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Research Update: Programmable tandem repeat proteins inspired by squid ring teeth

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Cephalopods have evolved many interesting features that can serve as inspiration. Repetitive squid ring teeth (SRT) proteins from cephalopods exhibit properties such as strength, self-healing, and biocompatibility. These proteins have been engineered to design novel adhesives, self-healing textiles, and the assembly of 2d-layered materials. Compared to conventional polymers, repetitive proteins are easy to modify and can assemble in various morphologies and molecular architectures. This research update discusses the molecular biology and materials science of polypeptides inspired by SRT proteins, their properties, and perspectives for future applications.
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INTRODUCTION

The mysterious world of underwater creatures has fascinated scientists for centuries. Aquatic animals often hide in the depths of seas and oceans, and the discovery of new species of deep-sea animals continues to this day. In the last two decades, for example, complete specimens of a colossal squid were found. There are over 350 different types of squids, which present some of the most extraordinary adaptation and survival strategies imaginable.[1] Squids have developed several features that allow them to be successful predators (Fig. 1), e.g., highly sophisticated nervous systems,[2] reflective tissue allowing camouflage,[3] gladii,[4] sharp and rigid beaks,[5] strong tentacles,[6] and numerous squid ring teeth (SRT) that line these tentacles.[7] The beak and SRT have attracted the interest of materials engineers due to their unique composite structure and extreme toughness.[8] The beak is a composite of histidine-rich protein matrix and chitin fibers.[5] Similar to the beak, SRT also exhibit interesting mechanical properties.[7] Squid tentacles allow fast and agile movement but also are used in defense and for predation.[6] The suction cups are aligned along the oral surface of the tentacles and act as anchors that strengthen the grip.[7] These cups contain an interior ring equipped with sharp teeth.[7] SRT are composed of a highly stiff, naturally occurring biomaterial with an elastic modulus (E) in the range of a 4-8 GPa.[9] SRT proteins are segmented co-polymers with a molecular structure based on alternating semicrystalline and amorphous domains.[10]

The first study that aimed to examine the amino acid sequences of proteins assembling into SRT utilized a combination of several techniques, i.e., high-throughput RNA-sequencing, proteomics, and advanced material characterization.[9] A protein's structure is determined by its amino-acid sequence, but the possible sequence space is astronomically large. Fibrous proteins (e.g., silk, collagen, elastin, SRT—squid ring teeth) have a reduced combinatorial search space due to repetitive sequences. For example, for a protein sequence of length L, the search space of a repetitive sequence drops from 20^{L} to $20^{L/N}$, where N is the repeat number (e.g., for a 100 amino acid sequence with 10 repeats, the number is $20^{10} \sim 10^{13}$ instead of $20^{100} \sim 10^{130}$). The simplified segmented molecular architecture of

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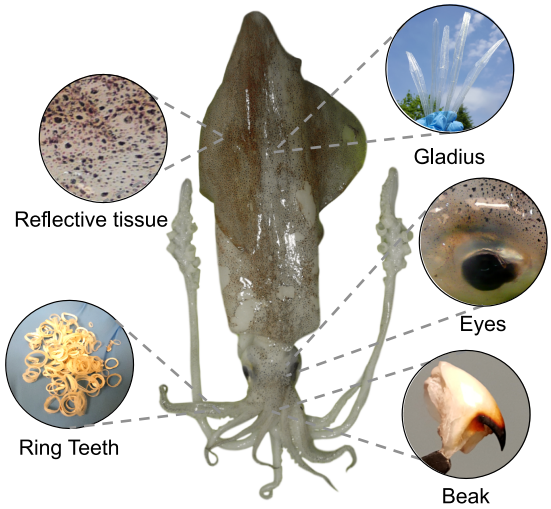

FIG. 1. Anatomy of the European common squid (*Loligo vulgaris*): *L. vulgaris* is a coastal cephalopod found in the Mediterranean and in the eastern Atlantic Ocean. The species has eight short arms and two long tentacles surrounding the mouth and beak. All ten limbs are lined with numerous suction cups equipped with squid ring teeth (SRT). The squid's body is supported by the gladius, a rigid internal structure composed of chitin. The eyes of *L. vulgaris* can detect polarized light, and its reflective tissue allows the manipulation of the overall body coloration. The beak, SRT, and gladius are often referred as the squid's "hard tissues" because of their excellent mechanical strength. These biological materials serve as inspiration to material scientists for the development of advanced functional materials.

fibrous proteins further simplifies the problem by constraining the sets of amino acids used in each segment of the repeating sequence. SRT proteins have highly modular sequences. The main repeat unit consists of a crystalline-forming region (β-sheets, which are stabilized by hydrogen bonds)[9] and an amorphous region, which appear alternately within the amino acid sequence. The crystalline-forming region is rich in Ala and His, while the amorphous region is rich in Tyr and Gly. The sequence linking both regions often contains Pro as the breaker between amorphous and semicrystalline regions.[12] This system allows a reduced amino-acid palette (A,S,V,T,H,G) for SRT proteins, which results in $\sim 10^7$ possible sequences for a crystal-forming region of length ten in a fibrous protein.

