Engineering β-sheet peptide coassemblies for biomaterial applications

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Abstract

Peptide coassembly, wherein at least two different peptides interact to form multicomponent nanostructures, is an attractive approach for generating functional biomaterials. Current efforts seek to design pairs of peptides, A and B, that form nanostructures (e.g., β-sheets with ABABA-type β-strand patterning) while resisting self-assembly (e.g., AAAAA-type or BBBBB-type β-sheets). To confer coassembly behavior, most existing designs have been based on highly charged variants of known self-assembling peptides; like-charge repulsion limits self-assembly while opposite-charge attraction promotes coassembly. Recent analyses using solid-state NMR and coarse-grained simulations reveal that preconceived notions of structure and molecular organization are not always correct. This perspective highlights recent advances and key challenges to understanding and controlling peptide coassembly.

Overview

In coassembly, at least two different peptide molecules spontaneously co-organize in solution to form multicomponent nanostructures. Peptides are polymers of typically fewer than 30 amino acids, small compared to proteins, but large enough to create limited numbers of secondary structural domains (β-strands or α-helices). This perspective focuses on β-strand coassembly, not self-assembly (nanostructure formation by a single peptide) or α-helical assembly; the latter topics are reviewed elsewhere.[1-2] Early examples of human-designed coassembling β-strand peptides consist of a self-assembling peptide (A) and a modified version (A*) having a functional group appended on either terminus. This type of peptide coassembly closely resembles self-assembly and is reviewed elsewhere.[2-3] This article focuses on coassembly that

requires the simultaneous presence of peptides with distinct amino acid sequences (A and B). Following the nomenclature of Gazit, we call this phenomenon "cooperative coassembly" because it requires interactions between distinct peptides (A/B), interactions that are clearly distinguished from interactions between molecules of the same peptide (A/A or B/B).[4] In contrast to proteins, which do coassemble in nature (e.g., chaplins and rodlins coassemble in bacteria,[5] and some virus capsid structures result from co-organized scaffolding and coat proteins[6]), peptides are not known to coassemble in nature. Nevertheless, several synthetic coassembling peptide pairs have been discovered in recent years, opening a new frontier in biomaterial design.

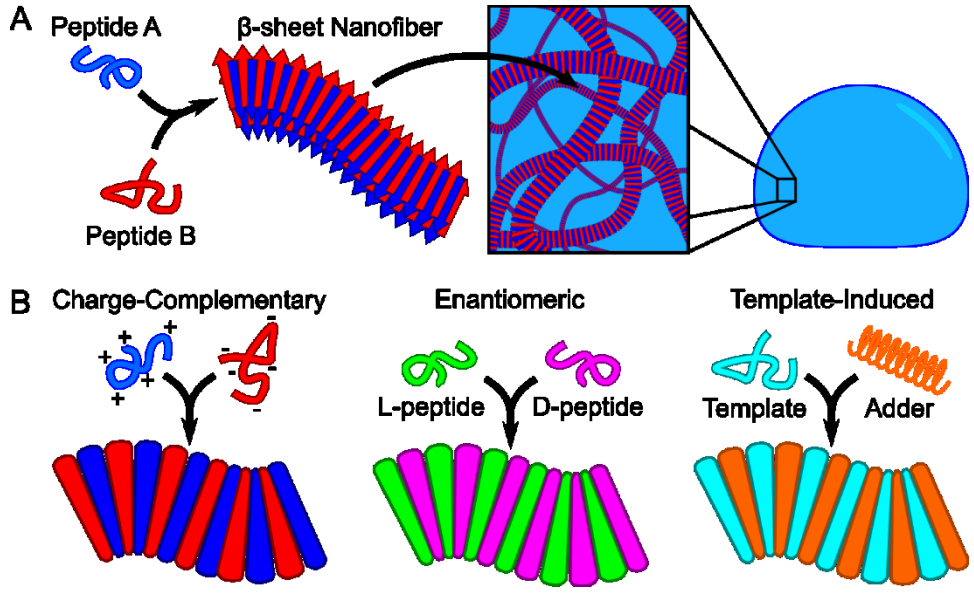

Figure 1. Overview of coassembling β-sheet peptides. A) Schematic illustrating an ideal cooperative coassembly of peptides A and B into β-sheet-rich nanofibers that physically entangle to form a hydrogel network. B) Cartoons representing the 3 categories of coassembly.

To guide our discussion of cooperative coassembly, we define the following ideal molecular-level characteristics (see Figure 1). We use the term "ideal" the way that it is used in

thermodynamics (e.g., ideal gas, ideal solution), to establish a basis for description. Properties of ideal β-strand coassemblies may or may not be desirable for material applications. An ideal coassembly would have a homogeneous molecular structure, with each peptide molecule in a well-defined conformation and β-sheets composed of A/B alternating β-strands with specific inter-strand organizations (e.g., antiparallel). In this configuration, nearest neighboring β-strands in a β-sheet are always complementary peptide molecules. Another characteristic of an ideal coassembly is selectivity: if peptides A and B selectively coassemble through specific interactions, then another peptide C cannot integrate into the coassembled structure. Selective coassembly would make it possible for multiple coassembling pairs to assemble independently and orthogonally (independent mechanisms even if in the same solution) from one another. For example, in an ideal mixture of peptides A, B, C, and D, peptide A would selectively coassemble with peptide B, and peptide C would selectively coassemble with peptide D, but A/C, A/D, B/C, and B/D coassemblies would not form. Deviation from an ideal cooperative coassembly would indicate a lack of specificity. We suggest that achieving a de novo coassembling design with these ideal characteristics is a fundamental challenge in biophysics.

In this perspective, we summarize recent progress in the application, design, and characterization of coassembling β-sheet peptides, emphasizing charge-complementary pairs. We begin by highlighting studies utilizing coassembling β-sheet peptides as functional biomaterials in biotechnological applications and studies demonstrating the ability to control material properties through systematic sequence changes. Next, the amino acid sequences that give rise to cooperative coassembly are categorized into three design classes: charge-complementary, enantiomeric, and template-induced (Figure 1b). We then describe our efforts to apply experimental and computational techniques to build molecular-level models of charge-

complementary peptides. Characterization performed to date reveals significant departures from ideal coassembly behavior. Thus, there are many opportunities to design and test more highly selective coassembling β-sheet peptides.

