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Design of self-assembling peptide hydrogelators amenable to bacterial expression

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ABSTRACT

Hydrogels formed from self-assembling peptides are finding use in tissue engineering and drug delivery applications. Given the notorious difficulties associated with producing self-assembling peptides by recombinant expression, most are typically prepared by chemical synthesis. Herein, we report the design of a family of self-assembling β-hairpin peptides amenable to efficient production using an optimized bacterial expression system. Expressing peptides, EX1, EX2 and EX3 contain identical eight-residue amphiphilic β-strands connected by varying turn sequences that are responsible for ensuring chain reversal and the proper intramolecular folding and consequent self-assembly of the peptide into a hydrogel network under physiological conditions. EX1 was initially used to establish and optimize the bacterial expression system by which all the peptides could be eventually individually expressed. Expression clones were designed to allow exploration of possible fusion partners and investigate both enzymatic and chemical cleavage as means to liberate the target peptide. A systematic analysis of possible expression systems followed by fermentation optimization lead to a system in which all three peptides could be expressed as fusions with BAD-BH3, the BH3 domain of the proapoptotic BAD (Bcl-2 Associated Death) Protein. CNBr cleavage followed by purification afforded 50, 31, and 15 mg/L yields of pure EX1, EX2 and EX3, respectively. CD spectroscopy, TEM, and rheological analysis indicate that these peptides fold and assembled into well-defined fibrils that constitute hydrogels having shear-thin/recovery properties.

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1. Introduction

Exploiting natural protein folds has proven useful in the design of self-assembled hydrogel networks. Peptides derived from the secondary structural units of globular proteins represent a rich source of building blocks for the construction of higher-order functional assemblies. β-strand [1–16], helical [17–19], β-hairpin [20–23] and sheet [24] secondary structural motifs have all found utility in the design of novel self-assembled biomaterials. Even very small peptides having only a few residues [25–29] and cyclic peptides [30–32] can assemble into complex architectures that support function.

Our lab has been developing shear-thin injectable gels from self-assembling β-hairpin peptides [33–47]. MAX8 is a twenty-residue peptide that when initially dissolved in aqueous solution at pH 7 and low ionic strength adopts an ensemble of random coil conformations rendering it fully soluble. The peptide contains seven lysine residues whose side chains are protonated under these solution conditions resulting in inter-residue charge repulsion, which favors the unfolded state of the peptide. However, intramolecular folding and consequent self-assembly of the peptide into a fibrillar network can be accomplished by increasing the solution pH to deprotonate some of the lysines or by simply increasing the ionic strength of the solution to screen the lysine-borne charge. In addition, increasing the solution temperature also promotes gelation by facilitating the desolvation of hydrophobic residues. Self-assembly results in the formation of a fibrillar network where each fibril is comprised of a bilayer of hairpins that have intermolecularly hydrogen bonded along the long axis of a given fibril. The association of the hydrophobic faces of the hairpin amphiphiles mediates bilayer formation, Fig. 1. During assembly, non-covalent branch points are formed in the fibril network, which serve as physical crosslinks that help define the mechanical properties of the gel. MAX8 gels display shear-thin/recovery behavior, which makes their delivery from simple syringe possible [36,40,45].

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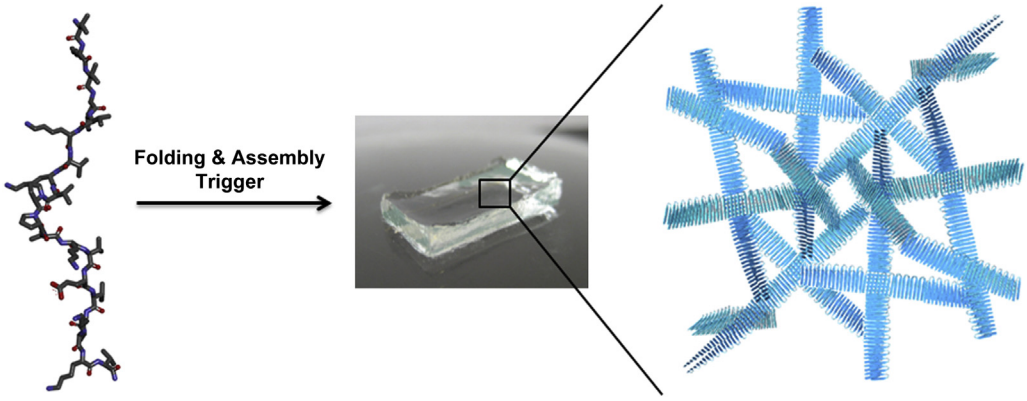

Fig. 1. Triggered folding and subsequent self-assembly of a β-hairpin resulting in the formation of a fibrillar hydrogel network.

Most self-assembling peptides are typically prepared by solid phase peptide synthesis [48]. This technique is rapid and amenable for small-scale batches. However, it can be limited by cost for scaled efforts and relatively low overall yields, especially if the peptide is purified to homogeneity. MAX8 is synthesized employing an amide solid support resin by Fmoc-based techniques, which results in a C-terminally amidated peptide and yields that hover around 10% after stringent purification.

Recombinant production is an alternative method that employs a host organism's machinery for peptide synthesis. Once optimized, fermentation represents a scalable, more cost-effective means to produce material-forming peptides. However, recombinant production of amphiphilic, self-assembling peptides is notoriously difficult and yields are typically low. With this said, work from the McPherson laboratory suggests that high yield production of self-assembling peptides is possible [49,50]. They showed that self-assembling β-strands could be recombinantly produced in optimized media as fusion protein constructs which are shunted to inclusion bodies.