Although composition of SRT proteins have been known since the 1970's,[7] they recently gained attention of several research groups, including ours, due to their unique behavior. We are exploring both native and recombinant SRT proteins and their biosynthetic variants in order to fabricate materials with tunable properties such as extensibility, stiffness, tensile strength, toughness, conductivity, optical transparency, and self-healing abilities.[11–13] These materials are highly desirable for wound dressing, electronic devices, adhesives, optics, sensors, and many more high-tech applications.[14–16]

This research update constitutes a compendium of knowledge gathered until now about the molecular composition and physicochemical properties of SRT-inspired proteins developed in our group. Moreover, we present recent progress concerning the production of recombinant SRT proteins and SRT-based tandem repeat polypeptides, with a view toward material fabrication and potential applications.

PRODUCTION OF RECOMBINANT AND SYNTHETIC SRT PROTEINS

Self-assembling proteins are valuable building blocks allowing the construction of materials with versatile chemical properties and functions based on their tertiary and quaternary protein structures.[17,18] The ability to produce such proteins via genetic engineering allows the design of new functions and chimeric structures.[16,19,20] Hence, protein engineering has widened the repertoire of building blocks beyond native sequences. Well-studied motifs from structural proteins such as silk, elastin, collagen, keratin, resilin, and recently SRT have been frequently used in combination to create multifunctional biomaterials for diverse applications.[12,21]

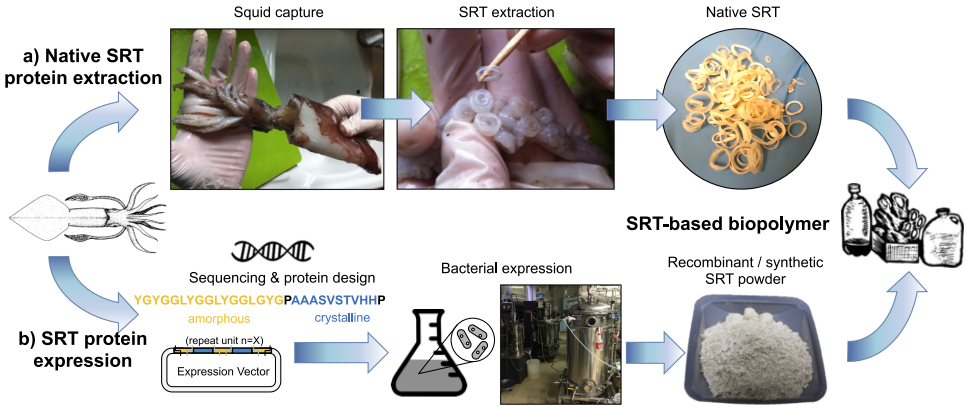

FIG. 2. Extraction and expression of SRT proteins: Native SRT protein complexes are extracted directly from squid's suction cups (upper arrow). Reproduced with permission from Pena-Francesch *et al.*, Adv. Funct. Mater. **24**(47), 7401 (2014).[12] Copyright 2014 Wiley. Biosynthetic routes (lower arrow) are used to obtain the recombinant and *de novo* designed proteins with particular molecular weights and sequence. The identified sequences of the protein of interest are produced in the chosen type of expression system, i.e., bacteria or yeast, using genetic engineering toolbox. Reproduced with permission from Jung *et al.*, Proc. Natl. Acad. Sci. U. S. A. **113**(23), 6478 (2016).[32] Copyright Author(s), licensed under a Creative Commons Attribution 4.0 License.

Besides the extraction from natural sources, structural proteins, including those derived from SRT, can be also produced on an industrial scale using genetically modified organisms (Fig. 2). From the perspective of material design, there are several advantages to the heterologous production of structural or fibrous proteins.[15,16,18] First, the composition and length of the designed protein sequence are genetically controlled (i.e., the process of protein expression in a host cell results in monodisperse products). Second, the molecular structure of a protein can be tuned by manipulating the amino-acid sequence. Next, utilizing functional groups of canonical (i.e., thiol, amine, phenol) or non-canonical (i.e., halide, azide, olefin, oxime, hydrazone, boronic ester) amino acids[22] allows highly specific conjugation of proteins to other molecules or synthetic polymers.[23] Last, functional polypeptides (e.g., antimicrobial, helix-coil transitions, surface adhesion) can be incorporated by *de novo* design of amino-acid sequences, resulting in a protein that exhibits properties tailored for the desired material type.[24] Beyond material-design concerns, protein-based materials are also

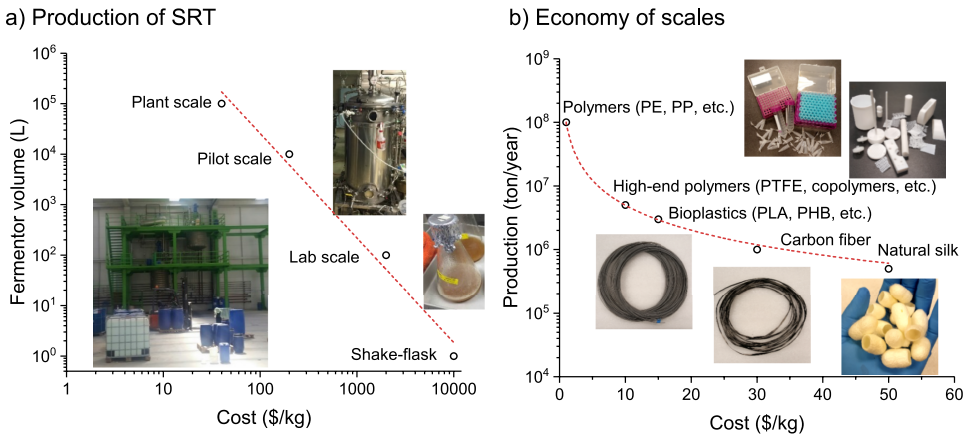

FIG. 3. Scalable production of structural proteins: (a) bioreactor size as a function of structural protein production. The numbers are estimated based on recombinant SRT production where the plant scale is an extrapolation based on smaller scale fermentation. (b) Economy of scales for polymeric materials. The cost of production of materials made of recombinant protein-based polymers decreases with the production scale up and this can constitute a cheaper and/or more environmentally friendly alternative for other material types.