Applications and Material Properties of Coassembled β-sheet Peptides

Design of coassembling peptides can be a means of overcoming challenges that would otherwise be faced with self-assembling peptides. Peptide coassembly is triggered by mixing rather than the physicochemical changes (e.g, pH, salt concentration, or temperature) used to trigger self-assembly of peptides. The following examples illustrate that designer coassemblies can exhibit improved material properties and biological effects in comparison to similar peptide self-assemblies, perhaps because of improved stability or better control of peptide structural and encapsulated cargo distributions. Previous work on charge-complementary coassembled peptide nanofibers demonstrated the encapsulation of a zwitterionic dye during assembly through electrostatic interactions between zwitterionic peptide nanofibers and a zwitterionic dye.[7] Electrostatic interactions between the dye and nanofibers led to approximately 94% of the dye, rhodamine B, persisting within the gel for 80 h. Coassembled nanofibers of these charge-complementary peptides exhibited equilibrium moduli an order of magnitude higher than that of gels formed from individual peptides. Nanoindentation measurements also showed increased adhesive forces. Coassembly by charge-complementarity improved the mechanical properties of peptides.

King et al. recently demonstrated the enzyme-controlled release of the dye dabsyl from a hydrogel matrix composed of charge-complementary peptides, p1 and p2, doped with dabsyl-FFK-PEG-p2.[8] Addition of the proteolytic enzyme trypsin resulted in cleavage of dabsyl-FFK-

PEG-p2 and release of dye molecules. In contrast, hydrogels only containing p1 and p2 were resistant to degradation by trypsin and elastase. The authors also demonstrated the use of the coassembled hydrogel as a cell-culture scaffold consistent with some prior studies.[7-11] Murine 3T3 fibroblasts favorably attached and proliferated on coassembled p1 + p2 hydrogels doped with a modified-p1 peptide as shown in Figure 2a.[8] Similar to early A+A* coassemblies,[2-3] the modified-p1 peptide incorporates a 3-amino acid motif, RGD, derived from fibronectin conjugated to the p1 peptide's C-terminus to facilitate integrin-mediated adhesion and improve cell viability within coassembled hydrogels.

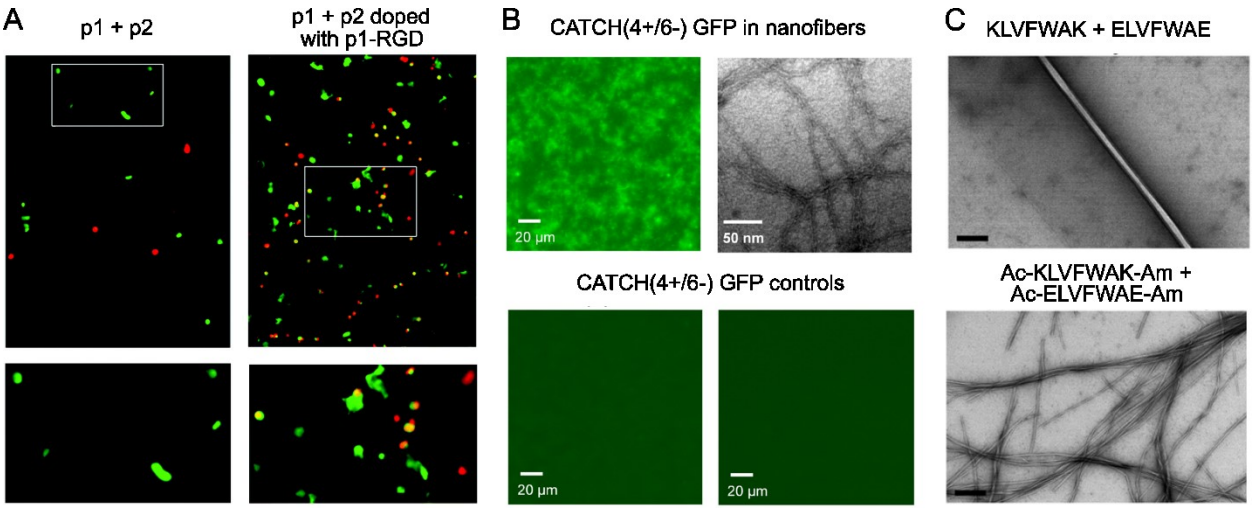

Figure 2. Example biotechnological applications utilizing coassembling β-sheet peptides. A) Confocal images of murine 3T3 fibroblasts stained with a Live/Dead assay on p1 + p2 peptide hydrogels and p1 + p2 hydrogels doped with modified p1 peptide expressing the RGD motif. Dead cells are stained with red fluorescing molecules and living cells are stained with green fluorescing molecules. Reproduced from Ref. 8 with permission from The Royal Society of Chemistry. B) Confocal and TEM images of nanofibers assembled from a mixture of CATCH(4+), CATCH(6-), and CATCH(6-)-GFP. Reprinted by permission from Springer Nature: Springer Cellular and Molecular Bioengineering, Co-Assembly Tags Based on Charge

Complementarity (CATCH) for Installing Functional Protein Ligands into Supramolecular Biomaterials, D. T. Seroski, A. Restuccia, A. D. Sorrentino, K. R. Knox, S. J. Hagen, G. A. Hudalla, Copyright 2016. C) TEM images of uncapped (top) and capped (bottom) amyloid-inspired peptides. Reproduced from Ref. 22 with permission from The Royal Society of Chemistry.

