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Modular peptides from the thermoplastic squid sucker ring teeth form amyloid-like cross-β supramolecular networks

Shu Hui Hiew [a,b], Paul A. Guerette [a,b,c], Ondrej J. Zvarec [a,b], Margaret Phillips [d], Feng Zhou [a], Haibin Su [a], Konstantin Pervushin [d], Brendan P. Orner [e], Ali Miserez [a,b,*]

[a] School of Materials Science and Engineering, Nanyang Technological University (NTU), Singapore 639798, Singapore
[b] Center for Biomimetic Sensor Science (CBSS), NTU, Singapore 637553, Singapore
[c] Energy Research Institute at Nanyang Technological University (ERI@N), 50 Nanyang Drive, Singapore 637553, Singapore
[d] School of Biological Sciences, Nanyang Technological University, Singapore 639798, Singapore
[e] School of Biomedical Sciences, King's College London, London SE1 1DB, United Kingdom

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ABSTRACT

The hard sucker ring teeth (SRT) from decapodiforme cephalopods, which are located inside the sucker cups lining the arms and tentacles of these species, have recently emerged as a unique model structure for biomimetic structural biopolymers. SRT are entirely composed of modular, block co-polymer-like proteins that self-assemble into a large supramolecular network. In order to unveil the molecular principles behind SRT's self-assembly and robustness, we describe a combinatorial screening assay that maps the molecular-scale interactions between the most abundant modular peptide blocks of suckerin proteins. By selecting prominent interaction hotspots from this assay, we identified four peptides that exhibited the strongest homo-peptidic interactions, and conducted further in-depth biophysical characterizations complemented by molecular dynamic (*MD*) simulations to investigate the nature of these interactions. Circular Dichroism (CD) revealed conformations that transitioned from semi-extended poly-proline II (PII) towards β-sheet structure. The peptides spontaneously self-assembled into microfibers enriched with cross β-structures, as evidenced by Fourier-Transform Infrared Spectroscopy (FTIR) and Congo red staining. Nuclear Magnetic Resonance (NMR) experiments identified the residues involved in the hydrogen-bonded network and demonstrated that these self-assembled β-sheet-based fibers exhibit high protection factors that bear resemblance to amyloids. The high stability of the β-sheet network and an amyloid-like model of fibril assembly were supported by *MD* simulations. The work sheds light on how Nature has evolved modular sequence design for the self-assembly of mechanically robust functional materials, and expands our biomolecular toolkit to prepare load-bearing biomaterials from protein-based block co-polymers and self-assembled peptides.

Statement of Significance

The sucker ring teeth (SRT) located on the arms and tentacles of cephalopods represent as a very promising protein-based biopolymer with the potential to rival silk in biomedical and engineering applications. SRT are made of modular, block co-polymer like proteins (suckerins), which assemble into a semicrystalline polymer reinforced by nano-confined β-sheets, resulting in a supramolecular network with mechanical properties that match those of the strongest engineering polymers. In this study, we aimed to understand the molecular mechanisms behind SRT's self-assembly and robustness. The most abundant modular peptidic blocks of suckerin proteins were studied by various spectroscopic methods, which demonstrate that SRT peptides form amyloid-like cross-β structures.

1. Introduction

Humboldt (*Dosidicus gigas*) squid have evolved robust sucker ring teeth (SRT) that are embedded within the suction cups coating

* Corresponding author at: School of Materials Science and Engineering, Nanyang Technological University (NTU), Singapore 639798, Singapore.
E-mail address: ali.miserez@ntu.edu.sg (A. Miserez).

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the inner surfaces of their arms and tentacles. Each appendage contains dozens of SRT [1], which are formidable biotools that are instrumental to the survival of this ancient predatory creature. SRT represent an intriguing biomaterial exclusively made of proteins called "suckerins" and they exhibit structural properties [2] that match those of robust synthetic polymers, which are based on the supramolecular assembly of suckerins. Inter-chain covalent cross-links are unusually absent in SRT, which are also devoid of the common building blocks observed in load-bearing natural materials such as minerals or chitin [3]. Because of this supramolecular assembly which is stabilized by cooperative weak interactions, SRT exhibit thermoplastic behaviour that make them a functional "bioink" for 3D bioprinting applications [4]. It has also been established that recombinant suckerins are versatile building blocks that can be used to engineer various structural and functional materials, including stable colloids [5], redox-active substrates for the growth of gold nanoparticles [6], and hydrogels whose elastic modulus can be modulated over several orders of magnitude [7].