highly desirable due to their favorable biocompatibility and biodegradability properties for medical applications.[16,25]

Although the progress of structural-protein-based biomaterials has gained significant momentum, several roadblocks still exist for this technology to reach its maximum potential. Specifically, the major challenge is the aggregation profile of SRT proteins expressed in bacterial expression systems (i.e., inclusion bodies), which limits the yield of production. In order to be industrially feasible, the production should have rates in the kilogram range rather than in grams. As shown in Fig. 3(a), SRT protein production is still performed in a university facility using an 80 L fermenter (protein purity of >90% and yield of ~0.05 g/l). These values could be increased in a pilot-scale run, but the development of the scale-up itself is expensive.[26] The high production cost (>$100/kg with <90% purity) does not only concern SRT proteins; this presents an even greater problem for high-molecular-weight repetitive proteins (including silk).[27] The economies of scale for industrial polymeric-material production (including natural cocoon silk) are summarized in Fig. 3(b). The graph highlights that the production cost could be reduced drastically as the scale of production goes up. We note that optimistic results with significantly higher protein production yields (>10 g/l) have been reported in expression systems other than bacteria (e.g., yeast) for production of collagen[28] and silk.[29]

TANDEM REPEAT PROTEINS DESIGN

Repetitive patterns are found in different classes of proteins including structural, membrane, and globular proteins. Tandem repetition intrinsically promotes stability through the periodic recurrence of favorable interactions; hence modular reuse allows for a stepwise increase in functionality in the biomaterial design. However, the design of highly repetitive sequences for structural proteins present challenges for protein engineering. For example, the construction of the synthetic genes encoding them can fail or generate nonspecific products.[16] Figure 4(a) summarizes three different methods of design strategy for producing tandem repeat genes. Conventional cloning, in which monomers are fused together into long concatemers in a step-by-step fashion, has been applied frequently in the

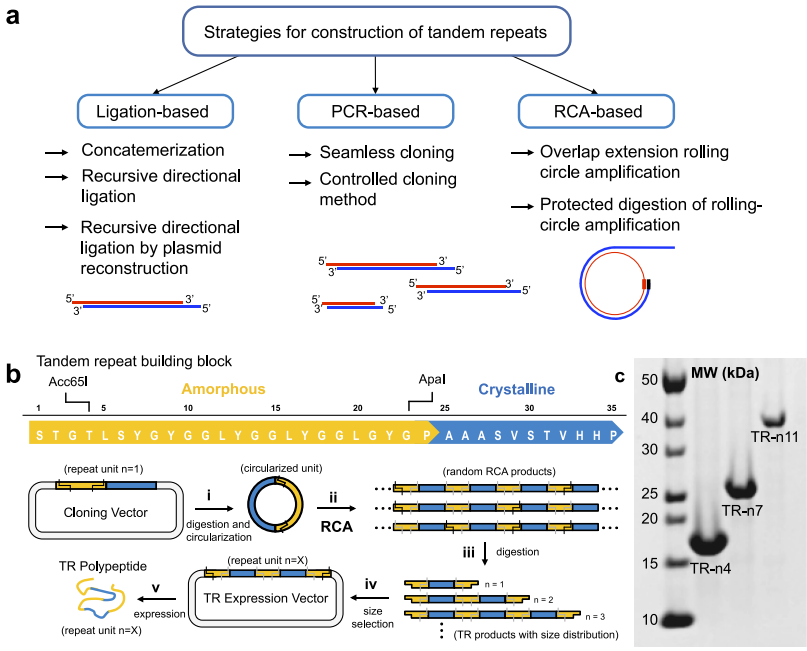

FIG. 4. Tandem repeat protein design: (a) three different strategies of tandem repeat gene synthesis. (b) PD-RCA method developed for creating tandem repeat proteins mimicking SRT proteins. (c) SDS-Page gel of biosynthetic SRT proteins with 4, 7, and 11 repeats. Reproduced with permission from Jung *et al.*, Proc. Natl. Acad. Sci. U. S. A. **113**(23), 6478 (2016).[32] Copyright 2016 Proceedings of National Academy of Sciences.

past[19] but is labor-intensive and time-consuming. Many newer protocols have been developed based on strategies such as seamless cloning and recursive directional ligation (RDL).[30] However, these methods still require numerous steps, are difficult to run in parallel, and do not provide tunable control over a range of molecular weights. The cloning strategy addressing these issues was reported earlier.[31] The authors used overlap extension rolling circle amplification (OERCA) to synthesize, in the parallel way, the genes encoding repetitive elastin-like protein-polymers. Recently, we proposed another method allowing construction of long repetitive sequences, denoted as protected digestion of rolling circle amplification [PD-RCA, Fig. 4(b)].[32] In PD-RCA, a circular repeat unit can be amplified continuously by the phi29 polymerase in the presence of both dCTP and methylated dCTP. Restriction enzyme sites (Acc65I in this case) containing cytosine can be digested while those containing 5-methylcytosine are protected, resulting in a distribution of tandem-repeat product sizes. One advantage of PD-RCA is the synthesis of different repeat-number of oligomer products in a single reaction. Hence, we demonstrated generation of a large library of the genes encoding proteins of different sizes with the same repeat unit. Using this method, we expressed tandem repeat proteins inspired by SRT [Fig. 4(c)] with the ultimate goal of revealing sequence-structure-property relationships in these proteins (e.g., identifying sequences that provides self-healing properties).[33] The major advantage of PD-RCA, unique among all competing methods of which we are currently aware, is the potential for tandem repetition of a large library of input sequences in a single reaction without the production of chimeric sequences. This feature enables large-scale evaluation of perfect-repeat sequences, given an analysis method with suitably high throughput.

