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STEM Degrees: 2 **STEM Participants:** 2

Major Goals: Fibrous proteins' material properties and self-assembly behavior can be manipulated over a wide response range through variations in their repeat-unit amino-acid sequences. High-throughput screening of tandem-repeat libraries will enable the discovery of sequences with distinctive physical properties and improve our understanding of self-assembly, leading to revolutionary advances in materials and life sciences. We will test this hypothesis via two distinct but interrelated specific aims:
Aim 1. Studying gene libraries of repetitive proteins using a label-free high-throughput technique
Aim 2. Understanding the relationship between repetitive protein sequence and structure

Accomplishments: PDF report attached

Training Opportunities: Nothing to Report

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Results Dissemination: 1. Vural, M., Colak, O., Mazeed, TES, Hamilton, R. F., Dong, L, Gao, H., Demirel, M. C. Bioinspired Stretchable Molecular Composites of 2D-Layered Materials and Tandem Repeat Proteins, Proceedings of National Academy of Sciences (PNAS), 2022
2. Dursun, B, Mazeed, TES, Colak, O, Boy, R, Demirel MC, Enhancing sustainability and elasticity of synthetic fibers by tandem repeat proteins Smart Materials and Structures 31 (4), 2022
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Honors and Awards: •Microfiber Challenge Award ($150k), 2022
- Member of National Academy of Inventors, 2019
- Tommy Hilfiger Social Innovation Challenge, Finalist, 2018
- H&M Foundation, Global Change Award, 2018
- Green Chemistry Council Travel Award, 2018

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Person Months Worked: 3.00 **Funding Support:**
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National Academy Member: N

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Participant Type: Graduate Student (research assistant)
Participant: Adam Alavi
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Article Title: Self-Assembly of Topologically Networked Protein?Ti3C2Tx MXene Composites
Authors: M Vural, H Zhu, A Pena-Francesch, H Jung, BD Allen, MC Demirel
Keywords: self-assembly, tandem repeat proteins, MXene, composites, electronic properties
Abstract: Hierarchical organization plays an important role in the stunning physical properties of natural and synthetic composites. Limits on the physical properties of such composites are generally defined by percolation theory and can be systematically altered using the volumetric filler fraction of the inorganic/organic phase. In natural composites, organic materials such as proteins that interact with inorganic filler materials can further alter the hierarchical order and organization of the composite via topological interactions, expanding the limits of the physical properties defined by percolation theory. However, existing polymer systems do not offer a topological parameter that can systematically modulate the assembly characteristics of composites. Here, we present a composite based on proteins and titanium carbide (Ti3C2Tx) MXene that manifests a topological network that regulates the organization, and hence physical properties, of these biomimetic composites. We designed, recombinant
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Article Title: Highly Conductive Self-Healing Biocomposites Based on Protein Mediated Self-Assembly of PEDOT:PSS Films
Authors: Yusuke Kikuchi, Abdon Pena-Francesch, Mert Vural, Melik C. Demirel
Keywords: conductive polymers, flexible electronics, structural proteins, biocomposites, self-healing
Abstract: Composites of conducting polymers offer a broad spectrum of materials for interfacing electronic devices with biological systems. Particularly, material systems based on poly(styrenesulfonate) doped poly(3,4-ethylenedioxythiophene) (PE- DOT:PSS) have found applications in many bioelectronic devices as biosensitive transistors, controlled drug delivery media, and strain, temperature, and humidity sensors. The biocompatibility, intercoupled electronic and ionic conductivity, and air stable electrical properties render PEDOT:PSS based material systems indispensable for bioelectronics. However, these materials are commonly used in thin film form since freestanding films of pristine PEDOT:PSS are considered mechanically brittle compared to biological tissues, and unlike biological systems these conductive films cannot restore/ heal their physical properties after excessive mechanical deformation. Here we report conductive biocomposites of PEDOT:PSS and tandem repeat proteins with the abilit
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Article Title: Dielectrophoretic separation of randomly shaped protein particles
Authors: Tae Joon Kwak, Huihun Jung, Benjamin D. Allen, Melik C. Demirel, Woo-Jin Chang
Keywords: Protein particle separationMorphology-based separationSize-based separationDielectrophoresis (DEP)Protein particlesProtein aggregates
Abstract: Recently, insoluble protein particles have been increasingly investigated for artificial drug delivery systems due to their favorable properties, including programmability for active drug targeting of diseases as well as their biocompatibility and biodegradability after administration. One of the biggest challenges is selectively collecting desirable self-repairable particles in the spherical morphology with monodispersity to enable consistent levels and rates of drug loading and release. Therefore, technology that allows sorting of protein particles with respect to size and morphology will enhance the design and production of next-generation drug delivery materials. Here, we introduce a dielectrophoretic (DEP) separation technique to selectively isolate spherical protein particles from a mixture of randomly shaped particles. We tested this approach by applying it to a mixture of precipitated squid ring teeth inspired tandem repeat protein particles with diverse sizes and morphologies.

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Article Title: Biosynthetic self-healing materials for soft machines
Authors: Abdon Pena-Francesch, Huihun Jung, Melik C. Demirel, Metin Sitti
Keywords: squid ring teeth, robotics, proteins
Abstract: Recently, insoluble protein particles have been increasingly investigated for artificial drug delivery systems due to their favorable properties, including programmability for active drug targeting of diseases as well as their biocompatibility and biodegradability after administration. One of the biggest challenges is selectively collecting desirable self-repairable particles in the spherical morphology with monodispersity to enable consistent levels and rates of drug loading and release. Therefore, technology that allows sorting of protein particles with respect to size and morphology will enhance the design and production of next-generation drug delivery materials. Here, we introduce a dielectrophoretic (DEP) separation technique to selectively isolate spherical protein particles from a mixture of randomly shaped particles. We tested this approach by applying it to a mixture of precipitated squid ring teeth inspired tandem repeat protein particles with diverse sizes and morphologies.

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Article Title: Hydration-Induced Structural Transitions in Biomimetic Tandem Repeat Proteins
Authors: Romeo C. A. Dubini, Huihun Jung, Chloe H. Skidmore, Melik C. Demirel, Petra Rovó
Keywords: solid state nmr, squid ring teeth, protein
Abstract: Recently, insoluble protein particles have been increasingly investigated for artificial drug delivery systems due to their favorable properties, including programmability for active drug targeting of diseases as well as their biocompatibility and biodegradability after administration. One of the biggest challenges is selectively collecting desirable self-repairable particles in the spherical morphology with monodispersity to enable consistent levels and rates of drug loading and release. Therefore, technology that allows sorting of protein particles with respect to size and morphology will enhance the design and production of next-generation drug delivery materials. Here, we introduce a dielectrophoretic (DEP) separation technique to selectively isolate spherical protein particles from a mixture of randomly shaped particles. We tested this approach by applying it to a mixture of precipitated squid ring teeth inspired tandem repeat protein particles with diverse sizes and morphologies.
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Article Title: Enhancing sustainability and elasticity of synthetic fibers by tandem repeat proteins
Authors: Burcu Dursun, Tarek El-Sayed Mazeed, Oguzhan Colak, Ramiz Boy, Melik C Demirel
Keywords: sustainability, squid, acrylic, protein-fibers, self-assembly, synthetic proteins
Abstract: Protein fiber production in heterologous organisms, such as bacteria, provides a new possibility for engineering high-performance materials and composites. The discovery and design of sustainable materials that are biological or inspired by biological principles are essential for the development and production of the next generation of circular bioeconomy. Here, we created a hybrid of biological and synthetic materials by combining bio-engineered proteins with synthetic acrylic polymers to enhance the sustainability and elasticity of the blend fibers. First, we developed an optimized expression (i.e. yield exceeding 1 g l?1) and purification method (>80% purity) for squid ring teeth inspired by tandem proteins at the facility scale. We showed that our protein-based powder, produced via industrial fermentation, can be manufactured into braided yarns with acrylic using wet-spinning. Our fibers have enhanced elasticity when hydrated due to the hydrogen network between the protein and acry
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Article Title: Bioinspired stretchable molecular composites of 2D-layered materials and tandem repeat proteins
Authors: Mert Vural, Tarek Mazeed, Dong Li, Oguzhan Colak, Reginald F. Hamilton, Huajian Gao, Melik C. Demi
Keywords: squid ring teeth, protein, composites
Abstract: Protein based composites, such as nacre and bone, show astounding evolutionary capabilities, including tunable physical properties. Inspired by natural composites, we studied assembly of atomistically thin inorganic sheets with genetically engineered polymeric proteins to achieve mechanically compliant and ultra-tough materials. Although bare inorganic nanosheets are brittle, we designed flexible composites with proteins, which are insensitive to flaws due to critical structural length scale (?2 nm). These proteins, inspired by squid ring teeth, adhere to inorganic sheets via secondary structures (i.e., ?-sheets and ?-helices), which is essential for producing high stretchability (59 ± 1% fracture strain) and toughness (54.8 ± 2 MJ/m3). We find that the mechanical properties can be optimized by adjusting the protein molecular weight and tandem repetition. These exceptional mechanical responses greatly exceed the current state-of-the-art stretchability for layered composites by over a f
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Article Title: Diffusive Dynamic Modes of Recombinant Squid Ring Teeth Proteins by Neutron Spectroscopy
Authors: Abdon Pena-Francesch, Huihun Jung, Madhusudan Tyagi, Melik C. Demirel
Keywords: squid ring teeth, neutron
Abstract: Stimuli-responsive structural proteins are emerging as promising biocompatible materials for a wide range of biological and nonbiological applications. To understand the physical properties of structural proteins and to replicate their performance in biosynthetic systems, there is a need to understand the molecular mechanisms and relationships that regulate their structure, dynamics, and properties. Here, we study the dynamics of a recombinant squid-inspired protein from Loligo vulgaris (Lv18) by elastic and quasielastic neutron scattering (QENS) to understand the connection between nanostructure, chain dynamics, and mechanical properties. Lv18 is a semicrystalline structural protein, which is plasticized by water above its glass transition temperature at 35 °C. Elastic scans revealed an increased protein chain mobility upon hydration, superimposed dynamic processes, and a decrease in dynamic transition temperatures. Further analysis by QENS revealed that while dry Lv18 protein dynamic
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as of 21-Nov-2022

Partners

,

I certify that the information in the report is complete and accurate:
Signature: melik demirel
Signature Date: 11/21/22 9:23PM

FINAL REPORT

This final report covers our studies in the sequence-structure domain (initial funding) and its application in optical fibers (add-on funding).