Peptide coassembly may reduce neurotoxicity by increasing fibrillization kinetics and altering the structure of toxic species. Incubation of coassembled mixtures of L-Aβ42 and D-Aβ42 with PC12 neuron-like cells resulted in suppression of cytotoxicity.[12] A reduced lag phase and faster assembly kinetics in coassembled mixtures of Aβ42 enantiomers is thought to reduce the number of toxic oligomeric species formed compared to single-peptide solutions of L-Aβ42.[12] Urban et al. observed rapid nanofiber formation in enantiomeric coassemblies of the Aβ fragment KLVFFAE and posit that these enantiomeric mixtures likely exhibit reduced neurotoxicity as well.[13] Charge-complementary peptides may show similar behavior to these enantiomeric coassemblies though studies have not examined this explicitly. While reduction in the number of toxic oligomeric species likely contributes to the reduction in neurotoxicity, we suggest that differences in coassembled oligomer and self-assembled oligomer structure may also contribute to the reduction in toxicity. Thus, changes in assembly pathways affected by peptide coassembly could inform our understanding of toxicity in amyloidogenic peptides. Favorable properties observed in coassembling peptide systems will likely expand their use as biomaterials for medical and biotechnological applications.

Coassembling peptides provide additional organizational control and complexity to multifunctional biomaterials not easily achieved with self-assembling peptides. Proteins can be attached to peptide termini and, upon assembly, immobilized onto the surface of nanofibers to

impart peptide hydrogels with desired biological functions. For example, Hudalla et al. demonstrated the gradated immobilization of multiple proteins onto coassembled nanofibers of a template-induced designed pair of peptides: β-tail (Ac-MALKVELEKLKSELVVLHSELHKLKSEL-Am) and Q11 (Ac-QQKFQFQFEQQ-Am).[14] Proteins, such as green fluorescent protein (GFP), enhanced GFP (eGFP), and red fluorescent protein (dsRED), were recombinantly expressed with β-tail peptide attached, and once mixed with Q11, the two peptides readily formed nanofibers incorporating the fluorescent proteins. Co-immobilization of multiple proteins onto a single structure at independently tuned ratios could be easily achieved using this coassembly approach. Seroski et al. developed CATCH(4+) (Ac-QQKFKFKFKQQ-Am) and CATCH(6-) (Ac-EQEFEFEFEQE-Am) peptides as an alternative nanofiber system to the Q11 and β-tail peptide pair.[15] Q11 and, to a lesser extent, β-tail are prone to self-assembly under certain conditions. This propensity for self-assembly could induce inclusion body formation and result in aggregation and misfolding of the protein of interest during recombinant expression in *E. Coli*. The net molecular charge of each CATCH peptide discourages self-assembly, which reduces the probability of inclusion body formation. As shown in Figure 2b, mixtures of CATCH(4+) and CATCH(6-)-GFP produce charge-complementary peptide nanofibers decorated with GFP.[15]

Conductive and photodegradable motifs have also been incorporated into coassembled hydrogels to impart photoactive functions. Manipulating the assembly process of coassembling peptides enables additional control over nanoscale organization compared to self-assembling peptides. Ardoña and coworkers synthesized conductive oligomers flanked by short peptide segments with different pK_a values that enabled peptide-mediated self-assembly and coassembly.[16] Photophysical properties of the peptide nanofibers were controlled by altering the

assembly conditions, which led to different arrangements of photoactive groups on nanofibers. Rapid addition of acid to mixtures containing both peptides resulted in the formation of randomly coassembled nanofibers. Alternatively, gradual decrease in pH by glucono-δ-lactone hydrolysis resulted in the higher pKa peptide assembling first, leading to self-sorted nanofibers. Wang et al. incorporated photodegradable molecules into the design of one of two charge-complementary peptide amphiphiles to add an additional mode of stimuli response beyond pH.[17] Reversible unbundling was observed when changing the solution pH. By incorporating a photodegradative motif into one of the peptide amphiphiles, peptide nanofibers could be unbundled into short nanofibers in response to ultraviolet or near-infrared light in addition to pH. Photochemical control of nanofiber association could be useful in potential drug delivery applications. Overall, coassembling β-sheet peptides allow additional control over nanoscale organization necessary for producing increasingly complex functional biomaterials.

Fiber morphology and rheological properties can also be tuned in coassembling β-sheet peptide nanofibers through the manipulation of intermolecular interactions. A small number of coassembling β-sheet pairs have been shown to form flatter, belt-like nanofibers compared to the twisted ribbons observed in their self-assembling counterparts.[18-21] Recently, Candreva et al. also demonstrated the ability to control fiber width and lateral association in a series of charge-complementary peptides derived from the amyloid β (Aβ) segment, KLVFFAE.[22] Capping of peptide termini by acetylation and amidation increased fiber widths and led to a higher degree of lateral association than with uncapped peptide variants as shown in TEM images (Figure 2c).[22] In addition, fiber morphology can be controlled by staging the assembly process. Using variants of the same Aβ segment, Li et al. produced heterogeneous nanotubes from mixtures of KLVFFAL and (pY)LVFFAL;[23] the peptides could also be assembled sequentially, resulting in

nanotubes with separate positive and negative layers. On a more macroscopic scale, changes to fiber structure may affect rheological behavior. For example, hydrogels formed from charge-complementary and enantiomeric peptides are stiffer than the self-assembled hydrogels formed by each component, as observed by rheological measurements.[7, 24] Recently, Nagy-Smith et al. demonstrated the ability to further tune mechanical rigidity in enantiomeric coassemblies through sequence mutations on the hydrophobic and hydrophilic faces of MAX1 and DMAX1 peptides.[24] The hydrogel rigidity can be reduced by replacing Val-Val interactions with less favorable Ile-Ile interactions in the nanofiber's hydrophobic core or increased with inter-guanidino hydrogen bonds formed between arginine residues on the hydrophilic exterior. This ability to control hydrogel rigidity more finely could be useful in regenerative medicine where it is important to be able to match the stiffness of natural extracellular matrices in different tissues. Soto Morales et al. evaluated the effect of charge-pairing on viscoelasticity, pore structure, and pore size in a set of 4 CATCH peptide variants, CATCH(4+), CATCH(4-), CATCH(6+), and CATCH(6-).[25] CATCH(4+/4-) peptides formed the stiffest hydrogels and showed 100% recovery of its initial stiffness within 132 seconds after high-strain disruption. In contrast, CATCH(6+/6-) formed the softest hydrogel and showed a lower percentage (63%) of shear recovery within 10 minutes compared to the other CATCH(+/-) pairs. Coassembled nanofibers were also co-administered with adjuvants to assess peptide biocompatibility and potential as drug-carriers for localized delivery. Subcutaneous injection with and without adjuvant did not elicit antibody response, which provides evidence for their biocompatibility.[25] In summary, researchers have demonstrated the sensitivity of fiber architecture and material properties to molecular interactions between complementary partners which may allow these properties to be finely tuned to suit a desired technological application.