HIGH-THROUGHPUT SCREENING OF TANDEM REPEAT PROTEINS

Design of novel *de novo* structural proteins from their primary amino acid sequence is an unsolved scientific problem. Moreover, most techniques that are used for predicting the protein structure rely on globular protein-structure databases and are rarely applicable to structural-protein assemblies.[34] Experimental characterization for this class of proteins often employs spectroscopic techniques including but not limited to Fourier transform infrared spectroscopy (FTIR), X-ray crystallography, Raman spectroscopy,[35] or light scattering.[36] Moreover, current high-throughput techniques based on flow cytometry or affinity assays can only screen protein libraries up to $10^{6\text{-}8}$ within a reasonable timeframe, which is inadequate to build a comprehensive sequence database for structural proteins. Furthermore, existing high-throughput fluorescence and affinity readouts are structure-specific and may miss materials with novel molecular morphologies.

We developed a network model based on entropic elasticity[37] to predict structure-property relationship for proteins [Fig. 5(a)]. Topological defects in protein networks have a strong impact in the physical properties. Therefore, investigation of topological network defect types as well as secondary structures will improve the prediction of physical properties of protein-based materials. The secondary structures of native and synthetic SRT proteins (mostly β-sheets and disordered random coils) have been analyzed by FTIR, showing evidence of secondary structure changes as a function of crystal-forming amino-acid sequence or mechanical or chemical stimuli (e.g., methanol treatment increases the crystallinity of SRT and silk fibroin).[38–40] A strong amide I band with a maximum peak centered between ~1650 cm^{-1} (majorly disordered) and ~1620 cm^{-1} (β-sheet rich) is usually leveraged for the secondary structure analysis in this class of proteins [Fig. 5(b)]. However, these structural-characterization methods have limited application in high-throughput polypeptide libraries.

Recently, we studied the structure-property relationship of silk and SRT proteins using ultrafast laser-probing spectroscopy.[38] We performed time domain thermal transmissivity (TDTT) experiments using purified SRT and silk proteins as well as overexpressed SRT recombinant proteins in *E. coli*, which clearly showed "proof of concept" quantification of protein crystallinity in real time [Fig. 5(c)]. Our novel TDTT technique theoretically enables screening of 10^{8}-10^{9} different structural polypeptide sequences for protein assembly in hours, a feat that would be impossible to achieve with existing screening tools such as fluorescence, immunostaining, or functional assays. We measured the thermo-optic properties of proteins using picosecond TDTT pump-probe measurements [Fig. 5(d)].[38] In these experiments, the pump is used to trigger a rapid thermal process in the sample and the probe beam

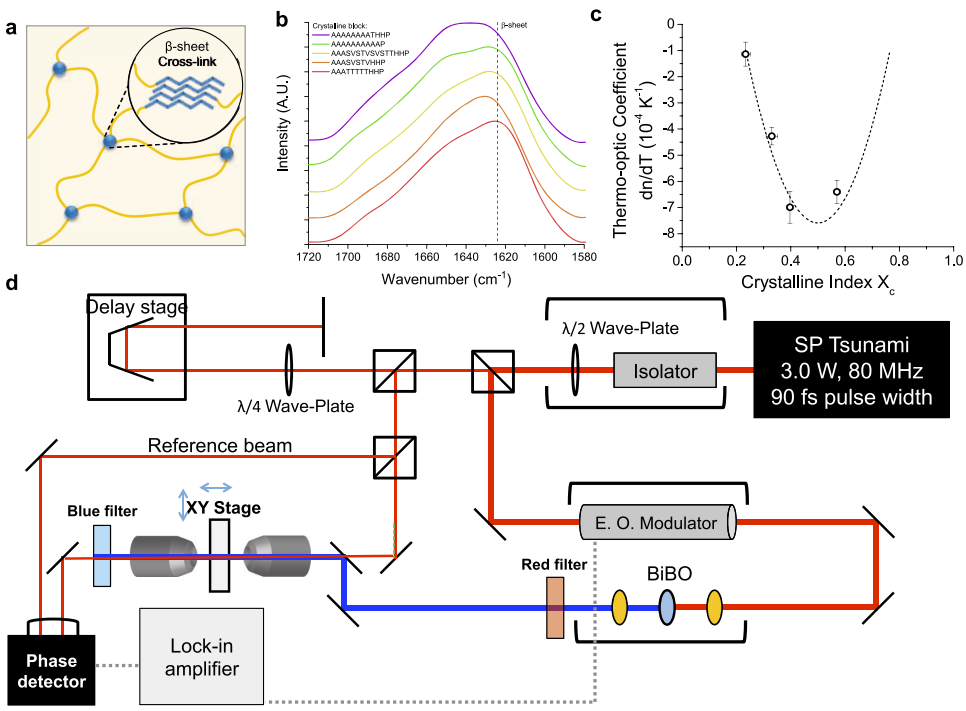

FIG. 5. Tandem repeat protein design: (a) schematic of self-assembled cross-linked proteins. (b) Fourier transform infrared spectroscopy (FTIR) confirms the secondary structure of selected polypeptides from libraries to validate the high-throughput approach. (c) Correlation between crystallinity as a function of thermo-optic coefficient obtained from TDTT measurements. Reproduced with permission from Jung *et al.*, Analyst **142**(9), 1434–1441 (2017).[38] Copyright 2017 Royal Society of Chemistry. (d) Schematic of time-domain transient thermal transmissivity (TDTT). The sub-picosecond resolution analyzes large sets of protein samples, allowing for the high-throughput screening of combinatorial libraries and directing the sequence design.

