT measurements (green curve in Figure 1C). The measured ^{13}C signal decay (Figure 1C) indicated that CATCH peptides do not form ideal in-register parallel β-sheets nor ideal in-register antiparallel β-sheets. Instead, a fraction of peptides arranges in-register parallel with their neighbors, and the remaining assembled peptides must adopt other structures. The exact percentage of in-register parallel β-strands will depend on whether CATCH peptides self-sort by β-sheet structure (fully parallel or fully antiparallel β-sheets) or coexist within a heterogeneous β-sheet (containing parallel and antiparallel nearest-neighbors), which will be considered in subsequent sections.

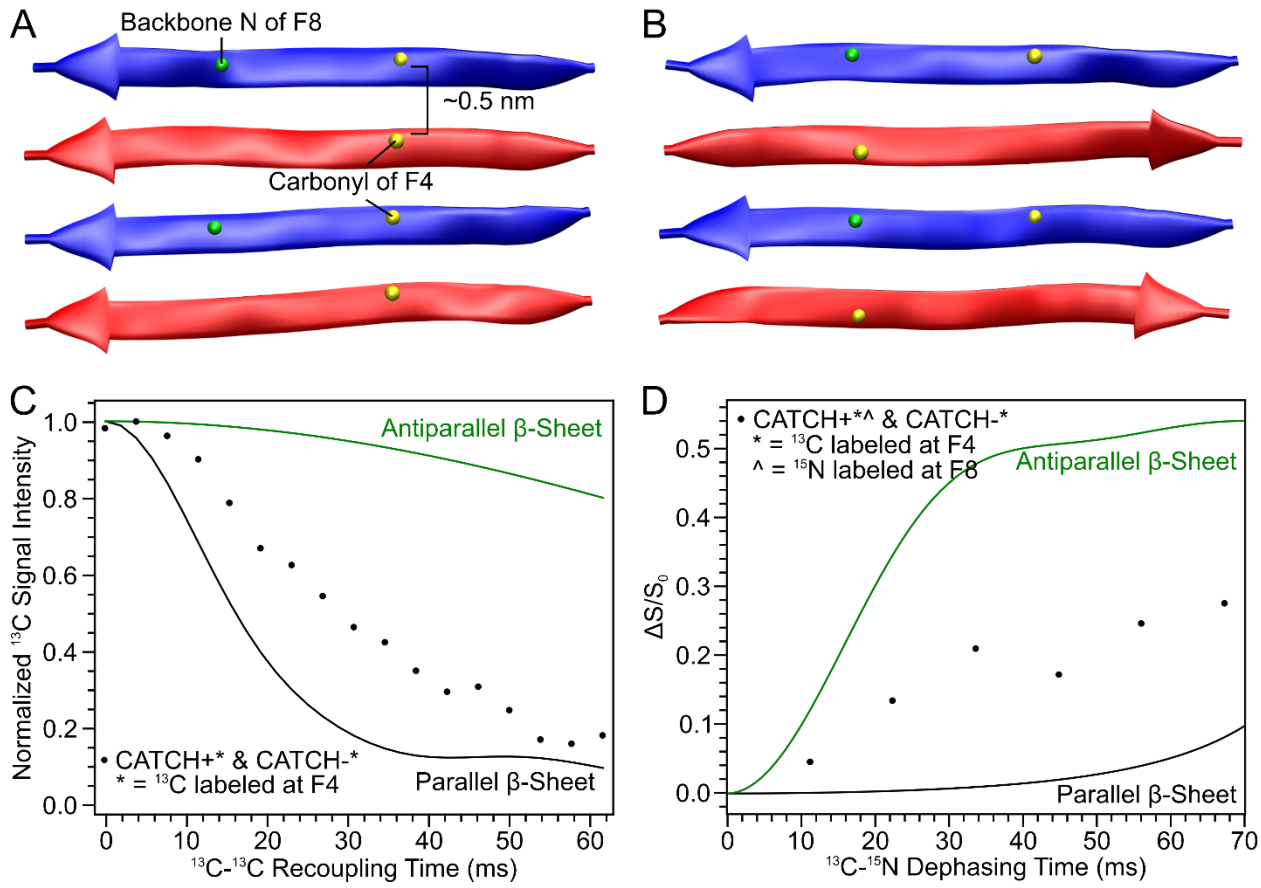


Figure 1. Dipolar recoupling NMR measurements detect in-register parallel and in-register antiparallel β-strand neighbors. A) An all-atom model with β-strands represented as ribbons, illustrating an ideal coassembled in-register parallel β-sheet labeled with ^{13}C at the carbonyl carbon of F4 (yellow spheres) and ^{15}N on the backbone nitrogen of F8 (green spheres). B) An ideal coassembled in-register antiparallel β-sheet with the same labeling scheme as in panel A. C) A ^{13}C-^{13}C PITHIRDS-CT NMR measurement on isotopically labeled coassembled CATCH peptide nanofibers. Decay of signal increases with closer ^{13}C-^{13}C proximity. Smooth black and green curves represent simulated ^{13}C signal decays for an in-register parallel β-sheet structure and in-register antiparallel β-sheet structure, respectively. D) A ^{15}N{^{13}C}REDOR NMR measurement on isotopically labeled coassembled CATCH peptide nanofibers. Increasing REDOR effect ($\Delta S/S_0$) corresponds to closer ^{13}C-^{15}N proximity. Smooth black and green curves represent

simulated ^{13}C dephasing for an in-register parallel β-sheet and in-register antiparallel β-sheet, respectively.

In-register antiparallel β-strand neighbors are also present in coassembled CATCH nanofibers as determined by the $^{15}N\{^{13}C\}$REDOR measurement on isotopically enriched samples. In the same sample as above, the backbone nitrogen at F8 on CATCH+ peptides was isotopically labeled with ^{15}N. Performing $^{15}N\{^{13}C\}$REDOR measurements on these nanofiber samples enables us to differentiate between in-register parallel and in-register antiparallel β-strand neighbors by reporting on distance-dependent dipolar couplings between ^{15}N- and ^{13}C-labeled sites. An ideal in-register parallel β-sheet would place ^{15}N sites greater than 1.0 nm in distance from the nearest ^{13}C sites. Resulting ^{13}C-^{15}N dipolar couplings would be relatively weak and would produce a minor increase in $\Delta S/S_0$ over increasing dephasing time in $^{15}N\{^{13}C\}$REDOR measurements (black curve Figure 1D). On the other hand, an ideal in-register antiparallel β-sheet places ^{15}N-labeled sites within 0.5 nm of ^{13}C-labeled sites on adjacent CATCH-strands. The closer proximity of ^{15}N sites to ^{13}C sites results in stronger ^{13}C-^{15}N dipolar couplings, which corresponds to a large dephasing or increase in $\Delta S/S_0$ value in the $^{15}N\{^{13}C\}$REDOR measurement (green curve Figure 1D). The measurement on coassembled CATCH nanofibers exhibited an intermediate dephasing and were consistent with the partial decay observed in the PITHIRDS-CT experiment. Thus, some CATCH peptides organize into in-register antiparallel, and others organize into in-register parallel with their neighbors. Again, the percentage of in-register antiparallel β-strand neighbors will depend on how departure from either ideal β-sheet structure affects $^{15}N\{^{13}C\}$REDOR dephasing curves. In-register antiparallel β-strand neighbors and in-register parallel β-strand neighbors may coexist within a single heterogeneous nanofiber, or they may self-sort by β-sheet structure into homogeneous parallel nanofibers and homogeneous antiparallel nanofibers.