PART-I

Fibrous proteins' material properties and self-assembly behavior can be manipulated over a wide response range through variations in their repeat-unit amino-acid sequences. High-throughput screening of tandem-repeat libraries will enable the discovery of sequences with distinctive physical properties and improve our understanding of self-assembly, leading to revolutionary advances in materials and life sciences. We will test this hypothesis via two distinct but interrelated specific aims:

Aim 1. Studying gene libraries of repetitive proteins using a label-free high-throughput technique

Aim 2. Understanding the relationship between repetitive protein sequence and structure

Aim 1. Studying gene libraries of repetitive proteins using a label-free high-throughput technique: We aim to produce repetitive protein libraries with precisely defined crystalline and amorphous regions. Using the crystalline domains of SRT sequences as templates (i.e., based on the considerable diversity of their variable AVSTH-rich domains), we propose constructing tandem-repeat gene libraries and analyzing the proteins they encode a high-throughput laser spectroscopy method. The sequence variation in our gene libraries will be based on (i) molecular weight (i.e., tandem-repeat number), (ii) amino acid content of the crystalline region, and (iii) the length ratio of the crystalline and amorphous domains. These parameters will directly affect crosslink and tie-chain densities of repetitive structural proteins, as shown in our preliminary data below.

Task 1.1. Design and construct gene libraries by cloning and propagating repetitive sequences: Effective body temperature maintenance represents a critical component of the survival and success of military service members in combat and other hazardous environments. Thermal storage technologies that absorb and release heat on demand can widen the acceptable range of working conditions and prolong the proper duration of exposure. To achieve the promise of thermal storage approaches on the battlefield, new technology is needed to improve the flexibility, weight, operating temperature, storage capacity, switching ratio, and safety of these materials. Our SRT-based block-copolymer biomaterials allow these properties to be tuned by manipulating the genetic coding sequence and the material processing strategies.

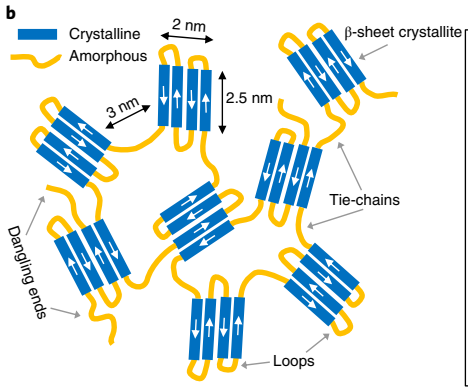


Figure 1: Network model of SRT-based semi-crystalline materials. Blue: crosslinking (crystalline) regions; arrows denote N-terminus to C-terminus direction of the polypeptide chain. Gold: tie-chain (amorphous) regions

Our working model of SRT-based semi-crystalline materials describes a network of physical crosslinks composed of hydrogen-bonded β-sheets connected by tie-chains of more loosely defined amorphous structure (**Figure 1**). This model suggests that manipulating the mass ratios of crosslinking regions and tie-chain regions in the block-copolymer architecture could enable tuning of the viscoelastic and thermal-storage behavior of these materials.

To investigate the contributions of the tie-chain (amorphous) regions to the material properties of SRT-based block copolymers, we constructed a series of tandem-repeat coding sequences that supply tie-chain regions with double or triple the length of those in our original designs. The new panels of sequences, dubbed the AM2 and AM3 series by analogy to the retroactively named original AM1 series, span a similar range of repeat numbers and molecular weights as our first series of constructs (**Figure 2**). In each case, the protein sequence for the more extended tie-chain region was designed by adding one or two perfect copies of the original tie-chain part in tandem. The coding sequences of the n=1 repeating units for the AM2 and AM3 series were optimized for maximum protein expression and to facilitate gene assembly using the Tang and Chilkoti method (Tang-NatMat-2016), ordered as synthetic gene fragments from IDT and cloned into plasmid vectors for long-term storage and further processing. These n=1 units were expanded into tandem-repeat coding sequences using our established PD-RCA method, cloned into expression vectors, and verified by Sanger sequencing. These vectors were then used to express and purify the encoded proteins in recombinant *E. coli* in a pET-derived system according to our previously published methods (**Figure 2**). (Jung-PNAS-2016) The identities of these protein samples were validated using standard MALDI-TOF mass spectrometry (**Figure 3**).

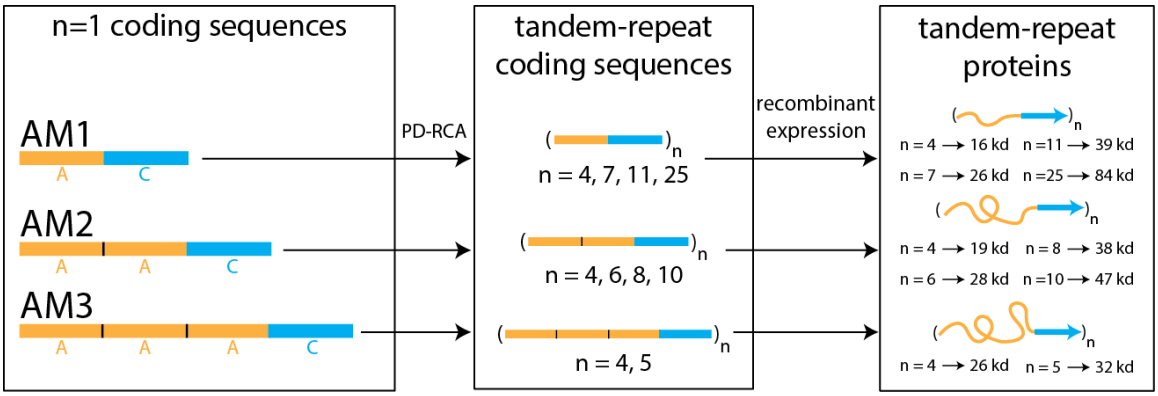


Figure 2: Construction AM series tandem-repeat block-copolymer proteins. A: amorphous (tie-chain) region. C: crystalline (crosslinking) region. n: tandem-repeat number. kd: protein molecular weight, kilodaltons).

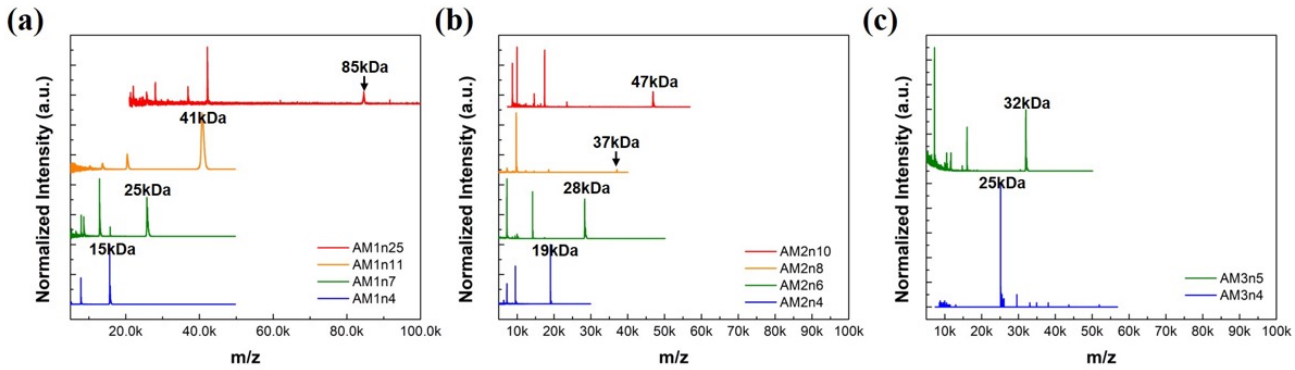


Figure 3: MALDI-TOF validation of molecular weight for purified protein materials from the (a) AM1, (b) AM2, and (c) AM3 series.