Amino Acid Sequence Pattern

Designs of early coassembling peptides relied on a heuristics-based approach in which positively and negatively charged variants of a β-sheet-forming sequence were designed to confer selective coassembly behavior through electrostatic interactions. Peptides designed in this manner were inspired by prior knowledge that alternation of hydrophilic and hydrophobic residues, i.e., an $(HP)_n$ pattern, promotes β-strand secondary structure.[2] Charged residues could replace residues at hydrophilic positions to create peptide variants with net negative or positive charges. Electrostatic repulsion between like-charged peptides (A/A and B/B) disfavors peptide self-association while electrostatic attraction between oppositely charged peptides (A/B) promotes co-association and fibrillization. For example, Seroski et al. designed the hydrophilic face of CATCH(4+) to contain only lysines and glutamines and the hydrophilic face of CATCH(6-) to contain only glutamic acids (Table 1).[15] These charge-complementary peptides cooperatively coassemble into β-sheet-rich nanofibers when combined in solution yet resist assembly in single-peptide solutions. This design was preceded by other designs of charge-complementary peptides based on a similar sequence pattern, such as P_{11}-13/P_{11}-14 and KVW10/EVW10.[9, 26] Wang et al. also employed charge-complementary amino acid sequences to bestow coassembly behavior in peptide amphiphiles, C_4-Bhc-EE-NH_2 and C_{14}-FKK-NH_2.[17] An alternative approach to designing charge-complementary coassembling peptides emphasizes the position of charged residues in addition to overall molecular charge. The p1 and p2 peptides by King et al. and P_{11}-4 and P_{11}-5 peptides by Aggeli et al. were developed along these lines; each molecule includes both positive and negative residues to give a lower overall charge than corresponding CATCH peptides.[8, 27] Recently, we investigated the role of charge in conferring

selective coassembly behavior on charge-matched pairs of CATCH(+/-) peptide variants. CATCH(4+/4-) and CATCH(6+/6-) peptide pairs successfully demonstrated selectivity towards cooperative coassembly. CATCH(2+) peptides showed some self-assembly, and thus, CATCH(2+/2-) mixtures do not exhibit selective coassembly behavior. Our results also revealed that CATCH(+) variants have a higher propensity for self-association within nanofibers than CATCH(-) variants.[28] The design of new charge-complementary peptides will require a detailed understanding of sidechain-sidechain interactions beyond net charge.

Table 1. Summary of Recent Coassembling β-sheet Peptide Designs

Peptide Names	Sequences
P_{11}-13	Ac-QQOFOWOFOQQ-Am (+4)[a]
P_{11}-14	Ac-EQEFEWEFEQE-Am (-6)
KVW10	Ac-WKVKVKVKVK-Am (+5)
EVW10	Ac-EWEVEVEVEV-Am (-5)
P_{11}-4	Ac-QQRFEWEFEQQ-Am (-2)
P_{11}-5	Ac-QQOFOWOFQQQ-Am (+3)
p1 (or KW-)	EEFKWKFKEE (-1)
p2 (or KW+)	KKEFEWEFKK (+1)
KLVFFAL	KLVFFAL (+1)
(pY)LVFFAL	(pY)LVFFAL (-1)
KLVFWAK	[Ac-]KLVFWAK[-Am][b] (+2)
ELVFWAE	[Ac-]ELVFWAE[-Am][b] (-2)
KF_4K	KFFFFK (+2)
EF_4E	EFFFFE (-2)
C_4-Bhc-EE-NH_2	C_4-Bhc-EE-Am
C_{14}-FKK-NH_2	C_{14}-FKK-Am

CATCH(2+)[c]	Ac-QQKFQFQFKQQ-Am (+2)
CATCH(2-)	Ac-QQEFQFQFEQQ-Am (-2)
CATCH(4+)	Ac-QQKFKFKFKQQ-Am (+4)
CATCH(4-)	Ac-QQEFEFEFEQQ-Am (-4)
CATCH(6+)	Ac-KQKFKFKFKQK-Am (+6)
CATCH(6-)	Ac-EQEFEFEFEQE-Am (-6)
MAX1	VKVKVKVKV^{D}*P*^{L}PTKVKVKVKV-Am
DMAX1	*VKVKVKVKV*^{L}P^{D}*PTKVKVKVKV*-Am[d]
L-Aβ(16-22)	Ac-KLVFFAE-Am
D-Aβ(16-22)	Ac-*KLVFFAE*-Am[d]
Q11	Ac-QQKFQFQFEQQ-Am
βTail	Ac-MALKVELEKLKSELVVLHSELHKLKSEL-Am
OPV3	DVV-oligo(p-phenylenevinylene)-DVV
OT4-NDI	KAA-quarterthiophene-KAA

[a] Net charges of charge-complementary peptides are shown in parentheses.

[b] KLVFWAK and ELVFWAE peptides were synthesized with capped ends (acetylated and amidated termini) and uncapped ends (standard termini).

[c] Multiple different combinations of CATCH(+) and CATCH(-) peptides have been investigated.

[d] Multiple different combinations of CATCH(+) and CATCH(-) peptides have been investigated.