DMD/PRIME20 simulations predicted heterogeneous nanofibers with a mixture of orientations and registry shifts

Coarse-grained DMD simulations of CATCH+ and CATCH- peptides were used to differentiate between coassembly into structurally heterogeneous nanofibers and self-sorting by β-sheet structure into different structurally homogeneous nanofibers. There are two possible models that rationalize the coexistence of parallel and antiparallel β-strand neighbors within coassembled samples. First, CATCH peptides may self-sort by structure into homogeneous parallel β-sheet nanofibers and homogeneous antiparallel β-sheet nanofibers. In other words, a portion of CATCH peptides nucleate and add onto the fiber ends in a parallel orientation while a separate population of CATCH molecules nucleate and propagate antiparallel β-sheet nanofibers. This coexistence of structurally self-sorted nanofibers has previously been observed in Alzheimer's amyloid β fibrils.[18] Alternatively, each nanofiber may be heterogenous, i.e., composed of a mixture of parallel- and antiparallel-oriented β-strand neighbors. In this model, CATCH peptides would add to the fiber end in parallel or antiparallel alignments without perfect selection of either orientation. We have previously observed this structural heterogeneity in King-Webb nanofibers.[9] For simplicity, we refer to the former case as the homogeneous β-sheet nanofiber model and the latter case as the heterogeneous β-sheet nanofiber model.

Coassembled CATCH peptide nanofibers in DMD/PRIME20 simulations are composed of β-strands arranged parallel and antiparallel as well as in- and out-of-register to nearest neighbors. The structure of coassembled nanofibers was analyzed in coarse-grained simulations of 48 CATCH+ and 48 CATCH- peptides from Shao et al.[11] The arrangement of β-strands in the final snapshots of 10 different simulation runs was determined by evaluating peak positions in interstrand distance distributions; carbon and nitrogen sites used in the interstrand distance calculations were chosen to match ^{13}C- and ^{15}N-labeled sites from dipolar recoupling measurements in Figure 1. Figure 2A shows distance distributions between the carbonyl carbon (CO) sites of F4 on CATCH+ and CATCH- peptides, which

report on parallel β-sheet content. Prominent peaks sitting around 0.49 nm and 0.98 nm match, respectively, to expected distances between nearest neighbor β-strands and next-nearest neighbor β-strands within ideal in-register parallel β-sheets (Supplemental Figure 1A). Minor peaks are also observed between 0.6 nm and 0.9 nm, which indicate the presence of out-of-register β-strand neighbors. Out-of-register β-strand neighbors occur when a peptide misaligns with its nearest neighbors such that the peptide does not satisfy all possible backbone intermolecular hydrogen bonds. Antiparallel β-sheet content was assessed through interstrand distance distributions between CO sites at F4 and backbone N sites at F8 (Figure 2B). Again, these sites were chosen to match the labeling scheme used in the $^{15}N\{^{13}C\}$REDOR NMR measurement. Two major peaks at 0.43 nm and 0.56 nm match expected distances between adjacent β-strands within ideal in-register antiparallel β-sheets (Supplemental Figure 1B). Another prominent peak was observed around 1.0 nm corresponding to the distance from one strand to its next-nearest neighbors. Smaller peaks between 0.6 and 1.0 nm match with interstrand distances expected in out-of-register β-sheets. Simulations predicted the formation of out-of-register β-strand neighbors, which are not explicitly accounted for by experiments shown in Figure 1. The percentage of in-register parallel, in-register antiparallel, and out-of-register β-strand neighbors determined from DMD/PRIME20 simulations are 41.6%, 33.8%, and 24.6%, respectively. As demonstrated in Figure 2C, all three β-strand arrangements coexist within a single coassembled CATCH nanofiber. Coarse-grained simulations provided evidence that CATCH peptides coassemble consistently with a structurally heterogeneous nanofiber model.

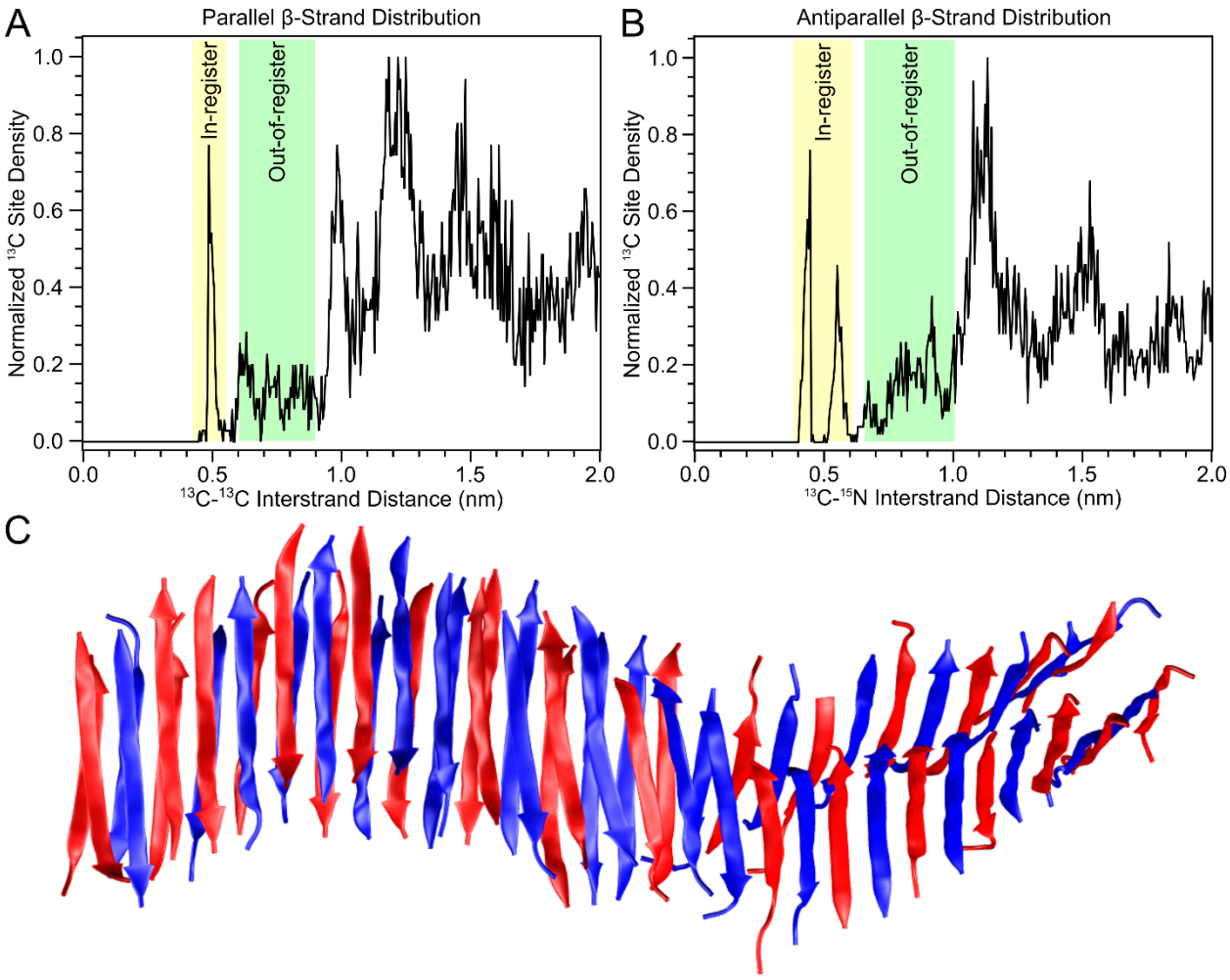


Figure 2. Structural analysis of CATCH nanofibers in DMD/PRIME20 simulations. A) Interstrand distance distribution between carbonyl carbon sites of F4 on CATCH peptides. Highlighted regions represent expected distances from in-register and out-of-register parallel β-sheet models shown in Supplemental Figure 3C. B) Interstrand distance distribution between carbonyl carbon sites of F4 and backbone nitrogen sites of F8 on CATCH peptides. Highlighted regions represent expected distances from in-register and out-of-register antiparallel β-sheet models shown in Supplemental Figure 3C. C) Snapshot of a coassembled CATCH nanofiber from a DMD/PRIME20 simulation. Red strands represent CATCH- molecules and blue strands represent CATCH+ molecules.