Task 1.2. High-throughput screening of gene libraries by TDTT to identify crosslink and tie-chain densities

We aim to screen structural proteins using the TDTT approach to understand the structure-property relationship. Directed evolution of structural-proteins properties will allow us to tune physical properties, which is vital for designing structural protein-based materials such as films or fibers. Merging materials science and directed evolution in synthetic biology will provide a versatile scientific pathway for creating new materials with specific tailorable properties. This new research direction could eventually lead to a portfolio of advanced properties (e.g., self-repairing, functionalized with biological moieties for chemical and biological defense).

We built the TDTT equipment through a DURIP grant. This technique is based on femtosecond pump-probe spectroscopy and requires precision optical components. We finally had the system up and running and started to collect proposed data for identifying crosslink and tie-chain densities of gene libraries. Our goal is to achieve high-throughput screening, but at this initial stage, we are only focusing on a low number of samples for calibration purposes (i.e., comparing the results with ssNMR results in collaboration with a group at the University of Leipzig) and making sure that the measurements generate consistent results under different sample processing conditions (e.g., chemically or thermally treated samples).

In the TDTT technique, we measure the group velocity of coherent acoustic phonons through pump-probe Brillouin scattering measurements using a two-tint version of our experimental setup. In pump-probe sizes, the initial pump pulse creates a heating event in the transducer; the energy imparted from the laser pulse paired with this temperature rise leads to the formation of coherent acoustic waves within the surrounding media. As the coherent acoustic wave traverses this media, in our case, the AM films, the probe beam is partially reflected due to the change in the refractive index associated with the pressure gradient; the transmitted portion of the beam continues towards the metal transducer and undergoes thermo-reflection. These two reflected beams will interfere and lead to periodic oscillations in the time-dependent reflectance signal, as shown in **Figure 4**.

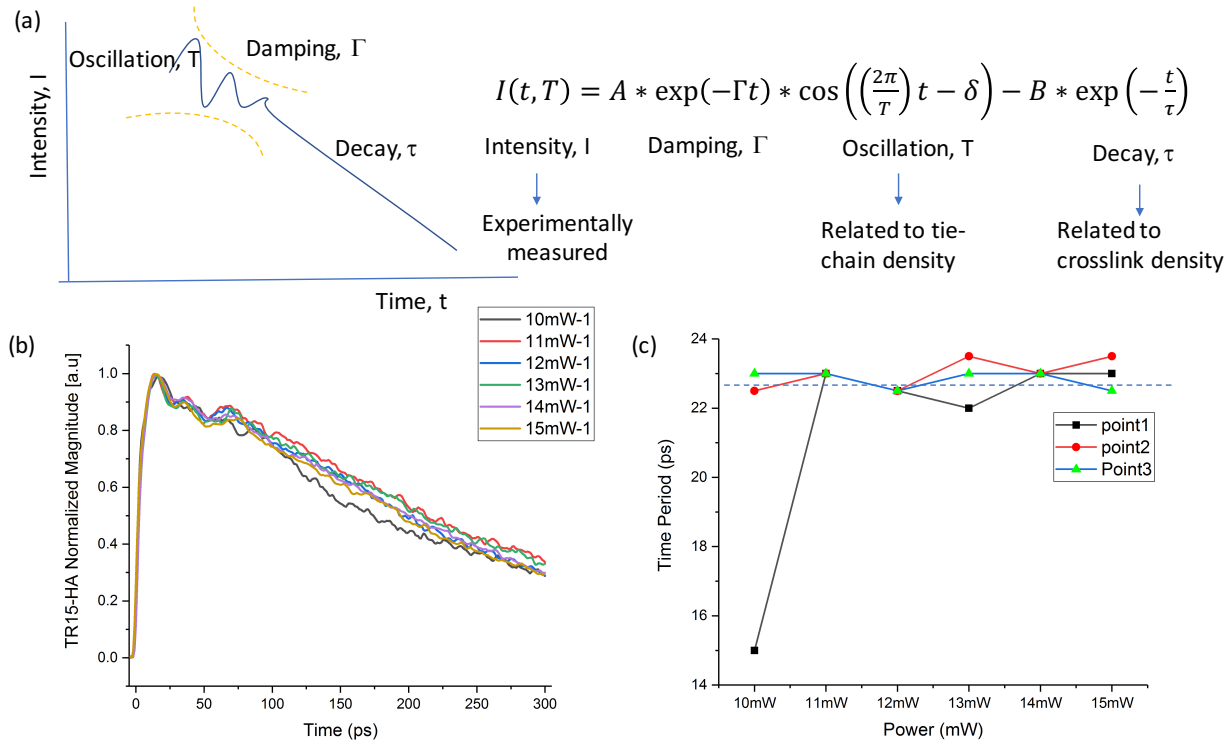


Figure 4. (a) Schematic of pump-probe data, where the intensity of this time-independent signal can be described via an exponential decay and oscillation equation. (b) Example data for AM1n4 sample as a function of laser pump power. (c) Oscillation (T) of the pump-probe experiment is obtained using the equation in (a). The result shows that T is independent of laser power, which is expected (i.e., T relates to tie-chain density, which is invariant for a given tandem repeat, "n")

We constructed synthetic versions of repetitive SRT genes with repeat number, n, and amorphous ratio f. However, larger values of *n* with larger values of *f* were challenging to express, possibly due to gene instability and protein toxicity. In **Figure 5**, we show the hydrophobicity values of SRT proteins as a function of repeat number and amorphous ratio, which shows that the toxicity of these proteins starts to impair cloning and protein expression (i.e., for reference, the most excellent hydrophobicity value observed in the E. coli proteome is 55 and the vast majority have values < 0). As for gene stability, we solved that problem using codon scrambling algorithms. When using the original, highly repetitive sequences, we found that plasmid sequences were altered by recombination when the proteins encoded that had hydrophobicity of 100 or greater. This could be due to repetitive sequences that allowed recombination, and protein toxicity could be a selective pressure that stopped/slowed the growth of all cells except those bearing recombined sequences. Although the scrambled sequences were prepared, we got almost no transformants. This suggests that the sequences remained toxic (due to their protein products), but recombination was reduced to a low background level, and the cells had no way to escape the toxicity. In summary, the scrambling algorithms effectively improve the genetic design (i.e., recombination was reduced to low background levels). However, the sequences were still toxic (i.e., due to the proteins they encode).

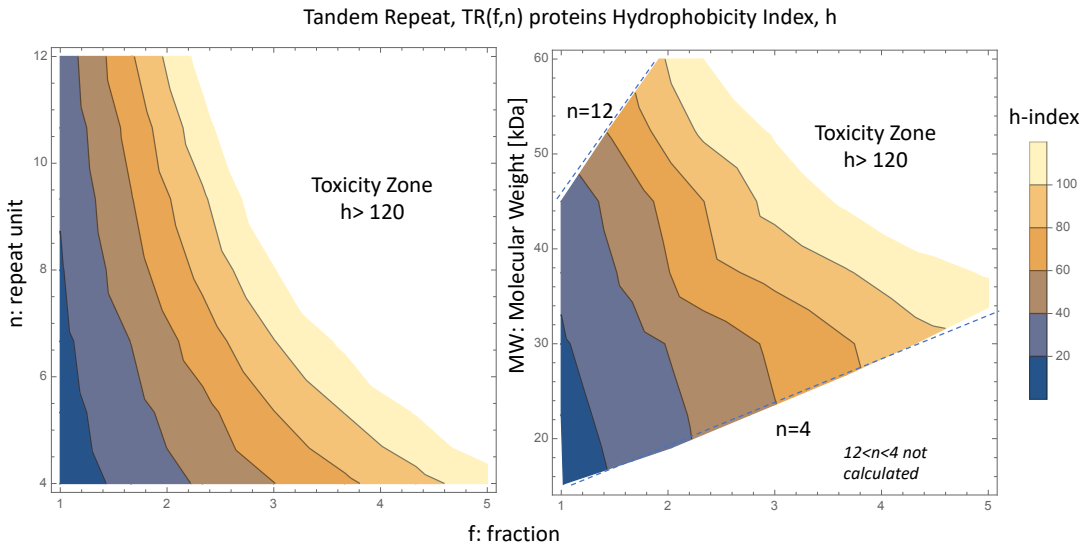


Figure 5. Hydrophobicity estimates of SRT-inspired tandem repeat proteins as a function of amorphous ratio, f, and repeat unit or molecular weight are plotted. Due to cell toxicity, it is challenging to express large n and f-numbered tandem repeat proteins.

<u>Aim 2. Understanding the relationship between repetitive protein sequence and structure</u>

The design of amino acid sequences for novel self-assembly and material properties is an unsolved scientific problem. Moreover, most techniques for predicting protein structure rely on globular protein-structure databases and are rarely applicable to fibrous-protein assemblies. Existing high-throughput fluorescence and affinity readouts are structure-specific and may miss materials with novel molecular morphologies. To address these challenges, we propose mapping sequence/structure/property relationships in repetitive structural-protein libraries by characterizing selected proteins discovered during high-throughput screening in depth.

<u>Task 2.1. Selection and expression of repetitive proteins from gene libraries</u>

Numerous features of SRT-based semi-crystalline materials render them an ideal platform for biomaterials science and engineering, including sequence-defined amorphous and crystalline domains, lack of posttranslational modifications, tractable sequence lengths, and advanced properties such as facile self-healing and switchable thermal storage. To explore fully the genetic programming of these materials and the breadth of properties this platform can enable, we require powerful new tools for high-throughput construction, cloning, expression, and screening of tandem-repeat coding sequences and their protein products.