is used to examine the excited relaxation dynamics and energy changes of excited volume. This technique will help us to identify structural proteins that have the ability to assemble and form cross-linked biomaterials.

PHYSICAL PROPERTIES OF SRT BASED MATERIALS

SRT provide a strong grip on a squid's prey and therefore require high mechanical strength. The segmented sequence (alternating segments of amorphous and crystalline domains) and semicrystalline morphology of SRT proteins facilitate the formation of a β-sheet-stabilized network that provides the necessary strength (Young's modulus of 1 GPa).[9,12] In the glassy state, the amorphous chains are locked in dense hydrogen bond interactions and cannot move past each other, resulting in a high strength and stiff material (GPa modulus) as shown in Fig. 6(a).[10] On the other hand, the amorphous chains are mobile in the rubbery state (i.e., above the glass transition temperature) while the crystalline β-sheet domains act as physical crosslinks. The resulting protein network gives elastomeric properties to the SRT materials, which have a plateau modulus in the MPa range and semi-reversible extensibility up to 300% strain [Fig. 6(b)]. The mechanical properties in both the glassy and rubbery states can be tailored to specific needs by varying the chain length, the protein morphology, and the cross-linking chemistry, which offers a wide range of design possibilities for SRT materials with moduli from kPa to GPa.[32]

The reported self-healing properties of SRT proteins also arise from the semicrystalline and amorphous morphology [Fig. 6(c)].[10,33] SRT materials can self-heal in their rubbery state; the hydrogen-bonded network that stabilizes the material is repaired under mild conditions of temperature (45-70 °C), hydration (20%-40% water content), and pressure (1 MPa).[10–12] The self-healed protein materials did not show signs of degradation or loss of properties after an undefined number of

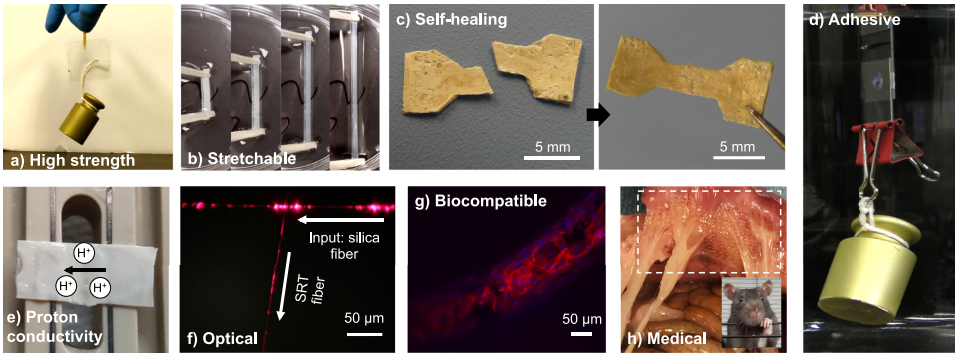

FIG. 6. Properties of SRT protein-based materials: SRT materials exhibit the following properties: (a) high mechanical strength (i.e., modulus in the GPa range in the glassy state). (b) Extensibility (i.e., up to 300% strain in the rubbery state). (c) Self-healing (i.e., broken SRT materials can be repaired without degradation). Reproduced with permission from Sariola *et al.*, Sci. Rep. **5**, 13482 (2015).[10] Copyright 2015 Nature Publishing Group. (d) Underwater adhesion (adhesion strength up to 2.5 MPa). Reproduced with permission from Pena-Francesch *et al.*, Adv. Funct. Mater. **24**(39), 6227 (2014).[11] Copyright 2014 Wiley. (e) Proton conductivity (1-5 mS/cm). (f) Optical. Reproduced with permission from Yilmaz *et al.*, ACS Photonics **4**(9), 2179 (2017).[48] Copyright 2017 ACS Publications. (g) Biocompatibility (support cell growth). (h) SRT are applied as a coating to augment mesh-tissue integration leading to improvements in abdominal wall stability in animal models. Reproduced with permission from Leberfinger *et al.*, Adv. Healthcare Mater. (submitted).[51] Copyright 2017 Wiley.

self-healing cycles, demonstrating that self-healing of SRT is a reversible process.[10–12] Furthermore, SRT can be used as additive or coating to provide self-healing properties to composite materials such as fibers and textiles.[33] SRT materials not only repair themselves but can also adhere to an array of substrates.[11] SRT show underwater reversible adhesion strength [Fig. 6(d)] up to 2.5 MPa over a wide range of pH and ion concentration.[11] The strength of underwater adhesion is significantly higher (i.e., at least ten times stronger) than that of other biological and bioinspired adhesives such as mussel adhesive plaque,[41] gecko footpad,[42] or sandcastle worm glue.[43]