Isotope-edited FTIR measurements support a heterogeneous β-sheet nanofiber model

Changes in amide I and amide II peak positions in FTIR measurements on a series of isotopically labeled CATCH nanofibers suggest a heterogeneous β-sheet nanofiber structure containing some β-strands aligned in-register parallel to one another. Introduction of ^{13}C isotopes at carbonyl carbon sites in β-sheet-forming peptides can affect CO stretching, which is observed as a change in the amide I peak position from 1628 cm^{-1} to 1611-1606 cm^{-1}.[29-31] The degree of downshift and the relative intensity of the ^{13}C-labeled amide mode depends on the relative position of the ^{13}C atoms, i.e. parallel vs antiparallel β-sheet structure and the number of strands in the sheet.[29, 31-32] In our previous study, CATCH nanofiber samples were prepared with CATCH+ and CATCH- peptides that were isotopically labeled with ^{13}C at the carbonyl carbon on F6.[11] By placing the ^{13}C atoms at the center of both peptides, the relative position of ^{13}C atoms to one another were insensitive to the alignment of β-strands (parallel vs. antiparallel) as depicted in Figure 3A. That is to say, each ^{13}C-labeled site is within 0.5 nm of ^{13}C sites on neighboring β-strands regardless of being oriented parallel or antiparallel to one another. Center-labeled CATCH nanofibers showed amide I peak splitting into two peaks at 1624 cm^{-1} and 1603 cm^{-1} (purple curve, Figure 3C). In this study, CATCH+ peptides were isotopically labeled with ^{13}C and ^{15}N at the carbonyl carbon of F4 and backbone nitrogen of F8 of CATCH peptides, respectively, and CATCH- peptides were isotopically labeled with ^{13}C at the carbonyl carbon of F4 as shown Figure 1A-B. By placing the ^{13}C and ^{15}N sites "off-center", the peak position and relative intensity of the ^{13}C-labeled amide I mode become sensitive to β-strand alignment.[31] For an in-register parallel β-sheet, distance between ^{13}C sites would be around 0.5 nm as shown in Figure 3B, and peak splitting similar to center-labeled sample would be expected. In contrast, an in-register antiparallel β-sheet positions ^{13}C sites around 1.0 nm in distance from one another, which would result in little to no change in peak position similar to the unlabeled nanofiber sample (black curve, Figure 3C).[32] The amide I peak is slightly downshifted to 1612 cm^{-1} in "off-center"-labeled CATCH peptide nanofibers (green curve, Figure 3C). Attenuation of peak downshifting has previously been observed in ^{13}C dilution experiments on self-assembling β-sheet peptides.[29]

Therefore, the downshifted peak suggests a structurally heterogeneous nanofiber with some β-strands having in-register parallel nearest neighbors and others having in-register antiparallel nearest neighbors as depicted in Figure 3B. If CATCH peptides had self-sorted by nanofiber structure into a mixture of homogeneous parallel nanofibers and homogeneous antiparallel nanofibers, moving the ^{13}C atoms to "off-center" positions would be expected to decrease the intensity of the 1603 cm^{-1} peak rather than upshift the ^{13}C-labeled peak. Similar site-dependent downshifting is seen in the amide II peak around 1550 cm^{-1}, which is affected by NH in-plane bending, CN stretching, and CO in-plane bending.[30] These experimental results are consistent with the prediction of structurally heterogeneous nanofibers from DMD/PRIME20 simulations.

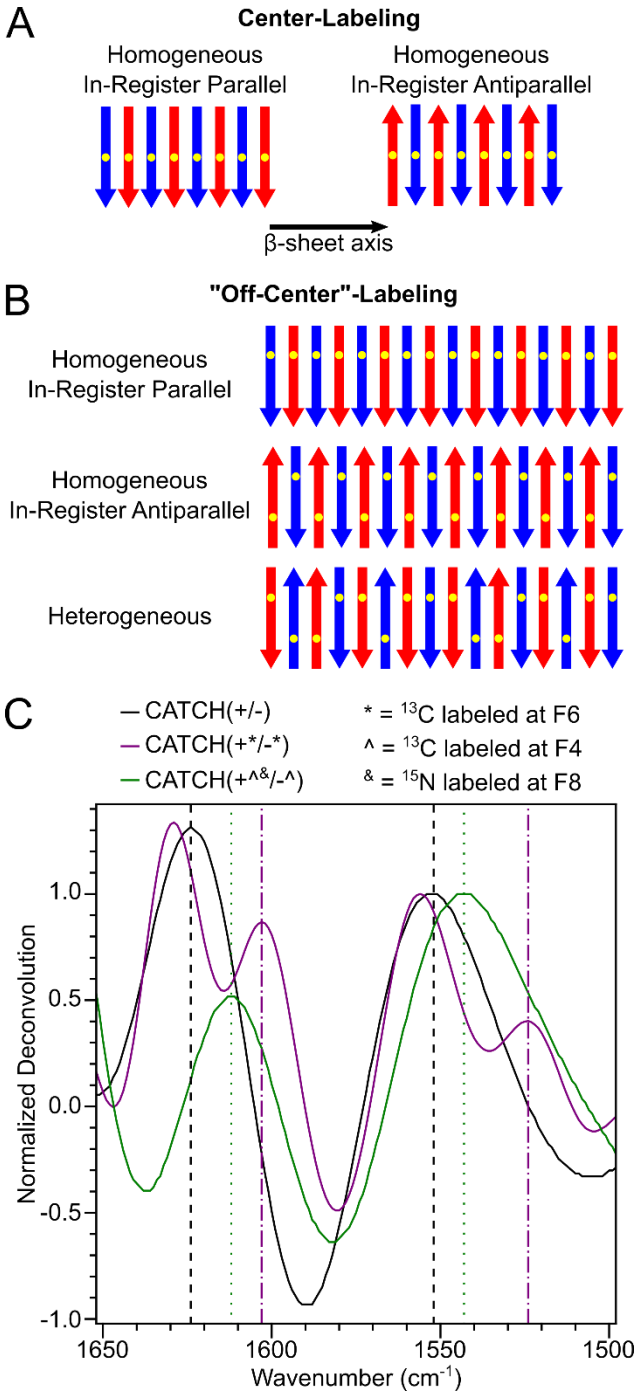


Figure 3. Effect of nanofiber structure on amide I and amide II peaks in isotope-edited FTIR spectra. A) Cartoons depicting the distribution of center-labeled ^{13}C atoms (yellow dots) for in-register parallel and in-register antiparallel β-sheet structures. B) Cartoons illustrating the effect of nanofiber structure on the distribution of "off-center" ^{13}C-labeled sites. B) FTIR spectra of equimolar mixtures of unlabeled

CATCH+ and unlabeled CATCH- (black), center-labeled CATCH+ and center-labeled CATCH- (purple), and "off-center"-labeled CATCH+ and "off-center"-labeled CATCH- (green) at 10 mM in 1X phosphate-buffered saline. Vertical lines show amide I and amide II peak positions for unlabeled (black), center-labeled (purple), and "off-center"-labeled (green) nanofiber samples.