Some charge-complementary designs utilize the naturally occurring β-sheet-forming sequence, KLVFFAE, derived from the Aβ(16-22) fragment, which begins to open up the range of possible architectures in coassembling peptide literature. KLVFFAE, which is thought to be the nucleating core of the Aβ protein, can form nanoribbons, nanotubes, or nanofibrils depending on assembly conditions.[29] In these designs, the first and/or last amino acids of the Aβ segment are replaced with either negatively or positively charged residues to create pairs of variants that

coassemble into two-component nanostructures.[7] As previously mentioned, Li et al. modified the first and last amino acids of the KLVFFAE fragment to produce two charged variants: KLVFFAL and (pY)LVFFAL.[23] In a mixture of the two peptides, heterogeneous coassembled peptide nanotubes formed rather than nanofibers. Each peptide was also able to self-assemble into leaflet nanotubes in single-peptide solutions, suggesting that this design is not selective towards cooperative coassembly. In the same vein, Candreva and coworkers developed the peptides KLVFWAK and ELVFWAE, which form coassembled nanofibers.[22] Each peptide was synthesized with unmodified N and C termini or capped via acetylation and amidation. Modification of the peptide termini alters the overall charge of each peptide and likely influences cooperative coassembly kinetics in addition to fiber morphology. Other sequences with a $P(H)_nP$ pattern like the Aβ(16-22) fragment show similar structures and behavior. Hu et al. observed a transition from belts to twisted fibrils in binary combinations of EF_4F, KF_4K, and EF_4K peptides.[18] The discovery of human-designed charge-complementary pairs based on patterns other than $(HP)_n$ will likely increase our ability to generate new coassembled nanostructures beyond nanofibrils.

Enantiomeric and β-sheet template-induced designs that do not rely on favorable electrostatic interactions have also been observed to impart coassembly behavior . Studies on mixtures of L- and D-chiral peptides confirmed Pauling and Corey's prediction of co-association into rippled β-sheets.[13, 20, 24, 30] As previously mentioned, Nagy-Smith et al. demonstrated enantiomeric coassembly through the formation of nanoribbons containing both L-MAX1 and D-MAX1 peptides or a series of variant pairs.[24] Racemic mixtures of KLVFFAE also coassemble into rippled β-sheets.[13] In contrast, template-induced designs, in which a β-sheet-forming peptide serves as a template, promote coassembly with an "adder" peptide that does not readily form β-

strands on its own or does so slowly. This templating approach has similarities to amyloid cross-seeding employed in α-synuclein and Aβ studies.[31] In the following examples, self-assembling peptide sequences or π-π stacking of aromatic groups are used to promote coassembly of other peptides, such as α-helical peptides. The self-assembling peptide Q11 facilitates the adoption of a β-strand conformation in β-tail peptides, and mixtures of Q11 and β-tail produce coassembled nanofibers.[14] In another example, Ardoña et al. manipulated the self-assembly and coassembly propensities of multi-chromophoric oligomers by appending highly aromatic molecules with short peptide segments.[16] Coassembly of di- and tri-peptides has also been facilitated by π-π interactions.[10, 32-33] Peptides belonging to enantiomeric and template-induced design pairs often do not resist self-assembly (i.e., are not inherently "selective"). Thus, alternative designs will likely be necessary to enable selective coassembly. For example, combining enantiomeric and template-induced design principles with charge-complementary sequences could further improve our ability to co-organize molecules.

Molecular-Level Analysis by Experimental and Computational Techniques

In this section, we review experimental and computational methods to probe coassembly kinetics and structures of coassembled β-sheets. When cooperative coassembly of peptides into β-sheets was first demonstrated in 2003,[27] experimental validation relied on spectroscopic methods and electron microscopy to report on β-sheet secondary structure and fiber formation. By analyzing single-peptide solutions and two-peptide mixtures using these methods, peptide coassembly could be inferred. However, these techniques do not provide details on assembly mechanism nor do they describe molecular-level structural information. More recently, high-resolution methods common in structural biology have allowed observation of structural

heterogeneity within coassembled nanofibers and the presence of oligomeric species during assembly processes. Insights from these studies have spurred our efforts to design selectively coassembling peptides that exhibit highly precise and targeted molecular recognition between complementary sequences.

Figure 3 illustrates how spectroscopic and imaging modalities designed for β-sheet self-assembly can be used to detect β-sheet coassembly. It is known that the β-strand secondary structure is unstable without inter-strand hydrogen bonding, meaning that peptides composed of ~15 amino acids or fewer are unlikely to adopt β-strand secondary structure without forming assemblies. Therefore, coassembly can be detected using spectroscopic methods that are sensitive to secondary structure, such as circular dichroism (Figure 3A) and Fourier-transformed infrared (Figure 3B) spectroscopies, or through fluorescence spectroscopy of dye molecules (most commonly thioflavin T) that bind to β-sheets. Furthermore, since coassembly is expected to produce structures with dimensions larger than molecular length scales, nanoscale imaging techniques such as transmission electron microscopy and atomic force microscopy can also detect coassembly (see Figure 3C). Most commonly, peptide coassembly is determined by comparing binary peptide mixtures to single-peptide solutions. For the particular case of selective coassembly, each peptide resists self-assembly, and thus, single-peptide solutions would not produce detectable β-sheet nanofibers. In contrast, solutions including both complementary peptides would form observable β-sheet structures. The ability to detect peptide coassembly is primarily limited by the sensitivity of each technique to observe structural changes of one peptide in the presence of its complementary partner.