Previously, we reported a new molecular biology method for the single-step construction of tandem-repeat coding sequences from circularized repeat-unit sequences: partial digestion of rolling-circle amplicons (PD-RCA, **Figure 6**). This method was designed to facilitate the single-tube tandem-repeat expansion of a pool of various repeat-unit sequences into tandem repeats without mixing (**Figure 7**). However, several technical problems have stymied the application of this method to our end goal of pooled tandem-repeat library construction. The critical issue has been the unavoidable production of single-stranded intermediates during rolling-circle amplification. Because many library members share similar or identical regions of sequence (e.g., constant tie-chain domains), opposite-sense single-stranded intermediates originating from

different repeat-unit templates can hybridize and ultimately generate chimeric products (**Figure 8**).

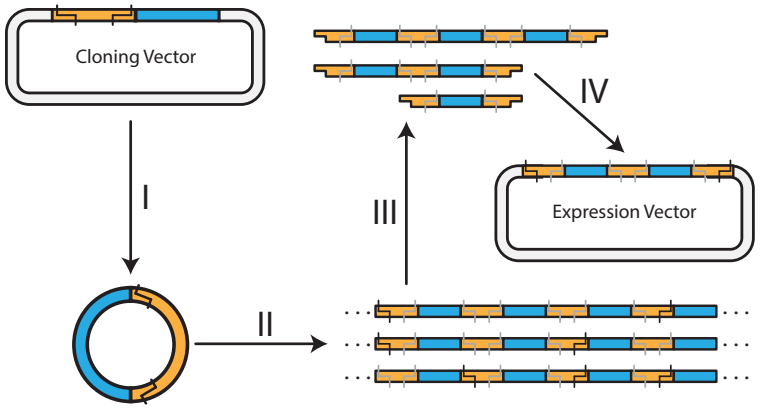


Figure 6. PD-RCA workflow. I: excision of repeat-unit coding sequence from a cloning vector and circularization. Restriction sites protectable by methylation are shown as black lines. II: Rolling-circle amplification in the presence of 5-methylcytosine to produce tandem-repeat products with some restriction sites protected from cleavage (grey lines). III: digestion at non-protected sites to yield tandem-repeat products of various sizes. IV: size selection and cloning of digestion products into an expression vector.

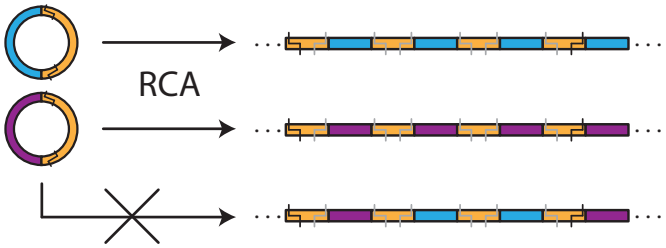


Figure 7. Because each circular template of a different sequence (blue, purple) yields a single tandem-repeat RCA product, chimeric tandem-repeats (blue and purple tandem repeat, bottom) should not be produced.

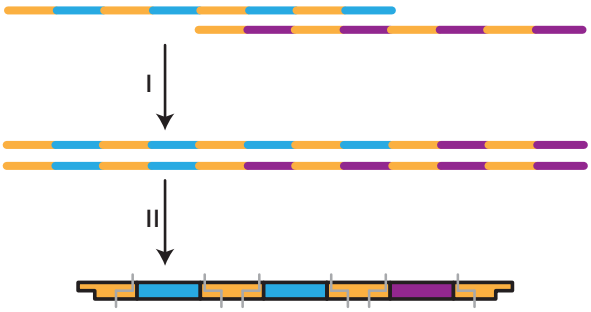


Figure 8. Single-stranded RCA products from different templates (blue, purple) hybridize due to sufficient complementarity at the reaction temperature. I: polymerase fills out double-stranded heteroduplex product. II: Double-stranded heteroduplex is partially digested, yielding clonable, chimeric tandem-repeat coding sequences.

Because many optimizations failed to yield a working protocol for pooled-library construction by PD-RCA, we exploited newly available technology for highly sequence-specific long-overlap

assembly to enable a controlled-doubling approach that is amenable to pooled libraries. In long-overlap assembly, overlap regions of 15-30 identical nucleotides at the ends of two double-stranded DNA fragments are subjected to mixtures of enzymes that chew back one strand of each, exposing single-stranded regions that hybridize and allow the fragments to be fused. In some implementations, assembly requires perfect hybridization between the exposed strands, and mismatches of even one base pair inhibit assembly, suggesting that this method can facilitate pooled doubling without unwanted chimera production.

However, we had previously rejected the possibility of using this approach for pooled-library tandem-repeat construction because methods available at the time failed to fuse fragments with small numbers of mismatches at their termini. Since practical approaches to producing linear fragments for subsequent assembly generate these small overlaps, we judged long-overlap assembly inappropriate for our purposes. Fortunately, a newer, proprietary version of the long-overlap assembly enzyme mix (NEB HiFi Assembly) now includes enzyme components that deletion mismatched ends, such as those produced by restriction digestion, while maintaining the remaining desirable qualities of the method. We have incorporated this technology into a facile two-step controlled-doubling method for tandem-repeat construction (**Figure 9**).

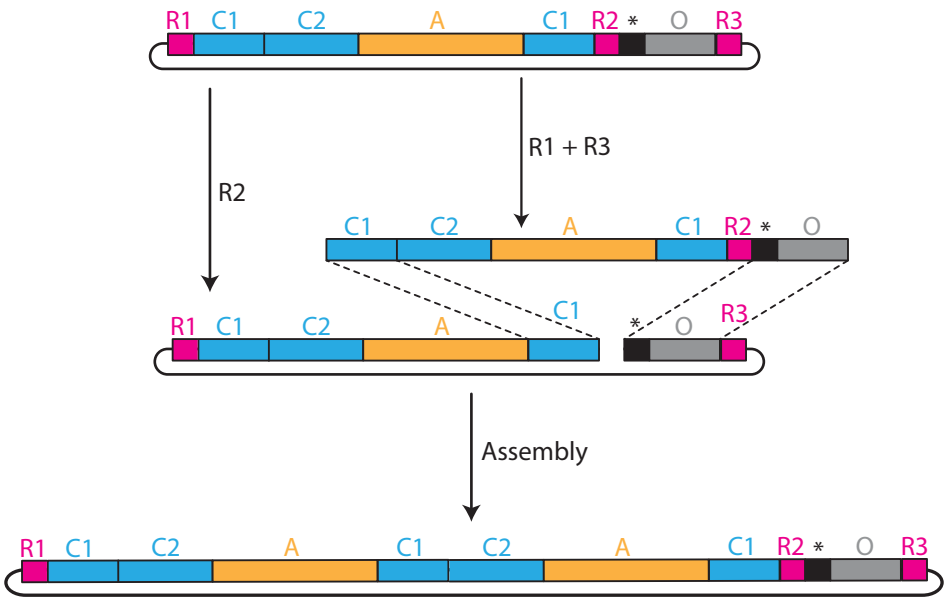


Figure 9. Controlled doubling of tandem-repeat coding sequences by digestion and fastidious long-overlap assembly. The region with sequence diversity (in this case, the crosslinking area, C) is split into two fragments, the first repeated at the end of the coding sequence. Three restriction sites are installed to facilitate the vector (R2) opening and excision of the coding sequence (R1 and R3). A noncoding overlap region (O) is included after the stop codon (*) to enforce specific assembly and avoid the production of chimeras. Separate digestion of the vector with two different sets of restriction enzymes yields an insert that can be assembled into the vector by long-overlap assembly using C1 and O as the overlap regions. This produces a sequence-doubled coding sequence that is amenable to later doublings by the same method.

To test the new method, we built a panel of 5 repeat-unit coding sequences that satisfy the scheme

in Fig 4 and differ minimally in their C1 and O overlap regions (**Figure 10**, top). These sequences were pooled and subjected to controlled doubling and cloning. Twenty colonies were sequenced to investigate the behavior of the doubling strategy when applied to a pooled library of near-identical sequences. Out of 19 successful sequencing runs, all but one of the input-sequence panels was represented in the doubled constructs (**Figure 10**, bottom left). Most of the sequenced clones reflect correctly assembled n=2 constructs, while the remainder were non-doubled n=1 input sequences (**Figure 10**, bottom right). Crucially, chimeric constructs containing fusions of sequences A-E were never observed, indicating that even one base pair of mismatch is sufficient to inhibit the fusion of chimeric constructs. These results give much confidence that the new method will apply to constructing large-pooled libraries of tandem-repeat coding sequences.