SRT proteins have programmable conducting and optical properties that are ultimately governed by their molecular structure. Proton conductivity in SRT proteins [Fig. 6(e)] has been investigated by impedance spectroscopy, revealing programmable conductivity in the 1-5 mS/cm range.[44] The proton conductivity values are among the highest reported for proton-conducting biological materials.[45–47] Moreover, SRT exhibit structure-dependent optical and thermal properties (thermo-optic response) that open up the possibilities in the design and fabrication of protein devices, such as waveguides, microresonators, add-drop filters, and switches [Fig. 6(f)].[13,38,48] Due to their structure-dependent properties and the simplicity of their fabrication, SRT materials show great potential in bioelectronics and biophotonics, allowing fine-tuning of the device's response.[49,50]

SRT proteins are also biocompatible and support cell growth [Fig. 6(g)],[51] which expands the applications of SRT-based materials into the biomedical field. The facile synthesis and processing of SRT materials become a major advantage when fabricating biocompatible materials with a desired set of properties. For example, surgical meshes are coated with SRT proteins for improvement hernia repair and showed increased tissue strength in animal models [Fig. 6(h)].[51]

PROCESSING AND APPLICATIONS OF SRT BASED BIOMATERIALS

Solution and thermal processing of structural proteins are extensively studied.[25,50,52] In most processing methods, proteins are exposed to various conditions (temperature, pressure, pH, presence of solvents, etc.). SRT proteins can be processed using both thermal and solution methods, which are common in the polymer industry. We highlight these processing and fabrication possibilities of SRT proteins below, and schematically describe in Fig. 7.

Solution-based processing is the most common method used in structural proteins. Roughly, it involves the solubilization of a protein in solvent and subsequent aggregation by removal of the solvent via ambient or vacuum assisted evaporation. Water-soluble proteins can be easily dissolved in aqueous buffers, but the solubilization of water-deficient structural proteins requires the disruption of

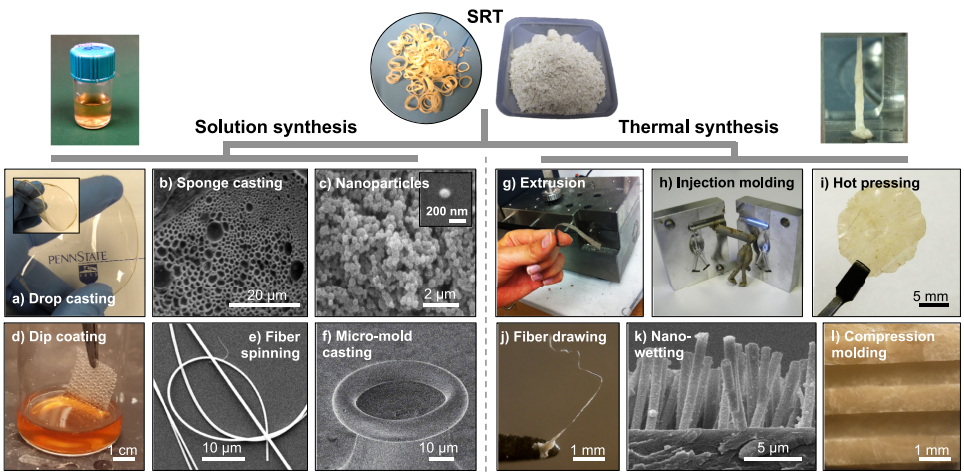

FIG. 7. Production of SRT protein-based materials: SRT materials are fabricated either by thermal or solution-based synthesis: (a) Thin films are fabricated by drop casting. (b) Sponges by particle-assisted casting and subsequent etching of the particles. (c) Nanoparticles are prepared by salting out or by addition of a surfactant. Reproduced with permission from Pena-Francesch *et al.*, Adv. Funct. Mater. **24**(47), 7401 (2014).[12] Copyright 2014 Wiley. (d) Coating of textiles and other substrates is performed by dip coating. Reproduced with permission from Leberfinger *et al.*, Adv. Healthcare Mater. (submitted).[51] Copyright 2017 Wiley. (e) Microfibers are prepared by electrospinning. Reproduced with permission from Pena-Francesch *et al.*, Adv. Funct. Mater. **24**(47), 7401 (2014).[12] Copyright 2014 Wiley. (f) Complex 3D geometries (such as WGM microresonators) are fabricated by mold casting. Reproduced with permission from Yilmaz *et al.*, ACS Photonics **4**(9), 2179 (2017).[48] Copyright 2017 ACS Publications. (g) fibers and rods are fabricated by extrusion, (h) complex 3D geometries are fabricated by injection molding, (i) thin films are fabricated by hot pressing, (j) fibers are fabricated by drawing, (k) nanoscale objects are fabricated by template-assisted nanowetting, and (l) patterned surfaces are fabricated by compression molding. Reproduced with permission from Pena-Francesch *et al.*, Adv. Funct. Mater. **24**(47), 7401 (2014).[12] Copyright 2014 Wiley.

aggregated protein structure. This step can involve acidic or basic conditions and the addition of salts or organic solvents. However, disruption of crosslinks is only possible for weakly bonded assemblies (e.g., hydrogen-bonded β-sheet proteins such as silk and SRT or hydrophobic interactions in helical polypeptides). For these proteins, aggregation or precipitation can be re-initiated by several methods such as salting out, isoelectric precipitation, addition of miscible solvents, or evaporation of the solvent.[52] These conditions can cause proteins to misfold or become kinetically trapped into undesirable assembly states; hence, the physical properties of these samples may vary significantly compared to native assemblies. Solution-based processing also involves the generation of solvent waste, increased processing time due to drying and purification steps, and the possibility of irreversible aggregation. For example, solution-based processing methods for the fabrication of silk-based materials have been developed,[52] but all require prior degumming and solubilization of silk fibroin, which involves additional dialysis steps and the use of harsh chemicals. Similarly, many solution-based methods have been proposed for the fabrication of aligned collagen, but the resulting mechanical properties of the reconstituted materials are significantly lower than that of natural collagen due to the lack of hierarchical structure.[53]