The recombinant production of amphiphilic self-assembling hairpins has not yet been reported and, with respect to the hairpins developed in our lab, is exceedingly challenging. This is due to their repetitive sequences, high hydrophobic content, and with respect to MAX8, it's C-terminal amide and the inclusion of D-proline at position 10 within its turn region. Residues having D-stereochemistry about their Cα carbon are not accommodated by the ribosomal machinery of bacteria.

Herein, we use *de novo* design principles to replace the four-residue turn sequence of MAX8 with sequences having high turn propensity and importantly, that contain residues of only L-stereochemistry. Turn replacement as well as the incorporation of a C-terminal carboxylate affords three new peptides (EX1, EX2 and EX3) that are amenable to bacterial expression. We initially prepare EX1-3 by solid phase peptide synthesis to quickly assess their material forming properties before the arduous task of developing an optimal expression system. As will be shown, all three peptides self-assemble into well-defined gel networks capable of shear thin-recovery rheological behavior. Finally, expression vectors were designed to allow rapid identification of a low molecular weight fusion partner and cleavage method that, along with optimized fermentation procedures, afforded effective production of these peptides.

2. Materials and methods

2.1. Materials

Fmoc-Val-Wang resin, Fmoc-Gly-Thr($\psi^{Me,Me}$pro)-OH dipeptide and Fmoc-protected amino acids were purchased from Novabiochem. 1H-Benzotriazolium 1-[bis(dimethylamino) methylene]-5chloro-hexafluorophosphate (1-),3-oxide (HCTU) was obtained from Peptides International. Acetonitrile was purchased from EMD Chemicals. Trifluoroacetic acid was purchased from Acros Organics. Dithiothreitol (DTT), thioanisole, anisole, Coomassie Brilliant Blue G-250, LB agar, LB broth, SOC medium, yeast extract, tryptone, spectinomycin, chloramphenicol, ampicillin were obtained from Sigma–Aldrich. 1,2-Ethanedithiol was purchased from Fluka. Diethyl ether was purchased from Fisher Scientific. Triton X-100 was obtained from Alfa-Aesar. Laemmli sample buffer, Polypeptide Standard, Tris/Glycine/SDS buffer (10×) and Criterion 18% Tris–HCl precast gel were purchased from Bio-Rad. The miniprep plasmid DNA purification kit was purchased from QIAgen. DNA constructs were ordered from Genscript. BP and LR Clonase, subcloning efficiency DH5α, Rossetta 2 cells, SeeBlue Plus2 pre-stained standard, NuPAGE Novex 4–12% Bis-Tris Gel 1.0 mm, NuPAGE MES-SDS running buffer (20×), SeeBlue Plus2 pre-stained standard and NuPAGE LDS sample buffer were purchased from Life Technologies. Carbenicillin was purchased from Teknova. pDonr253, pDest527, pDest532, pDest533, pDest546, pDest565 were kindly provided by Dominic Esposito (Protein Expression Laboratory, Leidos, NCI-Frederick). TevPep, MetPep, BadEX1, BadEX2 and BadEX3 genes were designed accordingly to be compatible for the Gateway cloning system by flanking attB sites at either ends and they were ordered from Genscript (See Supplemental Information). A cost analysis comparing the chemical synthesis and bacterial production of peptide EX1 is provided in the Supplemental Information.

2.2. Solid phase peptide synthesis and purification

An automated ABI 433A peptide synthesizer was used to synthesize the peptides. Fmoc-based solid peptide chemistry with HCTU activation was performed. A trifluoroacetic acid: thioanisole: 1,2-ethanedithiol: anisole (90:5:3:2) cocktail under Argon atmosphere for 2 h was used to cleave the dried resin-bound peptides from the resin and for simultaneous side-chain deprotection. Precipitation by cold diethyl ether and lyophilization of crude peptides were followed by peptide purification that was carried out by reverse phase-HPLC at 40 °C equipped with a semi-preparative Vydac C18 column. HPLC solvents A and B were 0.1% TFA in water and 0.1% TFA in 9:1 acetonitrile:water, respectively. For EX1 purification, a gradient was employed as 0% solvent B for 2 min, 0–10% solvent B over 10 min, 10–27% solvent B over 34 min and then 27–37% solvent B over 40 min. EX1 eluted at 28%B. For EX2 purification, a gradient was employed as 0% solvent B for 2 min, 0–15% solvent B over 13 min and then 15–35% solvent B over 40 min. EX2 eluted at 31%B. For EX3 purification, a gradient was employed as 0% solvent B for 1 min, 0–12% solvent B over 11 min, 12–25% solvent B over 26 min and then 25–35% solvent B over 40 min. EX3 eluted at 27%B. LC-MS (ESI-positive mode) was performed to verify the purity of the lyophilized peptides. (See Supplemental Information)

2.3. Cloning and transformation

The Gateway cloning system (Invitogen) was used to construct the expression clones used for transformation. Initial entry clones were generated by BP recombination of either an attB-flanked TevPep gene (encoding the TEV protease site + EX1, see Table S1 for DNA sequence) or an attB-flanked MetPep gene (encoding the methionine cleavage site + EX1) with the attP-containing donor vector, pDonr253. Subsequent LR recombination reactions between the entry clones and destination vectors containing the various fusion partners afforded the expression clones used for the transformation step. For example, inserts were supplied with 4 μg of lyophilized pUC57 plasmid DNA dissolved in 20 μL of water. For BP and LR reactions, the manufacturer's protocols were followed (Invitrogen). Briefly, 0.5 μL of 200 ng/μL pUC57 containing the TevPep insert, 1 μL of 120 ng/μL of pDONR253, 4.5 μL of TE buffer and 2 μL of BP clonase were mixed and left at room temperature for at least 1 h to generate the entry clones. *Escherichia coli* DH5α strain was used for cloning, and 1 μL of BP clonase reaction was transferred into 20 μL of