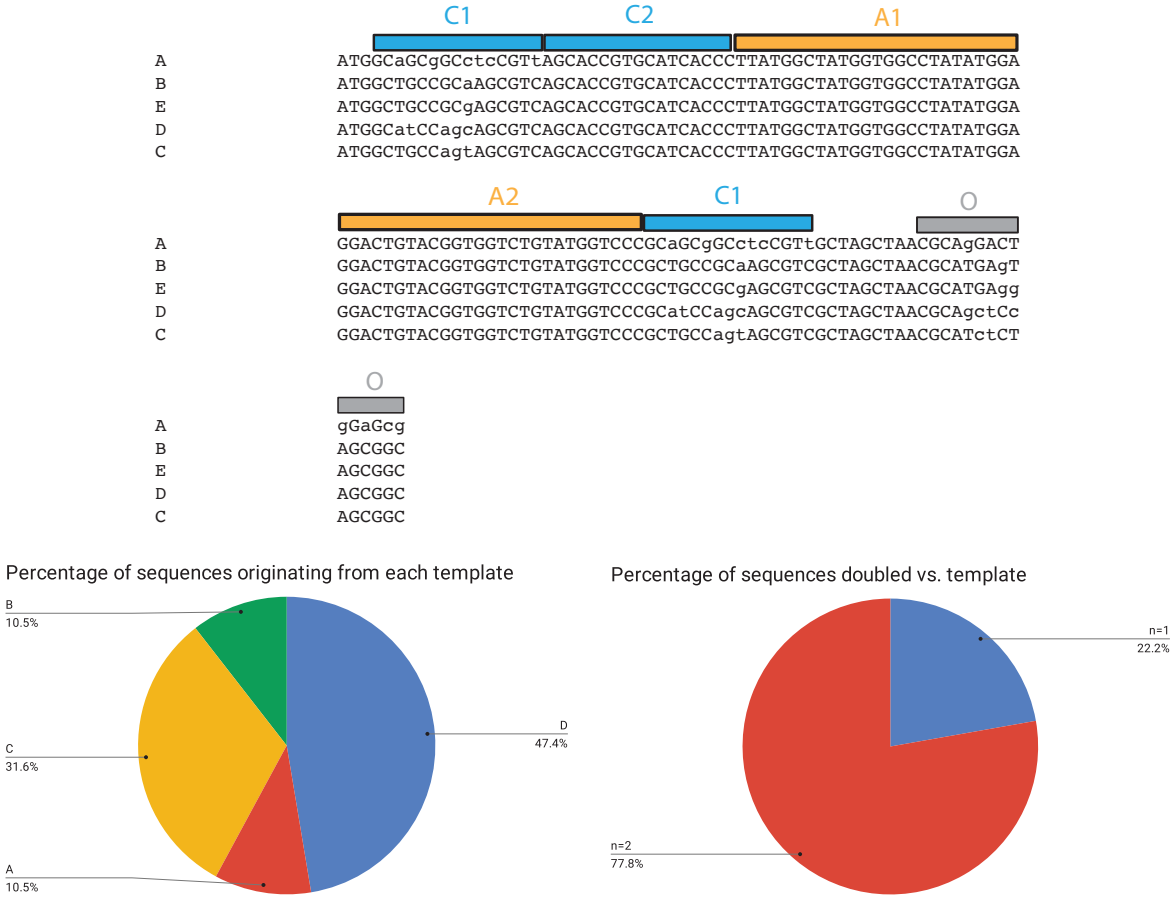
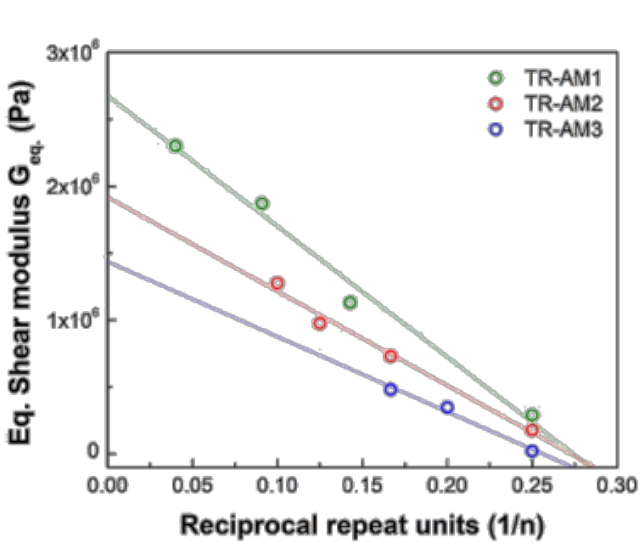


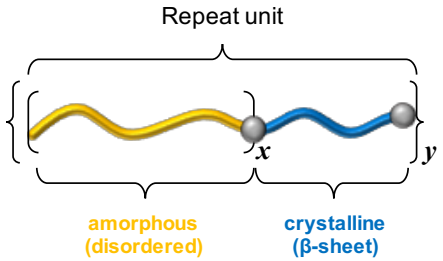
Figure 10. A panel of sequences was used to test tandem-repeat construction by pooled-doubling long-overlap-assembly. Top: Sequences A to E differ minimally in the C1 and O regions. Bottom: 19 sequenced clones show reasonable maintenance of library diversity, affordable conversion to n=2, and no evidence of chimera production.

Task 2.2. Elucidating material properties of selected repetitive proteins

Our goal is to produce repetitive SRT (Squid ring teeth) proteins with precisely defined crystalline and amorphous regions. Our gene design will be based on gene duplication as described in the previous aim with (i) molecular weight, M.W. (i.e., tandem-repeat number, n = 4-20), and (ii) the length ratio of the crystalline and amorphous domains, (i.e.1 to 3). As shown in **Figure 11**, these parameters directly control molecular scale distances of SRT variants, and hence mechanical

properties vary as a function of tandem repeat number and crystalline/amorphous ratio. The shear modulus, measured by rheology, of these samples for f=1-3 and n=4-25, are shown in **Figure 11**.





	AMxny		
Amorphous ratio number (x)	1	2	3
Repeat number (y)	4, 7, 11, 25	4, 6, 8, 10	4, 5, 6
Molecular weight (kDa)	15, 25, 41, 85	19, 29, 38, 47	26, 32, 39

Figure 11. The shear modulus of SRT proteins with an amorphous ratio of f=1-3 and varying repeat numbers of n=4-25 are shown (see table for the exact molecular weight of each polypeptide).

In **Figure 12**, we report conductive biocomposites of PEDOT: PSS and tandem repeat proteins with the ability to self-heal once plasticized via water. The tandem repeat proteins acquired from squid ring teeth (SRT) induce structural effects on PEDOT: PSS, including improved crystallinity and formation of fibrous network structures. These structural effects lead to electrical conductivity values reaching 120 S/cm for biocomposites with SRT protein concentrations below 20 wt %, which exceeds the conductivity of pristine PEDOT: PSS (~100 S/cm). More importantly, tandem proteins facilitate consistent self-healing of freestanding biocomposites with SRT protein concentrations beyond 40 wt %. The influence of structural changes on the electrical properties and the ability to heal sustained damage for conductive biocomposites are evaluated by performing four-point probe experiments on biocomposites before and after cutting the sample into two pieces (**Figure 12**). The electrical conductivity of biocomposites composed of different SRT concentrations corresponds with the structural changes observed in conductive biocomposites with altering SRT concentrations. In contradiction with percolation theory, the electrical conductivities of biocomposites increase, with increasing SRT concentration up to a threshold concentration of 20 wt % SRT. Improved crystallinity of PEDOT and the formation of fibrous network structures are known to generate pathways with higher mobility for electron transport. Low volumetric concentrations of SRT induce similar changes in the microstructure of PEDOT: PSS, as demonstrated during the structural characterization of biocomposites of SRT and PEDOT: PSS. The divergence of the conductivity values from the conventional percolation theory possibly originates from these structural effects for composites with SRT concentrations below 20 wt %. Beyond this concentration, percolative products dominate over constructive structural effects arising from the interactions between SRT and PEDOT: PSS.

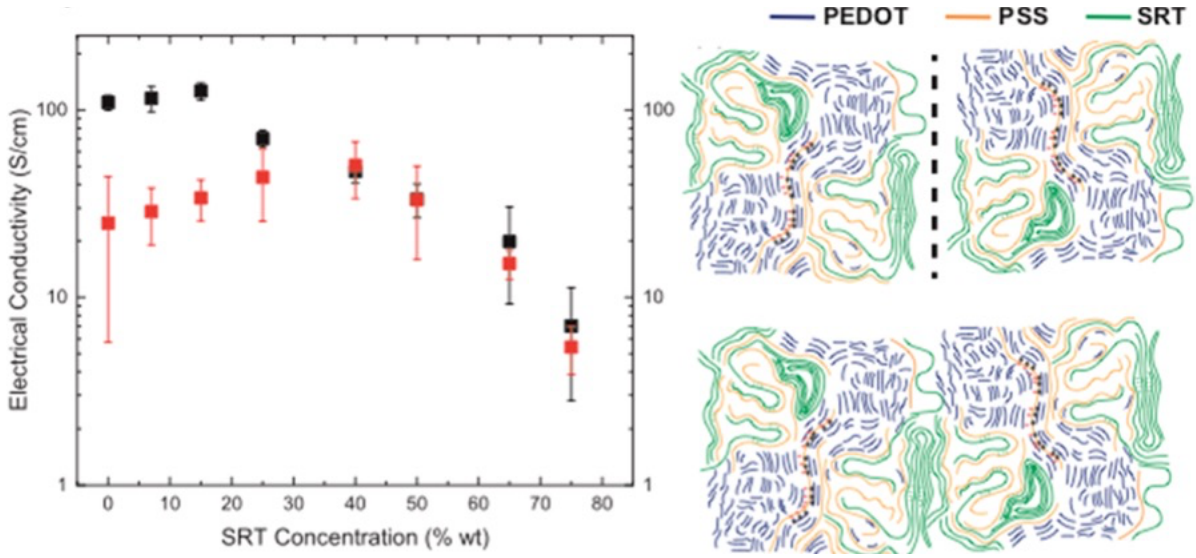


Figure 12. Electrical properties of conductive biocomposites before (black) and after (red) the cutting and healing process on the left. Schematic illustration of the healing process of conductive biocomposites after incision on the right.