A heterogeneous β-sheet nanofiber model is consistent with dipolar recoupling NMR results. Analysis of PITHIRDS-CT measurements of CATCH peptides slightly favored a heterogeneous β-sheet nanofiber model. We attempted to differentiate between a homogeneous nanofiber model and heterogenous nanofiber model in the PITHIRDS-CT measurement to further support our computational findings. If CATCH peptides self-sorted by β-sheet structure into homogeneous parallel nanofibers and homogeneous antiparallel nanofibers, "off-center" ^{13}C-labeled sites within in-register parallel β-sheet nanofibers would be uniformly spaced 0.5 nm apart along the nanofiber while "off-center" ^{13}C-labeled sites within in-register antiparallel β-sheet nanofibers would be dispersed such that sites are greater than 1.0 nm apart as shown by yellow dots in Figure 3B. In contrast, structurally heterogeneous nanofibers would place the "off-center" ^{13}C-labeled sites in small clusters along both sides of the β-sheet as depicted in Figure 3B. The difference in clustering of ^{13}C sites between the two models leads to different distributions of longer-range dipolar couplings, which produce different-shaped curves in dipolar recoupling NMR measurements (Supplemental Figure 2A). Best fits using simulated curves for the homogeneous nanofiber model (dashed black curve) and heterogeneous nanofiber model (solid red curve) are shown in Figure 4A. Experimental datapoints were fit for up to 47 ms of ^{13}C-^{13}C recoupling time to minimize error from relaxation effects observed at longer recoupling times. Both the heterogeneous nanofiber and the homogeneous nanofiber models appear to fit the data within measurement error. However, the heterogeneous β-sheet nanofiber model leads to a better fit than the homogeneous β-sheet nanofiber model. The best-fit model deviation from the experimental data ($\chi 2$)

for the heterogeneous nanofiber model fit is lower than that for the homogeneous nanofiber model. In conjunction with DMD/PRIME20 simulation predictions and isotope-edited FTIR measurements, quantitative analysis of PITHIRDS-CT measurements supports the structurally heterogeneous nanofiber model. The percentage of in-register parallel β-strand neighbors is 45.0 + 3.1% as calculated using the heterogeneous nanofiber model.

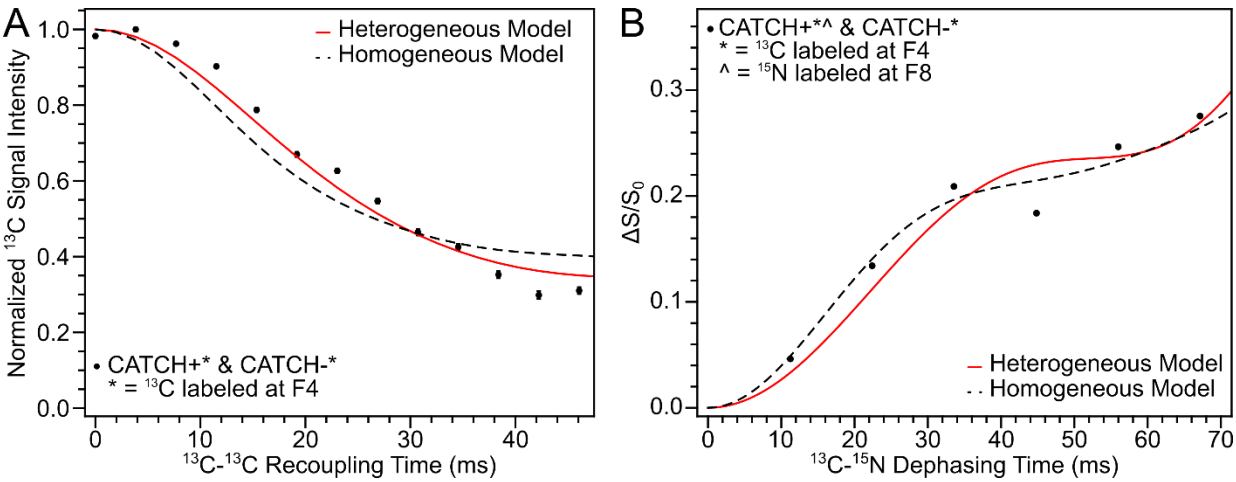


Figure 4. Quantitative analysis of in-register parallel β-sheet content and in-register antiparallel β-sheet content in coassembled CATCH peptide nanofibers. A) A ^{13}C-^{13}C PITHIRDS-CT NMR measurement on a nanofiber sample isotopically labeled at "off-center" residues against simulated curves representing the homogeneous nanofiber model (dashed black curve) and the heterogeneous nanofiber model (solid red curve). Error bars are smaller than the symbol size. B) A $^{15}N\{^{13}C\}$REDOR measurement on a nanofiber sample isotopically labeled at "off-center" residues against simulated curves representing a homogeneous nanofiber model (dashed black curve) and heterogeneous nanofiber model (solid red curve). Error bars are smaller than the symbol size.

Table 1. Comparison of estimated in-register parallel and in-register antiparallel β-strand neighbors from the homogeneous nanofiber model fit and the heterogeneous nanofiber model fit.

	Homogeneous Model Fit	Heterogeneous Model Fit
Estimated percentage of in-register parallel β-strand neighbor (PITHIRDS-CT)	63.6% (3.4%)*	45.0% (1.6%)
Estimated percentage of in-register antiparallel β-strand neighbors (^{15}N{^{13}C}REDOR)	40.1% (1.7%)	21.8% (1.8%)

* Standard error values are shown in parentheses.

The ^{15}N{^{13}C}REDOR measurements did not clearly differentiate between coassembly into structurally heterogeneous nanofibers and self-sorting by β-sheet structure into distinct structurally homogeneous nanofibers. Similar to the PITHIRDS-CT measurement, the shape of the fit depends on the nanofiber model (Supplemental Figure 2B). Figure 4B compares experimental ^{15}N{^{13}C}REDOR datapoints to fits using the homogeneous nanofiber model (dashed black curve) and the heterogenous nanofiber model (solid red curve). Both models provide comparable fits to experimental results, and the standard errors of estimated antiparallel β-strand neighbor content are nearly equivalent. Therefore, ^{15}N{^{13}C}REDOR measurements are unable to differentiate between the structurally homogeneous nanofiber model and structurally heterogeneous nanofiber model. We interpret our ^{15}N{^{13}C}REDOR measurements using the structurally heterogeneous nanofiber model given previously mentioned supporting evidence for this model. The percentage of in-register antiparallel β-strands was initially estimated to be 21.8%. However, we had to correct the calculated in-register antiparallel β-strand neighbors percentage for the presence of self-associated peptide neighbors, which reduce the observable number ^{13}C-^{15}N dipolar couplings and the amount of observed dephasing. ^{15}N- labeled sites are only found on CATCH+ peptides. Thus, CATCH-:CATCH- neighbors would reduce the number of ^{15}N sites near ^{13}C sites and the size of antiparallel β-strand clusters (Supplemental Figure 3). As detailed in the next paragraph, approximately 25.2% of CATCH- peptides form CATCH-:CATCH- neighbors. Therefore, the calculated value of in-register antiparallel β-strand neighbors was expected to be reduced by 12.5% (Supplemental Figure 4). The actual percentage of in-register antiparallel β-strand neighbors was expected to be 24.9 $\pm$ 4.1%.

