Journal of Structural Biology xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Journal of Structural Biology

journal homepage: www.elsevier.com/locate/yjsbi

Effect of sequence features on assembly of spider silk block copolymers

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ARTICLE INFO

Article history:
Received 26 December 2013
Received in revised form 1 March 2014
Accepted 3 March 2014
Available online xxxx

Keywords:
Silk
Block copolymers
Self-assembly
Modeling

ABSTRACT

Bioengineered spider silk block copolymers were studied to understand the effect of protein chain length and sequence chemistry on the formation of secondary structure and materials assembly. Using a combination of *in vitro* protein design and assembly studies, we demonstrate that silk block copolymers possessing multiple repetitive units self-assemble into lamellar microstructures. Additionally, the study provides insights into the assembly behavior of spider silk block copolymers in concentrated salt solutions.

1. Introduction

Spider silk motifs and their structure–function relationships have been a focus of research for almost two decades, due to the outstanding mechanical and biophysical properties of these protein fibers and their origins in an all aqueous, ambient spinning environment (An et al., 2012; Tokareva et al., 2014). Spider silks are remarkable natural polymers that consist of distinct peptide domains: repetitive middle core domains and two non-repetitive N-terminal and C-terminal domains (Bini et al., 2006). At least seven different types of silk proteins are known for some orb-weaving spiders (Lewis, 2006). These silks differ in primary sequence, physical properties and functions depending on the source species and the specific type of silk, as spiders use silk to build orb web frames and radii, for lifelines and for anchoring to substrates (Gosline et al., 1984). Spiders produce superior protein fibers compared to a well-known silkworm *Bombyx mori* (Lewis, 2006). Silk threads made of the major ampullate silks have a tensile strength (the maximum stress that a material can withstand while being stretched or pulled before failing or breaking) higher than steel and comparable to Kevlar, a material used in a bullet proof vests, and modest elasticity (Gosline et al., 1999). The exceptional mechanical properties of these spider silk fibers have inspired research into generating these proteins via recombinant DNA approaches in order to study material properties *in vitro*. In addition to engineering recombinant silk fibers, material scientists are interested in self-assembly of silk proteins into non-natural (non-fiber) material formats such as microspheres, films, hydrogels, and microcapsules.

The large core peptide domains in spider dragline silks are generally organized in block copolymer-like arrangements, in which two peptide sequences, crystalline (poly(A) and/or poly(GA)) and less crystalline (GGX or GPGXX) polypeptides alternate (Tokareva et al., 2014). The principles of the block copolymer designs from the study of synthetic polymers served as a guide for these protein systems, based on the sequence of the major ampullate dragline silk I (MaSp I) of the golden orb weaver, *Nephila clavipes*. Moreover, spider silks are useful models to study the relationship between amino acid sequences, block distributions and compositions, chain length, and functional properties such as mechanics. Predicting block copolymer self-assembly behavior *in vitro* is challenging. Such experimental techniques are important to provide insight into block copolymer behavior, yet insight into mechanisms that govern self-assembly of these types of structural proteins remains limited. The complexity of the self-assembly processes with multiblock copolymers, in particular proteins, is also exacerbated by the lack of theories to describe the morphology and structure from sequence data, except for the simplest synthetic diblock copolymers (Mai and Eisenberg, 2012). For example, the Flory–Huggins parameter (χ_{AB}) is typically used to quantify the degree of incompatibility between

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http://dx.doi.org/10.1016/j.jsb.2014.03.004
1047-8477/

the hydrophobic and hydrophilic blocks or domains. However, phase diagram predictions using the self-consistent mean-field (SCMF) theory and χ_{AB} parameters are still limited to diblock or triblock copolymers in bulk without solvents (Matsen and Schick, 1994). Therefore, advanced simulation approaches can fill the gap between experimental and theoretical studies with more complicated copolymers, such as those described in this work. This need is amplified with proteins, where complexity in sequence chemistry due to the 20 different amino acid chemistries, combined with the role of an aqueous environment, prohibit simple design rules and facile identification of fundamental rules that govern self-assembly.

Previously, we demonstrated the role of poly(alanine) distributions on β-sheet formation in spider silk block copolymers, using Fourier transform infrared spectroscopy (FTIR) and wide angle X-ray scattering (WAXS) to demonstrate that the number of poly(A) domains had a direct influence on the formation of crystalline β-sheets (Rabotyagova et al., 2010). In the present study, the goal was to understand the effect of peptide block length on the propensity to support self-assembly, a key step in formation of functional materials. Two different spider silk block copolymer designs were studied to provide this insight, copolymers $H(AB)_2$ and $H(AB)_{12}$. Here the A block is composed of a hydrophobic β-sheet forming poly(A) sequence, the B block consists of four hydrophilic GGX repeats, and the H block (a histidine tag) is made of six histidine residues and a short linker sequence to facilitate the purification of the recombinant proteins. In addition to the experimental approach to study assembly, a computational modeling tool based on coarse-grained (CG) dissipative particle dynamic (DPD) simulations (Groot and Warren, 1997) was employed to predict spherical aggregates formation *in silico* for comparisons to the experimental data. The DPD model is a representative CG approach that has been widely applied to study the structural evolution of polymer solutions. The results suggest that in addition to β-sheet content, the number of blocks (repeats) in a spider silk-like copolymer (e.g., polymer chain length), is an essential parameter that needs to be considered for successful materials formation.