In **Figure 13**, we present a composite based on proteins and titanium carbide ($Ti_3C_2T_x$) MXene that manifests a topological network that regulates the organization and hence physical properties of these biomimetic composites. We designed, recombinantly expressed, and purified synthetic proteins consisting of polypeptides with repeating amino acid sequences (tandem repeats) that can self-assemble into topologically networked biomaterials. We demonstrated that the interlayer distance between MXene sheets could be controlled systematically by the number of tandem repeat units. The conductivity values of MXene films vary as a function of the separation distance between MXene layers (**Figure 13**). MXene films prepared using larger intercalants demonstrated lower conductivity due to increased separation between sheets. Similar to the in-plane electrical conductivity characteristics of MXene films and MXene/TR composites, increased separation between MXene flakes results in lower out-of-plane electrical conductivity. However, unlike in-plane electrical conductivity, out-of-plane electrical conductivity has no dependence on the volumetric filler fraction of the composites; it simply scales with interlayer spacing between MXene flakes. This behavior is consistent with the formation of nacre-like structures in MXene/TR composites, as we proposed previously based on the structural characterization and in-plane electrical conductivity values of MXene/TR composites.

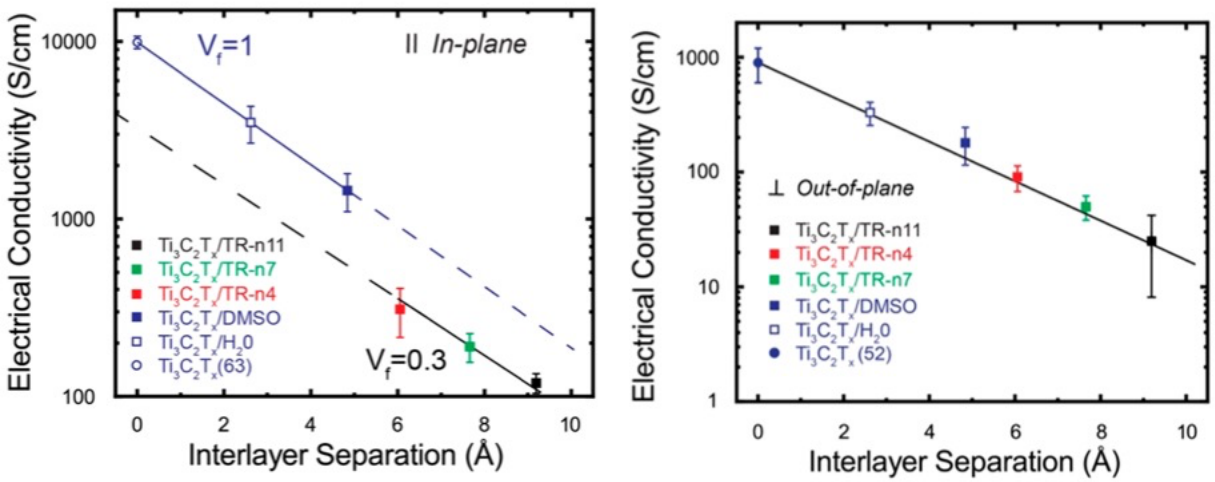


Figure 13. Left: Electrical conductivity values of MXene films and MXene/TR composites acquired from four-point probe measurements along with theoretical estimation lines calculated for two distinct filler fractions (V_f) of 1 (blue line) and 0.3 (black line) representing MXene films and MXene/TR composites, respectively. Right: Electrical conductivity values of MXene films and MXene/ TR nanocomposites acquired from four-point probe measurements along with theoretical estimation lines assuming volumetric percolation is equal to unity ($V_f = 1$).

In **Figure 14**, we introduce high-strength synthetic proteins that self-heal micro- and macro-scale mechanical damage within a second by local heating. These materials are systematically optimized to improve their hydrogen-bonded nanostructure and network morphology, with programmed healing properties (23 MPa strength after 1 second of healing) surpassing those found in other natural and synthetic soft materials by several orders of magnitude. This unprecedented healing performance opens new opportunities for bioinspired materials design and addresses limitations in self-healing materials for soft robotics and personal protective equipment. **Figure 14** shows biosynthetic SRT proteins with n = 4, 7, 11, and 25 tandem repetitions (TR) of the squid-inspired building block (TRn4, TRn7, TRn11, and TRn25, with molecular weight of 15, 25, 42, and 86 kDa, respectively). Driven by their segmented amino acid sequences, the TR-polypeptides self-assemble into supramolecular β-sheet-stabilized networks. Hence, by accurately controlling tandem repetition in our polypeptides (n = 4 to 25), we can systematically tailor the molecular defect density from "all-defective" networks (TRn4) to "close-to-perfect" networks (TRn25), optimizing the network morphology beyond that of native squid ring teeth protein complexes. Consequently, the physical properties of TR biosynthetic polypeptides, including protein cohesion at room temperature (i.e., protein-protein adhesion), surpass those of natural and recombinant squid-derived proteins of the same family. Such control of the molecular defects enables the programming of properties by sequence design, yielding protein materials with remarkable self-healing performance (superior to preceding work on self-healing proteins and other state-of-the-art synthetic self-healing materials), and provides an excellent platform to develop bio-based self-healing materials suitable for applications that require mechanical strength and fast healing kinetics.

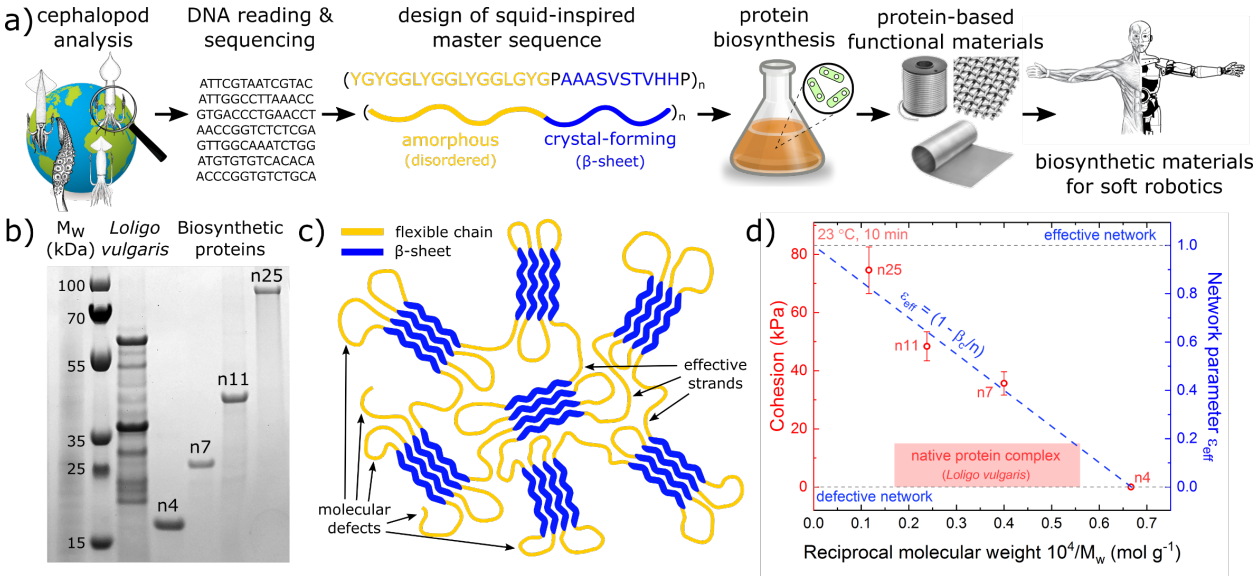


Figure 14. a) The analysis of squids, design of a squid-inspired master sequence, and biosynthesis of protein libraries yield protein-based functional self-healing materials for soft actuators and robotics applications. **b)** Protein sizes of native *Loligo vulgaris* protein complex and biosynthetic TRn4, TRn7, TRn11, and TRn25 polypeptides. **c)** Protein nanostructure is composed of a β-sheet nanocrystal network (blue) connected by flexible chains (yellow) with molecular defects (dangling ends and loops). **d)** Self-healing properties of squid-inspired proteins (room temperature) improved beyond native proteins due to an optimized network morphology. Error bars represent standard deviation.