We have previously sequenced dozens of genes encoding members of the suckerin protein family [8]. The suckerins are highly repetitive and modular, and assemble into a semi-crystalline supramolecular network consisting of an amorphous matrix reinforced with nano-confined β-sheets [4,8,9]. Almost all suckerins consist of peptide motifs that are reminiscent to those found in silk proteins. The first type of module is enriched with Alanine (Ala), Threonine (Thr), and Histidine (His) residues and is predicted to form β-sheet secondary structures, where the sheet length is precisely constrained by Proline (Pro) residues located on each side of the module, although it is not fully clear yet whether the β-sheets adopt a parallel or an anti-parallel orientation. The second type of module is dominated by Glycine (Gly), with a significant amount of Leucine (Leu) and Tyrosine (Tyr) residues present. These modules are generally longer and are predicted to form the amorphous phase of the network, though the presence of smaller β-sheets in these domains cannot be completely excluded. We hypothesized that the repetitive and modular units greatly contribute to the robust mechanical properties of SRT, and aimed to further understand their behaviour in different environmental conditions in order to unveil the physico-chemical factors that are responsible for the material's self-assembly and robustness. We dissected the primary amino acid sequence of the most abundant suckerin protein, suckerin-19 (molecular weight 39 kDa, Fig. 1A) to identify the key modular units and explore their fundamental interactions at the molecular scale. Importantly, the modular peptides found in suckerin-19 are conserved within most other members of the suckerin gene family [8], allowing us to investigate peptide sequence designs that are relevant to the entire SRT system.

A combinatorial peptide macroarray interaction assay was first designed to map out the interactions between the modular peptides, which was assessed by fluorescence intensity, whereas dynamic light scattering (DLS) experiments were used to monitor the kinetics of peptide self-assembly. Peptides that showed high relative interactions with this assay were selected for further secondary structure studies by CD and FTIR. Ala-rich peptides were observed to spontaneously self-assemble into micro-fibers and displayed amyloidogenic characteristics, which we suggest may play parallel roles in SRT self-assembly. The stability of these amyloid-like structures was then assessed by hydrogen/deuterium (H/D) exchange, which demonstrated a high protection factor of the residues involved in the hydrogen bonded network. Finally, *MD* simulations confirmed the propensity of suckerin-based Ala-rich peptides to form β-sheets, which were highly stable. In contrast, His-rich peptides had low propensity towards β-sheets formation. *MD* simulations also provided insights into the role of Pro on the stability of the modular β-sheet forming peptides.

2. Experimental details

2.1. SPOT synthesis

Peptides purchased from GL Biochem were verified with reverse-phase HPLC C-18 and LC-MS to have a high purity of greater than 98% before SPOT synthesis. The protocol for SPOT synthesis was partially modified from Hilpert et al. [10]; coupling cocktail was prepared peptide/HOBt/TBTU/DIEA (1:4.9:4.9:5, eq) in DMF and spotted at 1 μl per spot on Whatman Chr1 cellulose, and left to dry before respotting again for 3 cycles to maximize coupling. Thorough washing in DMF with agitation on a lab shaker was done before staining with bromophenol blue in methanol (20–50 mg in 1 L) to check for spots. After destaining of bromophenol blue in methanol, acetylation of the N-terminus α-amino group was done by immersing the cellulose sheet in capping solution of acetic anhydride/DIEA/DMF (1:1:8, vol/vol). Bromophenol blue staining was used to check for the completion of acetylation, and after final destaining with methanol, the cellulose sheet was left to dry under ambient conditions.

2.2. Preparation of dye-labeled peptides

Termini-modified peptides (N-terminus acetylated and C-terminus amidated) were labeled at their amidated C-terminus with Texas Red fluorescent dye (Invitrogen, Texas Red SE Mixed Isomers), according to labeling protocol from Invitrogen. Labeling mixture was incubated overnight on vortex and purified with dye removal column (Pierce™) before diluting with respective blocking buffers (at pH 4, 7 and 8.6) to concentrations of 0.1 μM, 0.5 μM and 1 μM prior to the macro array binding assay.

2.3. Peptide macro array binding assay

Spots were punched out and inserted into a 96-well microtiter plate. Contents of each well were hydrated with methanol for 1 h then washed 3× with respective buffers (at pH 4 (0.1 M Acetate buffer), pH 7 (0.1 M Phosphate-buffered saline) and pH 8.6 (0.1 M Tris-buffered saline)) prior to incubation with blocking buffers of the same pH with 0.2% Tween 20 and 0.1% BSA for 2 h on a rocker. Blocking buffer was removed and 40 μl of analyte solution containing fluorescently labeled peptides (0.1 μM, 0.5 μM and 1 μM) diluted in blocking buffer was added and incubated for 3 h on a rocker. Stringent washings were conducted at the end of the incubation (3× blocking buffer, 3× Tween containing buffer and 3× buffer) before imaging with Typhoon fluorescent imager. All steps were conducted at ambient temperature and in the dark. Each condition was done in quadruplicates to obtain a fair average.

2.4. Dynamic Light Scattering

Termini-modified peptides (N-terminus acetylated and C-terminus amidated) were dissolved in buffers of pH 4 (0.1 M Acetate buffer), pH 7 (0.1 M Phosphate-buffered saline) and pH 8.6 (0.1 M Tris-buffered saline), and in urea, at 50 μM prior to DLS measurements (Malvern Zetasizer Nano ZS). All buffers were double filtered prior to use. The Z-average size measurements were collected at intervals of 10 min, over a period of 24 h. Results are presented in 30 min segments and error bars are obtained and presented for the data obtained.

2.5. Circular dichroism and infrared spectroscopy

Termini-modified peptides (N-terminus acetylated and C-terminus amidated) were prepared at 2 mg/ml and a quartz