Solution-based processing is applicable to SRT proteins as it is to many other biological materials. SRT proteins are stabilized by β-sheet structures that act as physical crosslinks and are not water-soluble. Therefore, the β-sheet elements must be disrupted in order to solubilize the protein. To this purpose, aqueous and organic solvents including acidic/basic solutions (i.e., acidic pH below 3 and basic pH above 10 with solubility up to 1 mg/ml), salts (i.e., lithium bromide, calcium chloride, calcium nitrate, guanidinium chloride, etc.), surfactants (i.e., sodium dodecylbenzenesulfonate), and organic solvents (i.e., dimethylsulfoxide, hexafluoroisopropanol (HFIP) with concentrations up to 300 mg/ml) have been used to prepare SRT protein solutions depending on the solubility limit of the specific solvent cocktail (i.e., higher protein concentration results in high viscosity that may complicate the processing steps).[12,32] Transparent and flexible freestanding films (Young's modulus of 0.7-0.8 GPa) with thickness ranging from a few micrometers to several hundred micrometers can be easily fabricated by drop casting (e.g., 50 μm flexible SRT films were cast from 50 mg/ml protein HFIP

solutions), shown in Fig. 7(a).[12,32] SRT-based sponges are prepared by a particle-templating casting process, which consists of casting a SRT-microparticle/nanoparticle composite and subsequent etching of the particles [Fig. 7(b)].[52] This process allows for a broad range of particle materials because SRT are not soluble in water or most organic solvents, which facilitates particle etching. The pore size is designed by selecting the appropriate particle size and ultimately controls the mechanical properties of the final sponge. SRT nanoparticles are prepared by multiple aggregation methods based on salting out, solvent exchange, or isoelectric aggregation [Fig. 7(c)].[12] SRT proteins are used for biomolecule encapsulation and controlled release, such as the encapsulation of enzymes, DNA, and dyes.[25] Coating of complex substrates such as knitted or woven fabrics is performed by dip coating to fabricate advanced textiles that share SRT's properties [Fig. 7(d)].[33] Microfibers are spun by several methods including miscible solvent exchange and electrospinning [Fig. 7(e)].[12] Complex objects such as toroidal whisper-gallery-mode microresonators are made by micro-/nano-mold casting, enabling the fabrication of protein-based photonic devices [Fig. 7(f)].[48] Solution-based processes were used in both physically and chemically cross-linked proteins (e.g., silk and resilin, respectively). SRT-based materials also offer solution-based processes with several advantages including ease of processing (reduced solubilization and purification steps), recyclability (reversible cross-linking due to hydrogen bonds), and high-stability (in a wide range of temperatures—RT to 200 °C—and in aqueous and organic solvents).

Thermoplastic processing consists of softening the processed material with heat, forming it into a particular shape while soft, and hardening by cooling. These strategy methods offer a series of advantages over solution processing that primarily derive from minimization of solvent usage in the process. The structure and properties of the materials are usually conserved since defects arising from residual solvents are reduced. Due to the reversible nature of their thermal transitions, thermoplastic materials can be processed multiple times over their life cycle and therefore can be recycled. These aspects, together with the versatility and low cost of the production systems, make thermoplastic processing methods the most extensively used in the polymer industry.

Thermoplastic processing of protein-based biopolymers is limited by their thermo-mechanical properties. Structural proteins are typically processed above their glass transition temperature T_g, above which the polypeptide chains relax and the mechanical properties dramatically drop. However, the processing temperature should not exceed the denaturation temperature T_d, at which the protein unfolds and irreversibly aggregates (for globular proteins), or the thermal degradation temperature T_{deg}. The temperature range in which the protein can be processed (between T_g and T_d or T_{deg}) is typically very narrow and often limits the processing options. Hence, plasticizers such as water, or certain oils or alcohols (e.g., glycerol, butanediol) are mixed with protein to increase the mobility of the protein chains and therefore decrease T_g.[54] This results in a broader processing temperature range and expands the processing capabilities of the plasticized material system (i.e., the material is softer at lower temperatures, and therefore it is easier to shape). SRT proteins are stable up to temperatures of 200 °C and have a glass transition temperature in dry conditions of $T_g \sim 185$ °C.[11] Aided by plasticizers such as water, T_g is decreased to room temperature or even below 0 °C and the processing temperature range is significantly broadened.[12] This enables the thermoplastic processing of SRT proteins in mild conditions of hydration (5%–45% of water content), temperature ($T > 20$ °C), and pressure (1 MPa). These conditions facilitate easy processing, extend the manufacturing possibilities, and make SRT proteins a promising source for developing protein-based functional materials. Since SRT have thermoplastic properties, they can be processed with other biopolymers such as polylactic acid (PLA) or starch derivatives that can also be shaped by thermoplastic methods.[12] Conventional thermoplastic processing methods commonly used in polymer production systems have been successfully tested with SRT proteins. Extrusion [Fig. 7(g)], injection molding [Fig. 7(h)], hot pressing [Fig. 7(i)], and fiber drawing [Fig. 7(j)][12] are easily adapted to SRT by selecting the right plasticizing conditions, and a diversity of SRT-based micro-/macro-materials are successfully fabricated by these versatile methods. Likewise, the plasticizing of SRT allows for 3D protein printing, exploring new manufacturing possibilities for bioprinting and tissue engineering. In addition, the thermoplastic properties of SRT can also be exploited for the fabrication of nanomaterials such as thin films, nanotubes, nanoparticles, and complex nanoscale geometries and patterns for multiple applications in biotechnology (drug delivery, biosensing, surface wettability, etc.)