A fraction of in-register parallel β-strands formed like-peptide nearest neighbors (CATCH-:CATCH- and CATCH+:CATCH+ neighbors), which further demonstrated the degree of structural heterogeneity observed in coassembled CATCH nanofibers. Nanofiber samples were isotopically diluted by preparing coassembled mixtures with either CATCH- or CATCH+ peptides isotopically labeled with ^{13}C on the carbonyl carbon of F4. By labeling in this manner, the measurement of CATCH-:CATCH- and CATCH+:CATCH+ neighbors becomes sensitive to in-register parallel β-strand neighbors as illustrated in Figure 5A. PITHIRDS-CT measurements in Figure 5B showed a minor ^{13}C signal decay indicating some CATCH-:CATCH- and CATCH+:CATCH+ neighbors arrange in-register parallel to one another. The percentages of in-register parallel CATCH-:CATCH- and CATCH+:CATCH+ neighbors were 25.2 $\pm$ 3.4% and 21.1 $\pm$ 1.6%, respectively. In a previous study, we estimated the percentage of CATCH-:CATCH- and CATCH+:CATCH+ neighbors to range from 9.4% to 32.5% when samples were labeled at the center (insensitive to strand alignment).[11] The percentage of in-register antiparallel self-associated pairs is likely to be the same. It is important to note that relaxation effects and contributions from naturally abundant ^{13}C play a greater role in PITHIRDS-CT measurements on isotopically diluted nanofibers. Simulated PITHIRDS-CT curves do not adequately account for such effects and are also limited by the maximum possible simulated spin-system size.

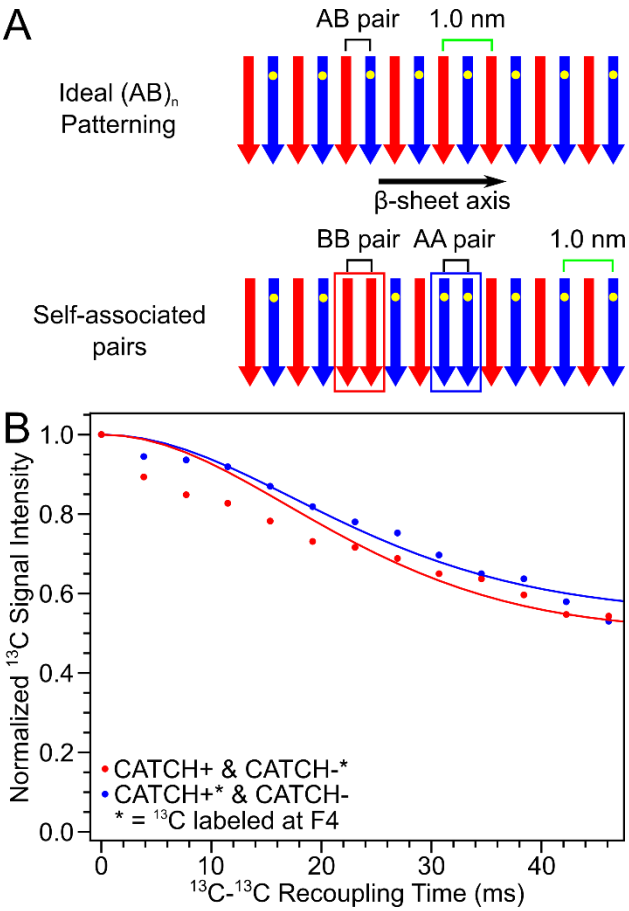


Figure 5. Quantitative analysis of like-peptide neighbors forming in-register parallel β-sheets within coassembled CATCH nanofibers. A) Cartoon illustrating the effect of like-peptide neighbors on isotopically diluted PITHIRDS-CT measurements. B) ^{13}C-^{13}C PITHIRDS-CT NMR measurements on isotopically diluted CATCH nanofibers. Solid curves represent simulations based on the heterogeneous nanofiber model. Error bars are smaller than the symbol size.

Estimation of out-of-register β-strand neighbors accounted for the remaining percentage of coassembled peptides

Some CATCH peptides must align out-of-register with neighboring strands to account for the remaining percentage of β-strands. In-register parallel β-strand neighbors account for 45.0% of peptides while in-register antiparallel β-strand neighbors account for 24.9% of peptides (summarized in Table 2),

which suggests a slight preference to arrange in-register parallel to neighboring β-strands. All isotopically labeled peptides adopt a β-strand conformation as indicated by comparing the chemical shift value of ^{13}C-labeled carbonyl carbon sites at F4 on CATCH+ and CATCH- to equivalent carbon sites in peptides adopting random coil conformations (Supplemental Figure 5).[33] Therefore, the remaining 30.1% of peptides must adopt a β-strand arrangement not accounted for by our isotopic labeling scheme shown in Figure 1A and 1B. Out-of-register β-strand neighbors observed in DMD/PRIME20 simulations likely account for the remaining peptides within nanofiber samples. Registry shifts would place isotopically labeled sites greater than 0.83 nm apart from one another and would not contribute significantly to PITHIRDS-CT or ^{15}N{^{13}C}REDOR measurements. Experimentally measured percentages also matched values calculated from DMD/PRIME20 simulations further corroborating the idea that CATCH peptides can arrange in-register antiparallel, in-register parallel, and out-of-register to nearest neighbors within a single heterogeneous nanofiber.

Table 2. Summary of experimentally and computationally estimated percentages of neighboring β-strand arrangements.

	Experimental	Computational
In-register Antiparallel β-strand Neighbors	24.9 $\pm$ 4.1%	33.8%
In-register Parallel β-strand Neighbors	45.0 $\pm$ 3.1%	41.6%
Out-of-register β-strand Neighbors	30.1 $\pm$ 7.2%	24.6%

Structural heterogeneity may influence nanofiber bundling

CATCH peptide nanofibers exhibit little lateral association compared to King-Webb nanofibers, which may result from differences in nanofiber structure. Figure 6A shows a TEM image of nanofibers formed in equimolar mixtures of CATCH+ and CATCH- peptides at 100 μM in 1X phosphate-buffered saline (PBS).[11] CATCH peptide nanofibers form a wiry mat of thin nanofibers with little nanofiber bundling. In contrast, Figure 6B shows a TEM image of King-Webb nanofibers at 10 mM in 10X PBS, which form large,

striated nanofiber bundles.[9] Morphological differences may result from differences in the degree of structural heterogeneity in CATCH nanofibers and King-Webb nanofibers. The higher degree of heterogeneity could prevent CATCH peptides from multiple β-sheet layers from forming. The high percentage of out-of-register β-strands neighbors could also provide an irregular interface that prevents nanofibers from bundling.