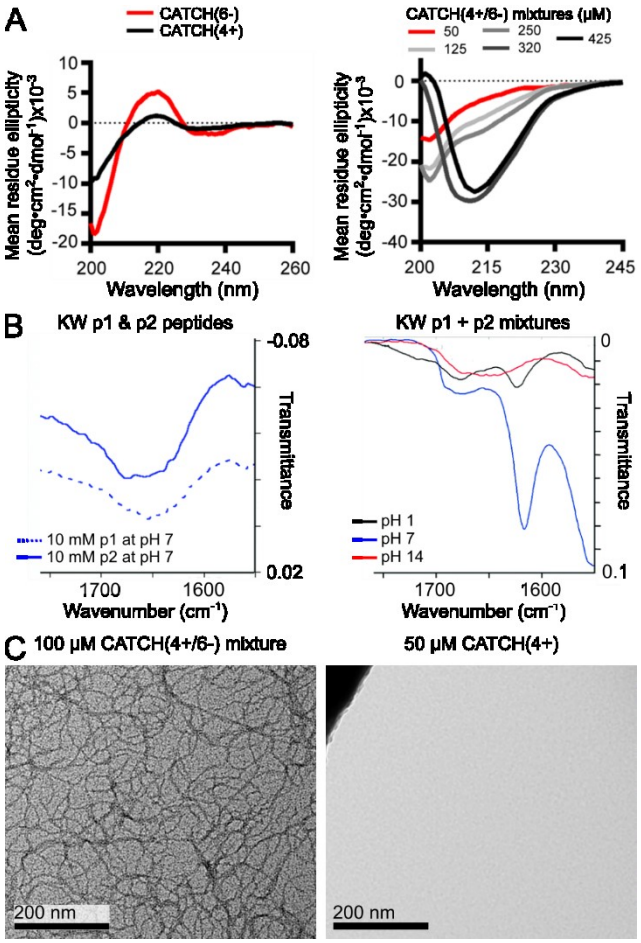

Figure 3. Spectroscopic and imaging methods for detecting β-sheet formation in binary peptide systems. A) CD spectra of CATCH(6-) and CATCH(4+) single-peptide solutions (left) and CATCH(4+/6-) mixtures at varying concentrations (right). Reprinted by permission from Springer Nature: Springer Cellular and Molecular Bioengineering, Co-Assembly Tags Based on Charge Complementarity (CATCH) for Installing Functional Protein Ligands into Supramolecular Biomaterials, D. T. Seroski, A. Restuccia, A. D. Sorrentino, K. R. Knox, S. J. Hagen, G. A. Hudalla, Copyright 2016. B) FTIR spectra of KW p1 and p2 peptides in single-peptide solutions (left) and an equimolar mixture (p1 + p2) at varying pH (right). Reproduced from Ref. 8 with permission from The Royal Society of Chemistry. C) TEM micrographs of a 50

μM CATCH(4+) solution (left) and a 100 μM mixture of CATCH(4+/6-) (right). Reprinted with permission from Ref. 43. Copyright 2020 National Academy of Sciences.

Figure 4 illustrates computational and experimental methods designed to probe unique molecular organizations specific to coassembled peptides. Figure 4A shows snapshots of 48 CATCH(4+) and 48 CATCH(6-) peptides in a discontinuous molecular dynamics (DMD) simulation. The DMD simulations utilize a four-bead-per-residue implicit-solvent coarse-grained protein model, known as the PRIME20 model. The knowledge-based geometric and energetic parameters for the PRIME20 model were calculated by fitting 711 native-state globular protein structures in the Protein Data Bank and 2 million decoy structures using a perceptron learning algorithm.[34-35] DMD/PRIME20 simulations have previously been applied to investigate the aggregation kinetics and fibril structure predictions of a broad range of amyloid-forming peptides including the tau fragments,[36] prion protein fragments,[37] and Aβ protein and its fragments.[38-42] DMD/PRIME20 simulations of coassembling peptides provide experimentally testable structural predictions. Figure 4B shows DMD-predicted distributions of distances between the carbonyl carbon atom of the F6 residue on CATCH(4+) and the F6 carbonyl site on neighboring CATCH(6-) and CATCH(4+) molecules. Figure 4C shows how the relative positions of these atoms can be probed using ^{13}C solid-state nuclear magnetic resonance (NMR) spectroscopy on samples with selective ^{13}C labeling. Figure 4D shows another solid-state NMR method, 2-dimensional (2D) NMR, that can identify interactions that are specific to the cooperative coassembly of complementary KW+ and KW- peptides developed by King et al.[8] In this 2D NMR spectrum, off-diagonal peaks (cross peaks) indicate magnetic interactions between ^{13}C-labeled sites, and colored circles indicate cross peaks between labeled sites on different peptide molecules. In a similar fashion, Li et al. used NMR measurements of ^{15}N-^{13}C dipolar couplings

to characterize the organization of KLVFFAL/(pY)LVFFAL coassemblies.[23] NMR is not the only spectroscopic technique that is capable of detecting intermolecular organization of complementary peptides. Isotopic labeling with ^{13}C at carbonyl sites can cause a shift in peaks in Fourier-transform infrared (FTIR) spectra in a way that is sensitive to nearest-neighbor molecular interactions.[24, 43-44] Finally, Forster resonance energy transfer (FRET) can be used to detect coassembly of complementary peptides tagged with fluorescent molecules.[20]