We employed state-of-the-art nuclear magnetic resonance (NMR) spectroscopy to link the atomistic structural and dynamic properties of an artificial bioinspired tandem repeat protein TR(1,11) to its stunning macroscopic properties including high elasticity, self-healing capabilities, and record-holding proton conductivity among biological materials. We show that the hydration-induced structural rearrangement of the amorphous Gly-rich soft segment and the ordered Ala-rich hard segment is the key to the material's outstanding physical properties. We found that in the hydrated state, both the Ala-rich ordered and Gly-rich disordered parts contribute to the formation of the nanoconfined β-sheets, thereby enhancing the strength and toughness of the material. This restructuring is accompanied by fast proline ring puckering and backbone cis−trans isomerization at the water−protein interface, improving the hydrated films' elasticity and thermal conductivity. In **Figure 15** below, we show the Ala Cα/Cβ region of TR(1,11) with distinct chemical shift regions for α-helical, β-sheet, random coil, and poly proline II helix conformations. Orange, green, and blue spectra are from the ambient powder (AP), ambient film (AF), and hydrated film (HF), respectively. The red dot indicates the average Ala Cα/Cβ shifts observed in the solution state. (Further details are available at *J. Phys. Chem. B* 2021, 125, 8, 2134–2145)

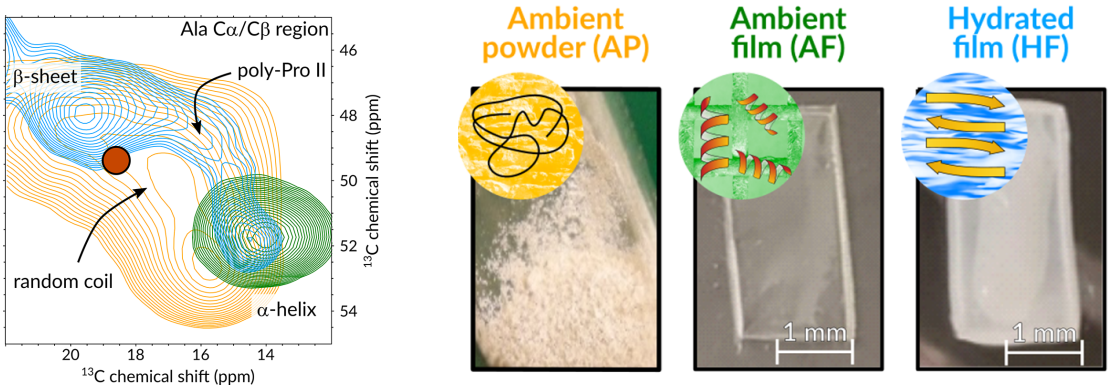


Figure 15. Solid state NMR studies of ambient powder, ambient, and hydrated film of SRT.

Protein materials endow biological systems with unique capabilities, including tunable control of structural, optical, electrical, self-healing, and thermal properties. Similarly, individual two-dimensional (2D) nanosheets show extraordinary mechanical, electronic, optical, and thermal properties. However, the brittle nature of 2D nanosheets drastically restricts the manufacturing of flexible and stretchable composites. Inspired by natural composites, we designed stretchable materials with a critical structural length scale insensitive to flaws (e.g., for nacre at 1micron, the bone at 100nm, whereas our approach at ~2 nm). Here, we developed molecular-scale control of inter-layer spacing of 2D nanosheets by self-assembling genetically engineered polymeric proteins to achieve mechanically compliant and ultra-tough composites. These engineered proteins adhere to 2D crystals via secondary structures (i.e., b-sheets and a-helices), which are essential to maintain high strength and stretchability (i.e., toughness values of 52.6 MJ/m^3 and 58.5% fracture strain simultaneously, as shown in **Figure 16**). We find that the mechanical properties can be optimized by adjusting the protein molecular weight and tandem repetition. These exceptional mechanical responses greatly exceed the current state-of-the-art stretchability for layered composites by over a factor of three, demonstrating the promise of engineering materials with reconfigurable physical properties. Thermal and mechanical properties are shown in **Figure 16**, which were recently submitted for publication.

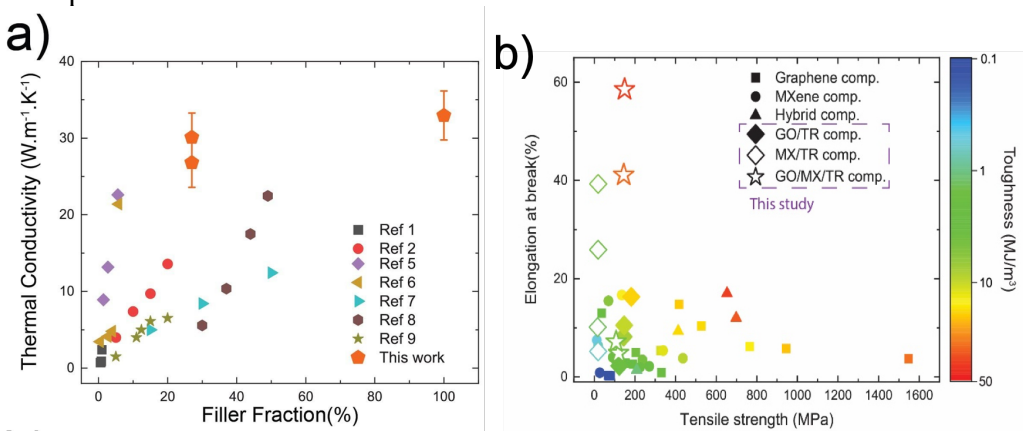


Figure 16. Examples of thermal (a) and mechanical (b) materials manufactured by our approach and existing technologies.

PART-II

Our goal in this proposal is to create revolutionary optical materials with unprecedented control over their visual, opto-thermal, and optomechanical responses using synthetic biology. Such power will enable the study of light-matter interactions in new regimes and the fabrication of new optical devices and structures. Detailed knowledge of structure-property relationships in proteins, acquired through this effort, will enable the design of optical materials and photonic devices with novel functionalities.

In **Figure 17**, we used multiscale simulations to parameterize and model the SRT protein systems to understand structure-property relationships in SRT-based proteins. First, we studied the parametrization of the amino acid and solvent molecules, and then we plan to compare the simulation results against the experimental data (**Figure 16**). We used Dissipative Particle Dynamics (DPD) simulations as our primary method of simulations, which is significantly faster compared to Molecular Dynamics (MD) simulations (e.g., one day of simulation time in a desktop server provides 100 ns with 200 chains in DPD compared to 0.1 ns with one chain) To parameterize the system, we studied binary MD simulations of each amino-acid at the atomistic detail. Then, we coarse-grained all the molecules into beads and used the cohesive energy density values from the MD simulations to construct Flory-Huggins interaction parameters for all pairs in our system. We aim to use DPD simulations to guide the experiments and eventually develop web-based tools for determining structure-property relationships for a given bio-elastomer sequence.

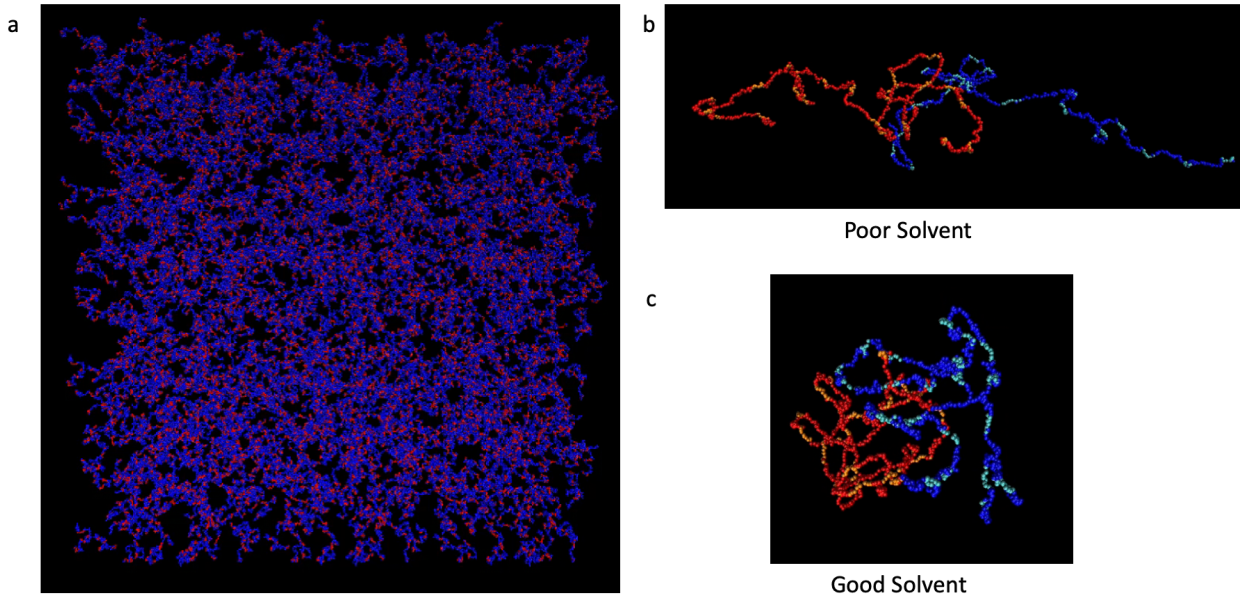


Figure 17. a) DPD (Dissipative Particle Dynamics) of 200 SRT protein chains dissolved in an organic solvent (e.g., HFIP, DMSO, water); Representative interactions of two chains (out of 200) in poor (b) and good (c) solvent.