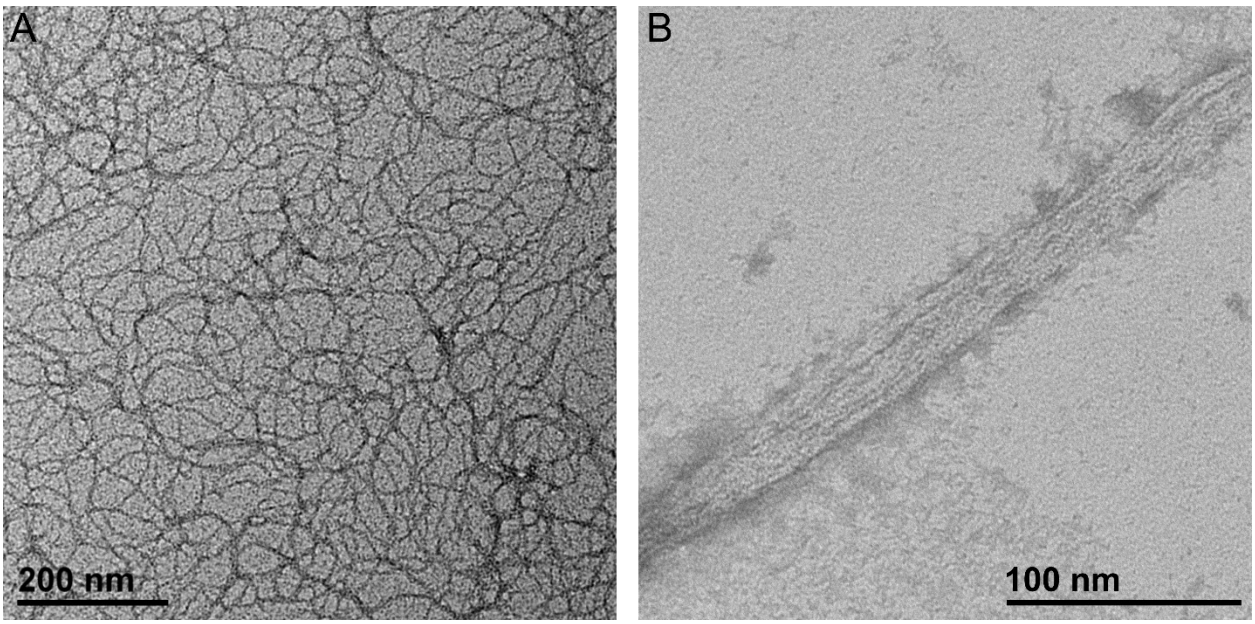


Figure 6. Comparison of nanofiber morphology in CATCH and King-Webb peptide systems. A) Transmission electron micrograph of equimolar mixtures of CATCH+ and CATCH- peptides at 100 μM in 1X phosphate-buffered saline. Copyright 2020 National Academy of Sciences. B) Transmission electron micrograph of equimolar mixtures of KW+ and KW- peptides at 10 mM in 10X phosphate-buffered saline. Reproduced from Ref. XX with permission from The Royal Society of Chemistry

Discussion

CATCH+ and CATCH- peptides coassemble into heterogeneous β-sheet nanofibers with parallel and antiparallel β-strand arrangements similar to King-Webb peptide coassemblies. Our previous study on

coassembled King-Webb peptide nanofibers observed the formation of heterogeneous β-sheets with 31.7% of peptides being in-register parallel to nearest neighbors and 56.4% of peptides being in-register antiparallel.[9] Mixtures of CATCH+ and CATCH- peptides behave in a similar manner by organizing into heterogeneous β-sheet nanofibers without a strong preference for a particular arrangement. However, CATCH peptides slightly favor a parallel orientation while King-Webb peptides had a slight preference for an antiparallel orientation. The coexistence of parallel and antiparallel β-strand arrangements within a nanofiber is distinct from polymorphism observed in self-assembling peptides. Multiple different structures have also been observed in self-assembled Aβ40 nanofiber samples, but each nanofiber had a single homogeneous structure as demonstrated from series of seeded nanofiber assembly experiments.[18] Similarly, DMD/PRIME20 simulations have previously observed structurally homogeneous nanofibers in some amyloids, such as Aβ(16-22) and tau protein fragments at higher simulation temperatures.[34-35] Prion protein peptides PrP(120-144) preferentially formed in-register parallel β-sheet structures in simulations.[36] In contrast, DMD/PRIME20 simulations of short designer hexapeptides formed heterogeneous nanofibers with a lack of preference towards antiparallel or parallel β-strand alignment.[37] Formation of heterogeneous nanofibers in CATCH peptide mixtures in simulations and experiments suggest structural heterogeneity results in part from the peptide sequences.

Amino acid sequence patterning and the role of electrostatic interactions during the coassembly process contribute to the structural heterogeneity observed in coassembled CATCH peptide nanofibers. Impurities remaining from peptide synthesis may also contribute to the formation of structurally heterogeneous nanofibers. However, the degree of structural heterogeneity is much greater than the percentage of peptide synthesis impurities (between 1- 4%). Therefore, the formation of heterogeneous nanofibers most likely arises from the CATCH+ and CATCH- peptide sequence design. Charge-complementary peptide pairs are likely to exhibit much faster assembly kinetics than self-assembling

peptides due to increased electrostatic attraction in addition to hydrophobic collapse; charge-complementary peptide mixtures can gel within minutes. Thus, structurally heterogeneous nanofibers may result from kinetically trapped peptides. Highly charged peptides, like CATCH+ and CATCH-, likely have a higher propensity to form kinetically trapped structures than peptides with an net molecular charges closer to neutral, like KLVFWAK and ELVFWAE;[38] increasing peptide charge increased coassembly kinetics in charge-matched variants of the CATCH peptides.[12] The contribution of electrostatic interactions may also overshadow other energetic contributions, such as hydrogen bonding, van der Wal's forces, and sidechain-sidechain complementarity. As a result, there may be little difference between the energy associated with adding a CATCH peptide to the fiber end in either an antiparallel or parallel orientation. The palindromic nature of the CATCH+ and CATCH- sequences likely adds to structural heterogeneity in nanofibers as well. Unmodified peptide termini, like in the King-Webb system, also have net charge, which would favor the formation of in-register antiparallel β-strand neighbors. CATCH peptides have acetylated and amidated termini, which may reduce their propensity to arrange in-register antiparallel β-strand neighbors. In contrast to charge-complementary peptides, repetitive self-assembling peptides, such as RADA16-I and MAX1, have been shown to form highly homogeneous nanofibers.[16, 19] Future charge-complementary designs may need to place charged residues on either end of the peptide to generate a bias towards a particular orientation. Increasing the diversity of amino acids within the hydrophobic core may also bias coassembly towards selecting a single structure. Coassembling peptides will require engineering of the energy landscape during peptide coassembly to form highly homogeneous nanofiber structures.

CATCH peptide nanofibers have a higher proportion of out-of-register β-strand neighbors than King-Webb nanofibers. Out-of-register peptides are registry shifted perpendicular to the fiber axis; potential hydrogen bonds are sacrificed in this configuration. Approximately 30.1% of CATCH peptides are out-of-register with their nearest neighbors compared to 11.9% of King-Webb peptides being out-of-register.

Out-of-register peptides may contribute to differences in morphological features between CATCH nanofibers and King-Webb nanofibers. Long, tortuous nanofibers with no fiber bundling are observed in TEM images of CATCH mixtures whereas large, striated fiber bundles have been observed in TEM images of King-Webb mixtures. An increase in out-of-register β-strand neighbors may inhibit the bundling of nanofibers though we note that fiber bundling could also result from artifacts in TEM sample preparation.[1, 9-11] A larger percentage of out-of-register β-strand neighbors may relate to differences in amino acid sequence patterning between CATCH and King-Webb peptides. CATCH+ and CATCH- peptides are mismatched in terms of overall peptide charge, and their hydrophilic faces are uniformly patterned with like-charged residues. Consequently, the 4 lysine residues on CATCH+ peptides can pair up with the 6 glutamic acid residues on the CATCH- peptide in multiple possible arrangements. Though acetylation and amidation increases the number of sacrificed hydrogen bonds by 1, the removal of charged end groups from CATCH peptide termini likely promote out-of-register β-strand arrangements. Contributions from electrostatic interactions would outweigh the loss from favorable hydrogen bonding, van der Waals, and dipole-dipole interactions when aligning out-of-register. King-Webb peptides have hydrophilic faces which alternate between positively and negatively charged residue blocks and match in terms of overall peptide charge. This patterning with alternating blocks of charged residues in King-Webb peptides may promote the in-register alignment of β-strands to nearest neighbors. The role of electrostatic interactions in influencing β-strand registry has previously been observed in self-assembling peptides. RADA16-I is known to form parallel β-sheet nanofibers with a two-residue registry shift, which aligns positively charged arginine and negatively charged aspartic acid residues.[19] Pairing of oppositely charged residues produce the greatest stabilizing interactions within β-sheets found in proteins.[39] A refined understanding of sidechain-sidechain interactions will aid in the control β-strand registry in coassembling peptides.