[Figs. 7(k) and 7(l)].[12] SRT-based materials can also be exposed to a series of post-processing treatments to control the nanostructure. These treatments include the exposure to temperature, humidity, pressure, and organic solvents (e.g., methanol) and have been used to induce crystallization and tailor the β-sheet content in SRT and other proteins up to ~60%-65%.[38,39,48] This last post-processing step provides additional customization of SRT materials since the physical properties (e.g., mechanical, thermal, optical) are ultimately governed by the nanostructure.

PERFORMANCE OF SRT-BASED MATERIALS

Due to their controllable sequence, programmable properties, and ease of processability, SRT proteins have shown exceptional potential in the development of electrical, thermal, and photonic devices. We reported the synthesis and fabrication of optical devices (i.e., whispering gallery mode resonator made of SRT proteins) as well as terahertz actuators and conductive composites using 2D layers of atomically thin crystalline inorganic materials. Figure 8 shows examples of SRT-based materials and devices for electronic (i.e., metallic 2D MXene composites and organic conductor composites),[55] terahertz (i.e., insulating 2D graphene oxide for actuators and bolometers),[56] and optics (i.e., protein-based waveguides, filters, and optical switches) applications.[48] 2D-layered materials establish the foundation of next-generation, programmable, flexible, optically superior, energy efficient, and mechanically strong materials and devices. Moreover, exploitation of unique material properties at the nanoscale opens up new doors for fundamental research. Recent advances in the nanotechnology of 2D materials combined with parallel improvements in biotechnology and synthetic biology have demonstrated that more complex composite materials with properties engineered precisely to optimize performance can be achieved.[55] Hence, we demonstrated the self-assembly of molecular composites of two-dimensional (2D) materials with the help of structural proteins. The amino-acid sequence of the proteins, which dictates the degree of crystallinity and alignment of the protein layers, can also be used to control the interactions at the 2D material/protein interface, ultimately dictating the functional physical properties (e.g., electrical resistivity and thermal conductivity) of these devices. Our work demonstrates the ability to use protein interfaces in contact

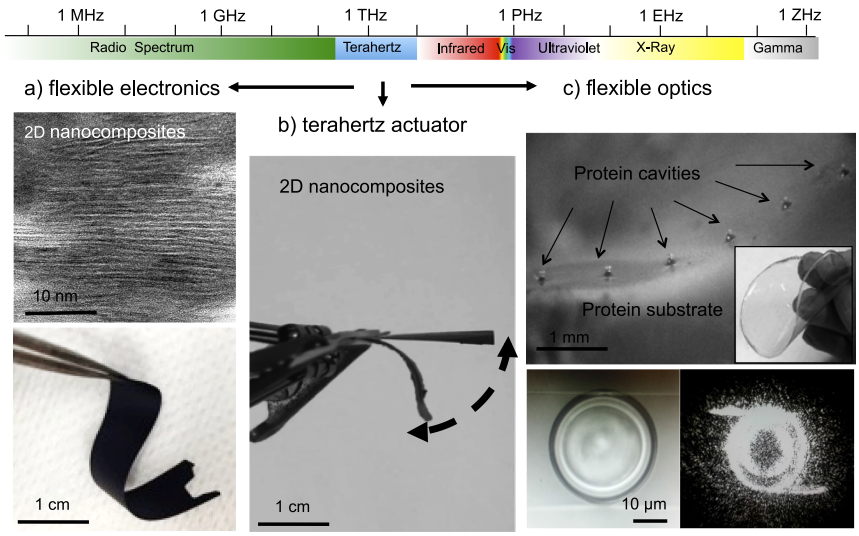

FIG. 8. Performance of SRT-based materials and devices: SRT-based molecular composites show potential use in flexible devices. SRT proteins act as assemblers in an array of 2D materials such as metallic titanium carbide (a), terahertz actuator applications made from layers of SRT and insulator graphene oxide (b), and protein-based resonant cavities for optical switch applications (c). Reproduced with permission from Yilmaz *et al.*, ACS Photonics **4**(9), 2179 (2017).[48] Copyright 2017 ACS Publications. Reproduced with permission from Demirel *et al.*, Adv. Funct. Mater. (published online 2017).[55] Copyright 2017 Wiley. Reproduced with permission from Vural *et al.*, Carbon **118**, 404 (2017).[56] Copyright 2017 Elsevier.

with 2D materials to control interfacial chemistry, electrical contact resistance, and thermal boundary resistance, which are nanoscale characteristics that are important to the operation of flexible 2D devices made from these materials. More importantly, our layered 2D systems, in which interlayer distances can be precisely and finely tuned by the molecular weight of the protein, offer a very interesting platform to study how optical, electrical, thermal, and mechanical properties can be controlled in a molecular composite. Successful development of programmable 2D composites will have a significant impact on multiple applications in various fields (e.g., synthetic biology, autonomy, nanotechnology, quantum materials and energy) and open new avenues of 2D material research.[55]

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