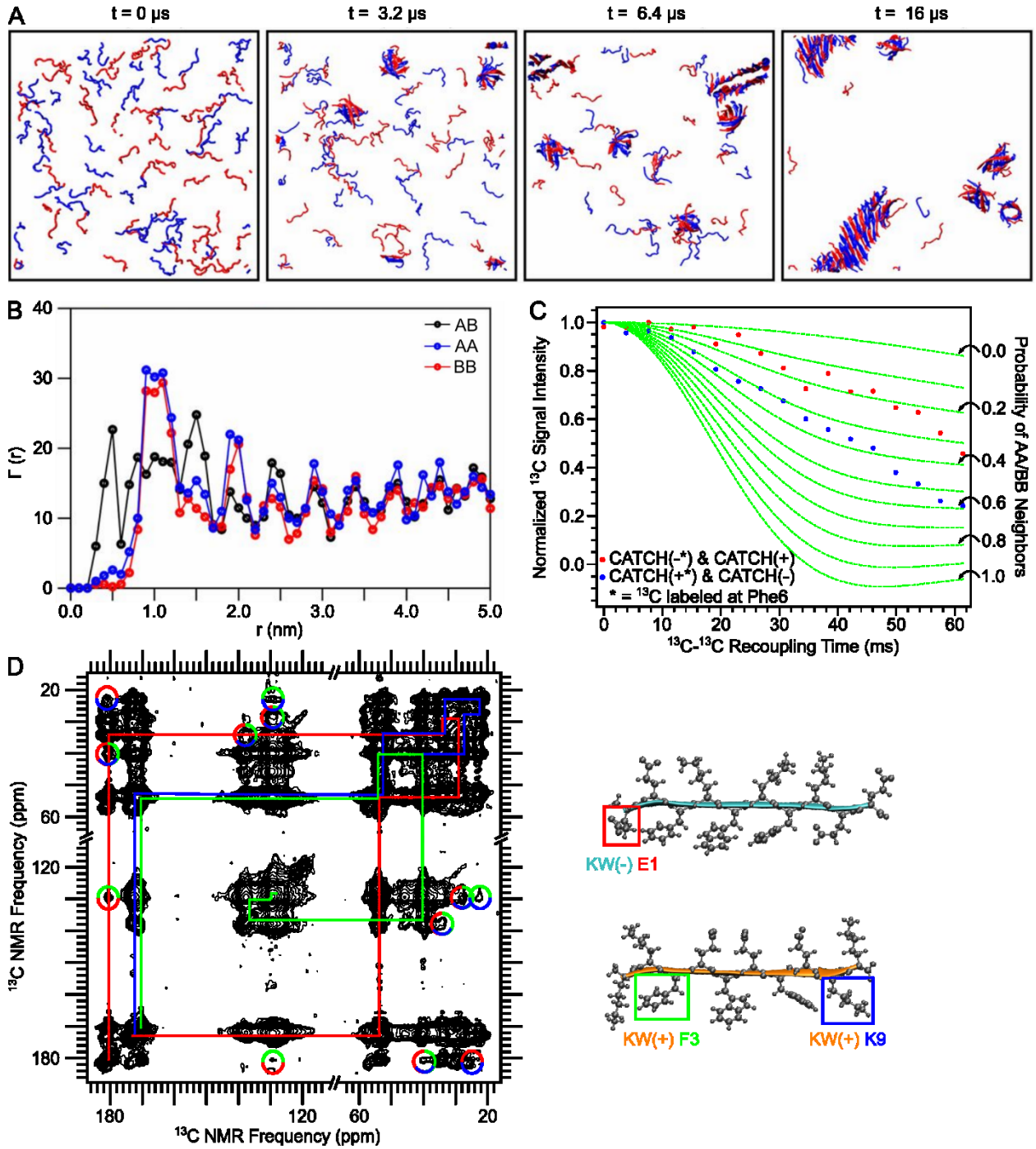

Figure 4. Computational and experimental measurements of molecular organization within selectively coassembled peptides. A) Snapshots of DMD/PRIME20 simulations of 48 CATCH(4+) and 48 CATCH(6-) peptides over simulation time. B) Nearest-neighbor distance distributions calculated from CATCH(4+/6-) nanofiber predictions. C) PITHIRDS-CT NMR

measurements on isotopically diluted CATCH(4+/6-) nanofibers. Reprinted with permission from Ref. 43. Copyright 2020 National Academy of Sciences. D) 2D DARR NMR spectra of coassembled KW(+) and KW(-) peptides uniformly ^{13}C labeled at indicated residue positions. Bi- and tri-colored circles indicate intermolecular contacts. Reproduced from Ref. 45 with permission from The Royal Society of Chemistry.

Experimental and computational analysis of peptide coassemblies at the molecular level has revealed both anticipated and unanticipated results. As expected, there is considerable evidence that charge-complementary peptides form nanostructures primarily via attractive interactions between partner molecules. Evidence of complementary molecules being nearest neighbors has been observed experimentally via NMR and FTIR spectroscopies and predicted theoretically using DMD simulations. However, experimental and simulation data have also revealed considerable departure from ideal cooperative coassembly behavior. In ideal coassembled β-sheets, complementary peptides are expected to alternate along a β-sheet. However, DMD simulations predicted and solid-state NMR detected that 9% to 33% of β-strands have like-peptide nearest neighbors.[43, 45] In addition, when organization of the King-Webb and CATCH(4+/6-) systems were probed in more detail, solid-state NMR measurements observed no strong preference for a specific inter-strand organization (parallel vs. antiparallel) or alignment of stacked β-sheets.[44-45] Some β-strands also had out-of-register nearest neighbors.[44-45] Differences in the relative amounts of these types of structural deviations between King-Webb and CATCH(4+/6-) nanofibers may explain differences in nanofiber morphology, which highlights the importance of sequence-to-structure relationships in peptide coassemblies. Finally, DMD simulations of 48 CATCH(4+) and 48 CATCH(6-) peptides predicted an unanticipated nanostructure: while CATCH(4+/6-) mixtures formed mostly β-sheet nanofibers, they also

coassembled into size-limited β-barrel oligomers that do not convert to nanofibers in simulation.[43] Experimental efforts to observe oligomeric species, based on electron microscopy and light scattering, did provide evidence of oligomers, but the structure of these oligomers remains unknown. In our recent study of charge-matched CATCH(+/-) peptide pairs, oligomeric species persisted longer in CATCH(4+/4-) mixtures than in CATCH(6+/6-) mixtures as observed in time-series TEM images.[28] These oligomeric species may point to a two-step nucleation process within the coassembly pathway similar to aggregates observed during peptide self-assembly.

Evidence that nanofiber structure is not well-controlled in β-sheet peptide coassemblies inspired recent efforts to design new pairs computationally. Xiao et al. developed a Monte Carlo (MC)-type peptide coassembly design (PepCAD) algorithm to discover potential selectively coassembling peptide pairs quickly and efficiently.[46] Development of this algorithm follows recent success in computational designs of α-helical assemblies.[47] The PepCAD algorithm proceeds by modifying amino acid sequences while evaluating a score that depends on the binding free energy and intrinsic self-aggregation propensity for a pair of peptides coassembled into a β-sheet scaffold. Trial sequence moves, based on intra-chain residue mutation, intra-chain residue exchange, or inter-chain residue exchange, are accepted or rejected based on the computed score using the MC Metropolis sampling method. A low negative score during sequence evolution means that the peptide pair is more likely to form fibril-like coassemblies but not fibril-like self-assemblies. In applying the PepCAD algorithm, Xiao et al. started with a pool of around 1×10^6 possible peptide pairs and identified six pairs of charge-complementary 11-mer peptides.[46]

Experimental and computational analysis of the computationally designed peptide pairs mostly indicate highly ordered β-sheet nanofibers, but the PepCAD algorithm is not completely predictive of the final structure. The top six candidate peptides identified by the PepCAD algorithm were tested computationally and experimentally for coassembly. Five out of the six peptide pairs were confirmed to selectively coassemble, with four forming β-sheet nanofibers and one forming a stable non-fibrillar aggregate.[46] NMR spectra of the four nanofiber-forming peptide pairs indicated a higher degree of structural order than had been observed for previous β-sheet coassemblies. The increased structural control resulting from computational optimization may be essential to achieving selectivity in coassembling β-sheet peptide pairs.