By combining computational simulations and experimental measurements, the structural heterogeneity within CATCH nanofibers was resolved at a molecular level. Coarse-grained simulations (DMD/PRIME20) predicted the arrangement of β-strands in both parallel and antiparallel orientations indicating a structurally heterogeneous nanofiber. FTIR spectra on isotopically labeled nanofiber samples showed site-dependent changes, which support the heterogeneous nanofiber model. Nuclear spin simulations combined with dipolar recoupling NMR measurements further supported computational predictions. DMD/PRIME20 simulations also predicted the formation of nanofibers with some β-strands lying out-of-register to adjacent strands. Out-of-register β-strands were not explicitly accounted for in the isotopic labeling scheme employed in our dipolar recoupling NMR measurements. Experimentally measured percentages of in-register parallel and in-register antiparallel β-strand neighbors qualitatively agreed with values estimated from DMD/PRIME20 simulations, which provided further evidence of out-of-register β-strands within nanofiber samples. Previous work examining structural heterogeneity within King-Webb nanofibers and like-peptide neighbors within CATCH nanofibers also showed consistency between computational and experimental assessments.[9, 11] These results demonstrate DMD/PRIME20 simulations' ability to capture experimentally relevant structural features in coassembling peptides despite vast differences in timescale. Overall, the enhanced resolution of our data analysis by combining computational tools with solid-state NMR and isotope-edited FTIR measurements has provided deeper insights into structural features observed in coassembled CATCH nanofibers.

Conclusions

Existing charge-complementary peptides have successfully demonstrated coassembly, yet precise programming of β-sheet nanofiber structure has not been realized. Charge-complementary peptides form polymorphic coassembled nanofibers and do not appear to favor any single structure during

assembly. Solid-state NMR measurements and computational simulations detected CATCH peptides that aligned parallel and antiparallel to nearest neighbors within coassembled β-sheet nanofibers. The mixture of β-strand orientations may result from the palindromic amino acid sequence patterning of the CATCH peptides. CATCH+ and CATCH- peptides mostly aligned in-register with one another, which maximizes the number of hydrogen bonds formed with nearest neighbors, but approximately 30.1% of β-strands were out-of-register to adjacent strands. Misalignment of CATCH peptides with nearest neighbors may sacrifice potential hydrogen bonds in favor of electrostatic interactions. Structural heterogeneity observed in coassembled CATCH nanofibers was consistent with our previous findings on coassembling King-Webb peptides despite differences in their amino acid sequence patterning. Our findings also demonstrated the benefits of a combined computational and experimental approach to elucidate molecular-level details within coassembled nanostructures. DMD/PRIME20 simulations predicted the formation of structurally heterogeneous CATCH nanofibers rather than multiple distinct structurally homogenous nanofibers. DMD/PRIME20 simulations were also able to identify out-of-register β-strands within coassembled CATCH nanofibers, which were consistent with experimental NMR results. Future coassembling β-sheet designs will need to consider more complex sequence patterns that leverage sidechain-sidechain interactions to encode precise nanostructures.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at [insert link].

Interstrand distance distributions of ideal in-register parallel and in-register antiparallel β-sheets (Supplemental Figure 1). Comparison of homogeneous nanofibers and heterogeneous nanofibers at varying parallel and antiparallel β-sheet content (Supplemental Figure 2). Effect of like-peptide nearest

neighbors on cluster size distributions of in-register antiparallel β-strands (Supplemental Figure 3). Effect of CATCH-:CATCH- neighbors on measured in-register antiparallel β-sheet content (Supplemental Figure 4). 13C CPMAS spectra of isotopically labeled CATCH nanofibers (Supplemental Figure 5). Examples of all-atom in-register parallel and in-register antiparallel β-sheet nanofibers (Supplemental Figure 6). (PDF)

AUTHOR INFORMATION

Corresponding Author

Anant K. Paravastu - School of Chemical and Biomolecular Engineering, Georgia Institute of Technology, Atlanta, GA 30332, United States; Email: anant.paravastu@chbe.gatech.edu

ACKNOWLEDGMENT

This research was supported by funds from the National Science Foundation Grants CBET-1743432 and OAC-1931430. The authors acknowledge the use of instruments in the NMR Center at the Georgia Institute of Technology.

REFERENCES

1. Seroski, D. T.; Restuccia, A.; Sorrentino, A. D.; Knox, K. R.; Hagen, S. J.; Hudalla, G. A., Co-Assembly Tags Based on Charge Complementarity (CATCH) for Installing Functional Protein Ligands into Supramolecular Biomaterials. *Cell. Mol. Bioeng.* **2016**, *9* (3), 335-350.

2. Hudalla, G. A.; Sun, T.; Gasiorowski, J. Z.; Han, H.; Tian, Y. F.; Chong, A. S.; Collier, J. H., Gradated assembly of multiple proteins into supramolecular nanomaterials. *Nature Materials* **2014**, *13* (8), 829-836.

3. Chang, R.; Zou, Q.; Xing, R.; Yan, X., Peptide-Based Supramolecular Nanodrugs as a New Generation of Therapeutic Toolboxes against Cancer. *Advanced Therapeutics* **2019**, *2* (8), 1900048.

4. Ouberai, M. M.; Dos Santos, A. L. G.; Kinna, S.; Madalli, S.; Hornigold, D. C.; Baker, D.; Naylor, J.; Sheldrake, L.; Corkill, D. J.; Hood, J.; Vicini, P.; Uddin, S.; Bishop, S.; Varley, P. G.; Welland, M. E., Controlling the bioactivity of a peptide hormone in vivo by reversible self-assembly. *Nature Communications* **2017**, *8* (1), 1026.

5. Eskandari, S.; Guerin, T.; Toth, I.; Stephenson, R. J., Recent advances in self-assembled peptides: Implications for targeted drug delivery and vaccine engineering. *Adv. Drug Del. Rev.* **2017**, *110-111*, 169-187.

6. Tostanoski, L. H.; Jewell, C. M., Engineering self-assembled materials to study and direct immune function. *Adv. Drug Del. Rev.* **2017**, *114*, 60-78.

7. Sun, T.; Han, H.; Hudalla, G. A.; Wen, Y.; Pompano, R. R.; Collier, J. H., Thermal stability of self-assembled peptide vaccine materials. *Acta Biomater.* **2016**, *30*, 62-71.

8. Chen, J.; Pompano, R. R.; Santiago, F. W.; Maillat, L.; Sciammas, R.; Sun, T.; Han, H.; Topham, D. J.; Chong, A. S.; Collier, J. H., The use of self-adjuvanting nanofiber vaccines to elicit high-affinity B cell responses to peptide antigens without inflammation. *Biomaterials* **2013**, *34* (34), 8776-8785.

9. Wong, K. M.; Wang, Y.; Seroski, D. T.; Larkin, G. E.; Mehta, A. K.; Hudalla, G. A.; Hall, C. K.; Paravastu, A. K., Molecular complementarity and structural heterogeneity within co-assembled peptide β-sheet nanofibers. *Nanoscale* **2020**, *12* (7), 4506-4518.

10. King, P. J.; Giovanna Lizio, M.; Booth, A.; Collins, R. F.; Gough, J. E.; Miller, A. F.; Webb, S. J., A modular self-assembly approach to functionalised beta-sheet peptide hydrogel biomaterials. *Soft Matter* **2016**, *12* (6), 1915-23.

11. Shao, Q.; Wong, K. M.; Seroski, D. T.; Wang, Y.; Liu, R.; Paravastu, A. K.; Hudalla, G. A.; Hall, C. K., Anatomy of a selectively coassembled β-sheet peptide nanofiber. *Proceedings of the National Academy of Sciences* **2020**, *117* (9), 4710-4717.