2. Materials and methods

2.1. Materials

The pET30a(+) vector was obtained from Novagen (Madison, WI). Synthetic oligonucleotides were purchased from Invitrogen (Carlsbad, CA). Restriction enzymes, calf intestinal phosphatase (CIP), T4 ligase, and Quick ligation kits were purchased from New England Biolabs (Beverley, MA). DNA purification was performed using kits from Qiagen (Valencia, CA). Ninitrilotriacetic acid (Ni–NTA)-agarose resin for protein purification was acquired from Qiagen (Valencia, CA). Slide-A-Lyzer dialysis cassettes were obtained from Pierce (Rockford, IL). The 1 kb DNA ladder, NuPAGE 4–12% Bis-tris protein gels, SeeBlue Plus 2 protein standard, and T1 phage-resistant cells were purchased from Invitrogen (Carlsbad, CA). All cloning steps were performed in *Escherichia coli* strain DH5a from Invitrogen (Carlsbad, CA). Protein expression was carried out in *E. coli* strain BL21DE3 (Invitrogen, Carlsbad, CA). Kanamicine and imidazole were purchased from Sigma (St. Louis, MO). Silicon wafer chips were obtained from Ted Pella, Inc. (Redding, CA). Other chemicals with the highest grade of purity were purchased from Fisher Scientific (Pittsburg, PA).

2.2. Cloning, expression, and purification of spider silk block copolymers

The spider silk block copolymers were constructed and cloned into a commercially available expression pET30a(+) vector in a similar fashion to the procedure we have described previously (Rabotyagova et al., 2009; Tokareva et al., 2013). The amino acid sequence of the A and B blocks were derived from major ampullate dragline silk I of *N. clavipes* (Accession Number: P19837). The coding sequences of two spider silk-like blocks were AB and AB_3, where are A is a hydrophobic block and B is a hydrophilic block, were designed to carry *SpeI* and *NheI* restriction sites at the ends of the coding sequences for the ligation of the blocks into a modified pET30a(+) vector. By using a step-by-step directional ligation approach, direct control over the assembly of monomeric genes into complex sequences was achieved. Ligation reactions were carried out with T4 DNA ligase (Invitrogen, Carlsbad, CA) at 16 °C.

The cloned constructs were transformed into the bacterial host for protein expression. Larger-scale expression was performed using a fermentor (BioFlow3000, New Brunswick Scientific Co., Edison, NJ). Minimal medium supplemented with 1% yeast extract was employed (Bini et al., 2006). Ammonia was used to maintain the pH at 6.8. When the pH exceeded 6.88 a feed solution (50% glucose, 10% yeast extract, 2% $MgSO_4 \times 7H_2O$) was added. All culture media contained kanamycin (50 μg/mL). Expression was induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Fisher Scientific, Hampton, NH) when the optical density, OD_{600} was between 10 and 15.

Protein purification was performed under denaturing conditions on Ni–NTA resins (Qiagen, Valencia, CA) using our previously published procedure (Rabotyagova et al., 2009). The spider silk block copolymers were eluted using urea-based buffer (pH 4.5). The proteins were dialyzed against water using Slide-A-Lyzer Cassette (Pierce, Rockford, IL) with MWCO of 2000 Da. Dialyzed proteins were lyophilized. The purity of expressed proteins was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) followed by Colloidal Blue staining. Protein identity was confirmed by matrix-assisted laser desorption ionization mass spectrometry (MALDI-TOF; Tufts Core Chemistry Facility, Boston, MA) and amino acid sequencing (MALDI-TOF; Tufts Core Chemistry Facility, Boston, MA).

2.3. Dynamic light scattering (DLS)

Concentrated and dilute aqueous solutions of spider silk block copolymers were analyzed by DLS (Brookhaven Instruments Corporation, Holtsville, NY). DLS was performed using a 532 nm laser at room temperature with a scattering angle of 90°. To prepare concentrated silk solutions, lyophilized recombinant silk proteins were dissolved in 9.3M LiBr at various concentrations followed by dialysis against DI water.

2.4. Scanning electron microscopy (SEM)

SEM was used to assess morphological characterization of the spider silk block copolymers. The experiments were performed using a Zeiss 55Ultra and a Zeiss Supra Systems (Harvard University Center for Nanoscale Systems, Cambridge, MA). Lyophilized spider silk block copolymers were dissolved in water to a final concentration of 1 mg/mL and dried on a silicon chip in a closed container at room temperature. Images were taken using InLense and SE2 detectors at 5.00 kV.

2.5. Atomic force microscopy (AFM)

AFM imaging and force spectroscopy measurements were performed on an Asylum Research MFP-3D-Bio Atomic Force Microscope (Asylum Research, an Oxford Instruments Company, Santa Barbara, CA). For imaging in fluids, 80 μL sample was deposited as a drop on a silicon substrate and imaged. IgorPro 6.22A image analysis software was used to determine the heights of observed

Please cite this article in press as: Tokareva, O.S., et al. Effect of sequence features on assembly of spider silk block copolymers. J. Struct. Biol. (2014), http://dx.doi.org/10.1016/j.jsb.2014.03.004