Summary and Future Outlook

Existing peptide designs have successfully demonstrated cooperative coassembly, but more work is needed to program peptides for perfectly "ideal" selective coassembly and demonstrate precise control of nanostructure. Structural heterogeneity in existing coassembling β-sheet peptides contrasts with efforts in α-helical coiled-coils where precise control over the intermolecular packing and structural order have been demonstrated.[48-50] This difference may result from the fundamental interactions underlying β-sheets and α-helices. While hydrophobic collapse and sidechain-sidechain interactions heavily contribute to assembly processes in α-helical coiled coils and β-sheet nanofibers, β-strands also require intermolecular hydrogen bonds between adjacent peptide backbones. On the other hand, α-helices are stabilized by intramolecular hydrogen bonds, which may simplify the programming of precise nanostructures. Further exploration of the vast sequence space may identify coassembling β-sheet designs capable of encoding a homogeneous nanostructure. Algorithms, like PepCAD, can facilitate the

discovery process, while molecular-level techniques, such as DMD/PRIME20 simulations and solid-state NMR, provide molecular-level insight into next-generation designs. By identifying designs with specific intermolecular complementarity, selectivity among coassembling peptide pairs will likely improve as well. Combining computational and experimental tools will bring us closer to understanding the biophysics of encoding highly selective and highly precise coassembled nanostructures.

Computational simulations and experimental measurements have also led to the observation of early oligomeric aggregates, which may play an important role in structural heterogeneity within coassembled nanofibers. Characterization of oligomeric species in self-assembling peptides has advanced alongside the development of various techniques, such as fluorescence correlation spectroscopy, cryo-electron microscopy, and dynamic nuclear polarization-enhanced solid-state NMR.[51-53] Applying these techniques to understand the structure and role of oligomeric species, i.e. on-pathway or off-pathway, will shed light on future cooperative coassembly design. By better understanding early aggregates formed during coassembly, off-target nanostructures may be avoided, improving structural uniformity. It is also possible that intentionally introducing folded aggregates along the assembly pathway could better steer resulting nanostructures. Recently, Roberts et al. observed a solid-state transition from α-helices to β-sheets in coassembled coiled-coil peptides, SAF p1 and p2a.[54] Designing coassembling α-helices to undergo this structural transition would benefit from the precision of α-helical coiled-coil designs and place us a step closer towards our goal of exquisite control of nanostructure. Unfolding and folding events that mimic the process observed in amyloidogenic peptides may also modulate the energetic landscape, and bias coassembly towards selection of a single

structure. Understanding the structure and evolution of early aggregates will paint a fuller picture of structural polymorphism and approaches for mitigating heterogeneity.

Compared to the numerous studies examining thermodynamic and kinetic behavior in amyloids such as Aβ and α-synuclein,[55-56] there are only a few studies that include thermodynamic or kinetic data on coassemblies. Among the limited studies on co-assembly, most focus on charge-complementary designs and none to our knowledge focus on thermodynamics or kinetics. Nevertheless, some published data suggest interesting behavior. For example, Candreva et al. saw no β-sheet formation in equimolar KLVFWAK and ELVFWAE after 1 day of incubation at 80°C, but upon cooling to 50°C, β-sheets were detected by CD spectroscopy.[22] The lack of coassembly at elevated temperatures contrasts with similar studies on Aβ where increasing temperature drove self-assembly by enhancing the hydrophobic effect.[57] In addition, salt concentration, which influences the degree of electrostatic screening, has been shown to increase assembly kinetics in some charge-complementary peptides and decrease it in others.[22, 28] One might expect nanofiber structure to be highly sensitive to physicochemical factors such as temperature, salt concentration, pH, and solvent composition, but we are not aware of any systematic examination of such molecular-level details. The rapid kinetics of coassembly, particularly when driven by electrostatics, raises the question of whether the heterogeneity in β-sheet structure observed in coassembled β-sheets results from kinetically trapped states. Observation of oligomeric states may be evidence of kinetic trapping.[43] A better understanding of the thermodynamics and kinetics of coassembly will improve our ability to design coassembling peptides and perhaps target specific structures.

While coassembling β-sheet peptides provide additional complexity and control to functional biomaterials, the effects from attaching biomolecules on cooperative coassembly behavior and

the functionality of immobilized biomolecules have not been fully characterized. Intuitively, the overall charge and size of attached biomolecules will influence cooperative coassembly kinetics. On the other hand, coassembling peptides may impact protein folding and lead to a loss of function in immobilized proteins. Understanding these impacts is necessary for improving the design of coassembling peptides for various biotechnological applications. Again, computational and experimental tools developed to examine coassembled peptide nanostructure will aid in assessing coassembly behavior and the structure of any immobilized biomolecule. In addition, insights gained from studies characterizing sequence-to-structure relationships in coassembling designs and formation of intermediates during coassembly outlined above will likely guide application-oriented designs. Coordinated efforts in computational and experimental biophysics to study coassembling β-sheet peptides in isolation and in applications will advance our biosynthetic capabilities and broaden our understanding of protein folding.

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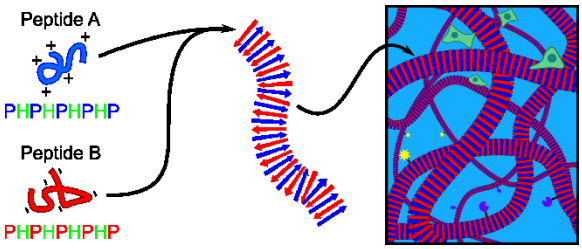

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