12. Seroski, D. T.; Dong, X.; Wong, K. M.; Liu, R.; Shao, Q.; Paravastu, A. K.; Hall, C. K.; Hudalla, G. A., Charge guides pathway selection in β-sheet fibrillizing peptide co-assembly. *Communications Chemistry* **2020**, *3* (1), 172.

13. Miller, Y.; Ma, B.; Nussinov, R., Polymorphism of Alzheimer's Aβ17-42 (p3) Oligomers: The Importance of the Turn Location and Its Conformation. *Biophys. J.* **2009**, *97* (4), 1168-1177.

14. Ma, B.; Nussinov, R., Polymorphic Triple β-Sheet Structures Contribute to Amide Hydrogen/Deuterium (H/D) Exchange Protection in the Alzheimer Amyloid β42 Peptide. *J. Biol. Chem.* **2011**, *286* (39), 34244-34253.

15. Miller, Y.; Ma, B.; Nussinov, R., Polymorphism in Self-Assembly of Peptide-Based β-Hairpin Contributes to Network Morphology and Hydrogel Mechanical Rigidity. *The Journal of Physical Chemistry B* **2015**, *119* (2), 482-490.

16. Nagy-Smith, K.; Moore, E.; Schneider, J.; Tycko, R., Molecular structure of monomorphic peptide fibrils within a kinetically trapped hydrogel network. *Proceedings of the National Academy of Sciences* **2015**, *112* (32), 9816-9821.

17. Meier, B. H.; Riek, R.; Böckmann, A., Emerging Structural Understanding of Amyloid Fibrils by Solid-State NMR. *Trends Biochem. Sci.* **2017**, *42* (10), 777-787.

18. Tycko, R., Physical and structural basis for polymorphism in amyloid fibrils. *Protein Sci.* **2014**, *23* (11), 1528-1539.

19. Cormier, A. R.; Pang, X.; Zimmerman, M. I.; Zhou, H.-X.; Paravastu, A. K., Molecular structure of RADA16-I designer self-assembling peptide nanofibers. *ACS Nano* **2013**, *7* (9), 7562-7572.

20. Tycko, R., Symmetry-based constant-time homonuclear dipolar recoupling in solid state NMR. *J. Chem. Phys.* **2007**, *126* (6), 064506-064506.

21. Gullion, T.; Baker, D. B.; Conradi, M. S., New, compensated Carr-Purcell sequences. *Journal of Magnetic Resonance (1969)* **1990**, *89* (3), 479-484.

22. Sinha, N.; Schmidt-Rohr, K.; Hong, M., Compensation for pulse imperfections in rotational-echo double-resonance NMR by composite pulses and EXORCYCLE. *J. Magn. Reson.* **2004**, *168* (2), 358-365.

23. Rance, M.; Byrd, R. A., Obtaining high-fidelity spin-12 powder spectra in anisotropic media: Phase-cycled Hahn echo spectroscopy. *Journal of Magnetic Resonance (1969)* **1983**, *52* (2), 221-240.

24. Fung, B. M.; Khitrin, A. K.; Ermolaev, K., An Improved Broadband Decoupling Sequence for Liquid Crystals and Solids. *J. Magn. Reson.* **2000**, *142* (1), 97-101.

25. Veshtort, M.; Griffin, R. G., SPINEVOLUTION: A powerful tool for the simulation of solid and liquid state NMR experiments. *J. Magn. Reson.* **2006**, *178* (2), 248-282.

26. Humphrey, W.; Dalke, A.; Schulten, K., VMD: Visual molecular dynamics. *J. Mol. Graphics* **1996**, *14* (1), 33-38.

27. Phillips, J. C.; Braun, R.; Wang, W.; Gumbart, J.; Tajkhorshid, E.; Villa, E.; Chipot, C.; Skeel, R. D.; Kalé, L.; Schulten, K., Scalable molecular dynamics with NAMD. *J. Comput. Chem.* **2005**, *26* (16), 1781-1802.

28. Nelson, M. T.; Humphrey, W.; Gursoy, A.; Dalke, A.; Kalé, L. V.; Skeel, R. D.; Schulten, K., NAMD: a Parallel, Object-Oriented Molecular Dynamics Program. *The International Journal of Supercomputer Applications and High Performance Computing* **1996**, *10* (4), 251-268.

29. Moran, S. D.; Zanni, M. T., How to Get Insight into Amyloid Structure and Formation from Infrared Spectroscopy. *The Journal of Physical Chemistry Letters* **2014**, *5* (11), 1984-1993.

30. Tatulian, S. A., Structural characterization of membrane proteins and peptides by FTIR and ATR-FTIR spectroscopy. *Methods Mol. Biol.* **2013**, *974*, 177-218.

31. Decatur, S. M., Elucidation of Residue-Level Structure and Dynamics of Polypeptides via Isotope-Edited Infrared Spectroscopy. *Acc. Chem. Res.* **2006**, *39* (3), 169-175.

32. Bouř, P.; Keiderling, T. A., Ab Initio Modeling of Amide I Coupling in Antiparallel β-Sheets and the Effect of 13C Isotopic Labeling on Infrared Spectra. *The Journal of Physical Chemistry B* **2005**, *109* (11), 5348-5357.

33. Wishart, D. S., Interpreting protein chemical shift data. *Prog Nucl Mag Res Sp* **2011**, *58* (1-2), 62-87.

34. Cheon, M.; Chang, I.; Hall, C. K., Influence of temperature on formation of perfect tau fragment fibrils using PRIME20/DMD simulations. *Protein Sci.* **2012**, *21* (10), 1514-1527.

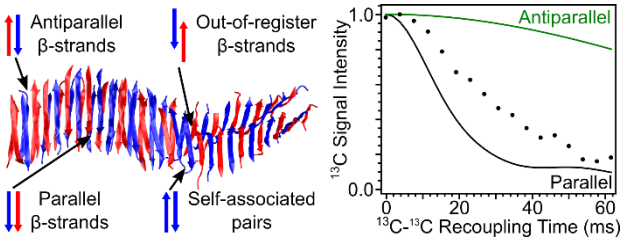
35. Cheon, M.; Chang, I.; Hall, C. K., Spontaneous formation of twisted Aβ(16-22) fibrils in large-scale molecular-dynamics simulations. *Biophys. J.* **2011**, *101* (10), 2493-2501.

36. Wang, Y.; Shao, Q.; Hall, C. K., N-terminal Prion Protein Peptides (PrP(120–144)) Form Parallel In-register β-Sheets via Multiple Nucleation-dependent Pathways. *J. Biol. Chem.* **2016**, *291* (42), 22093-22105.

37. Wagoner, V. A.; Cheon, M.; Chang, I.; Hall, C. K., Fibrillization propensity for short designed hexapeptides predicted by computer simulation. *J. Mol. Biol.* **2012**, *416* (4), 598-609.

38. Candreva, J.; Chau, E.; Aoraha, E.; Nanda, V.; Kim, J. R., Hetero-assembly of a dual β-amyloid variant peptide system. *Chem. Commun.* **2018**, *54* (49), 6380-6383.

39. Merkel, J. S.; Sturtevant, J. M.; Regan, L., Sidechain interactions in parallel β sheets: the energetics of cross-strand pairings. *Structure* **1999**, *7* (11), 1333-1343.


Antiparallel
β-strands
Out-of-register
β-strands
Parallel
β-strands
Self-associated
pairs
1.0
0.0
^{13}C Signal Intensity
Antiparallel
Parallel
0
20
40
60
^{13}C-^{13}C Recoupling Time (ms)

TOC Figure