In **Figure 18**, we focused on producing optical fibers processed via solution processing. We used two approaches, split film, and wet spinning, to obtain protein fibers. Typically, proteins are exposed to various conditions such as temperature, pressure, pH, and the presence of solvents to process them from viscous solutions to solid fibers. In the split film fiber approach, TR-n11 is dissolved in hexafluoroisopropanol (HFIP), as shown in **Figure 18a**. The protein solution is

introduced into a saturated aqueous sodium chloride solution. The protein solution produced a thin film on the surface of the aqueous solution since SRT proteins are not water-soluble. The protein film is twinned using a metal rod, then washed and stretched until the material can be converted into a fiber. TR-n11 is dissolved in DMSO in wet spinning to convert the protein powder into a solution, as shown in **Figure 18b**. The solvent is extruded through a needle injector by simply washing it out in an aqueous bath. After extrusion, the solvent is removed, and the filament is stretched to improve the pliability of the fiber. **Figure 18c-d** show examples of TR-n11 fibers produced using split film fiber and wet spinning techniques.

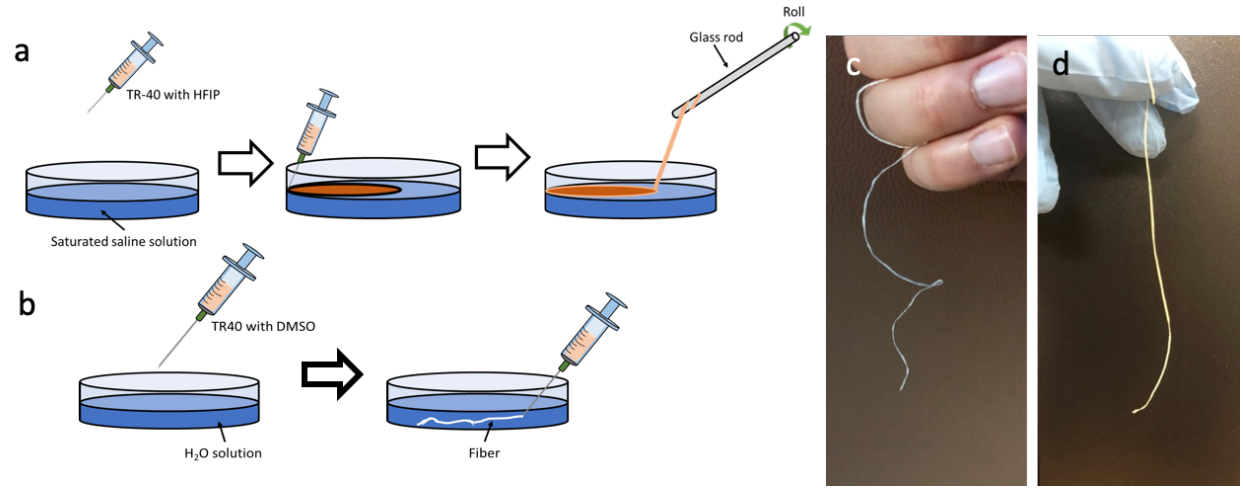


Figure 18. TR-n11 fibers and films are produced using solution processes: Schematic of (a) split film fiber and (b) syringe-based wet-spinning, and corresponding optical images of fibers are shown in c) and d), respectively.

Our goal in this proposal is to create revolutionary optical materials with unprecedented control over their optical, opto-thermal, and optomechanical responses using synthetic biology. This approach will enable the study of light-matter interactions in new regimes and the fabrication of new optical devices and structures. Detailed knowledge of structure-property relationships in proteins, acquired through this effort, will enable the design of optical materials and photonic devices with novel functionalities. Protein fiber production in heterologous organisms, such as bacteria, provides a new possibility for engineering high-performance materials and composites. The discovery and design of sustainable materials that are biological or inspired by physical principles are essential for developing and producing the next generation of circular bioeconomy.

We developed an optimized expression and purification method for squid ring teeth inspired by tandem proteins at the facility scale. The designed building block for the tandem repeat proteins was based on the cross-linked crystal-forming sequence PAAASVSTVHHP and amorphous sequence YGYGGLYGGLYGGLGY observed in native SRT (**Figure 19a**). We used the E. coli strain BL21(DE3) to produce these proteins. Since our protein is intrinsically insoluble under physiological conditions, our chosen production strategy allowed the slow accumulation of the TR protein in inclusion bodies using the low-level expression provided by the pET14b plasmid in our selected strain. Our initial approach to purify recombinant TR proteins was to wash bacterial inclusion bodies using a variety of detergents and solvents after microfluidizer disruption. Although this led to modest increases in purity over cell lysate pellets, the purity was still less than

80%, as determined by SDS-PAGE (**Figure 19b**). Therefore, we developed an organic extraction method in which the inclusion body pellet was dissolved in DMSO and then re-precipitated using purified water as a counter solvent. This approach is based on the observation that TR proteins are highly soluble in pure DMSO but become highly insoluble after adding small amounts of water. Smaller protein domains, typical of the significant contaminating proteins in the cell lysate, are expected to remain soluble in DMSO up to reasonably high concentrations of water (up to 200 mg/ml), which would afford significant purification (**Figure 19b**). As expected, this approach led to a substantial increase in purity, exceeding 80% (**Figure 19c**), as well as a high yield of production.

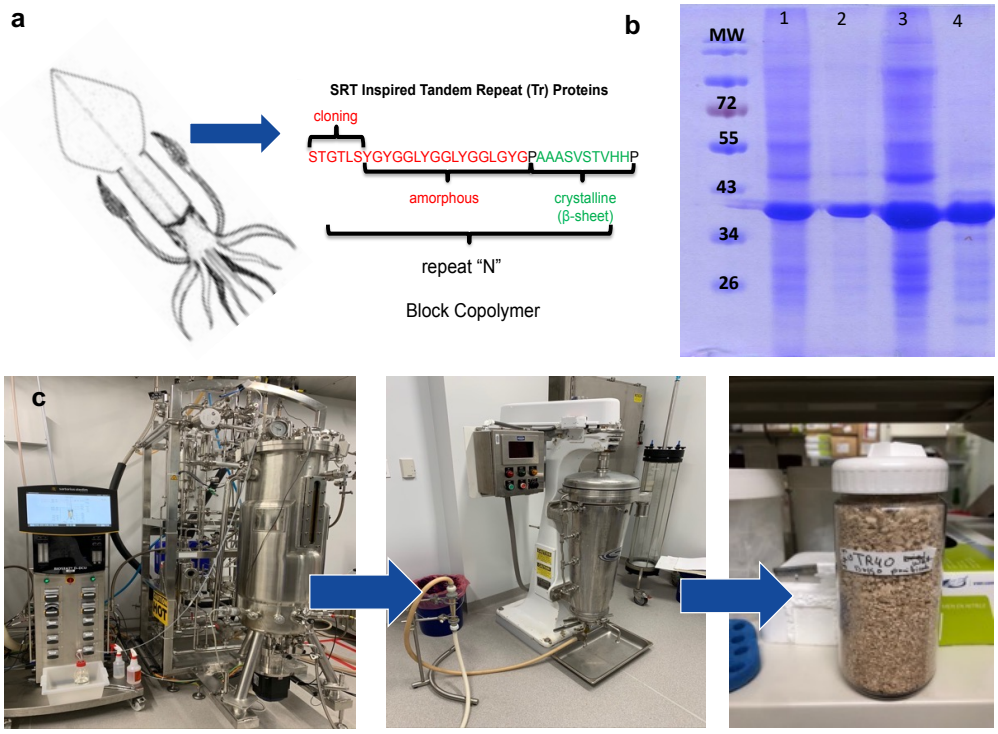


Figure 19. a) Design of a squid-inspired tandem protein sequence; b) SDS protein gel of TR-n11 shows ~40 kDa protein with different purification methods: 1) Buffer purification with 1xLB feedstock, 2) DMSO purification with 4xLB feedstock, 3) Buffer purification with 4xLB feedstock and 4) DMSO purification with 4xLB feedstock; c) 100L fermentation and downstream processes of centrifugation and purification, which yields of 1.5 g/L dry protein TR-n11 powder.

We used a laboratory-scale wet spinner to form fibers of acrylic and TR proteins. The spinning dope was prepared from lyophilized powder of TR protein (39.8 kDa, n=11 repeats) and acrylic (~120 kDa) polymers. **Figure 20a** shows a schematic illustration of the spinning process of the TR/acrylic blend. The powder solution was mixed in DMSO and deaerated before flowing through the spinneret, with holes of approximately 100 μm diameter. DMSO was removed from the forming fiber in an aqueous coagulation bath and further washed in a secondary bath (**Figure 20b and 20c**). The maximum solubility of TR proteins in DMSO was ~ 200 mg/ml; however, by increasing the temperature above 60 °C, TR protein/acrylic blends were successfully spun with optimum flow conditions using aqueous coagulation and washing baths at room temperature. Multifilament-blend TR-acrylic fibers were wound around a plastic dish (**Figure 20d**) for drying

and tested for tying a knot. These filaments are interlaced by hand to demonstrate the first example of a braided yarn made of TR-acrylic blends (**Figure 20e**).

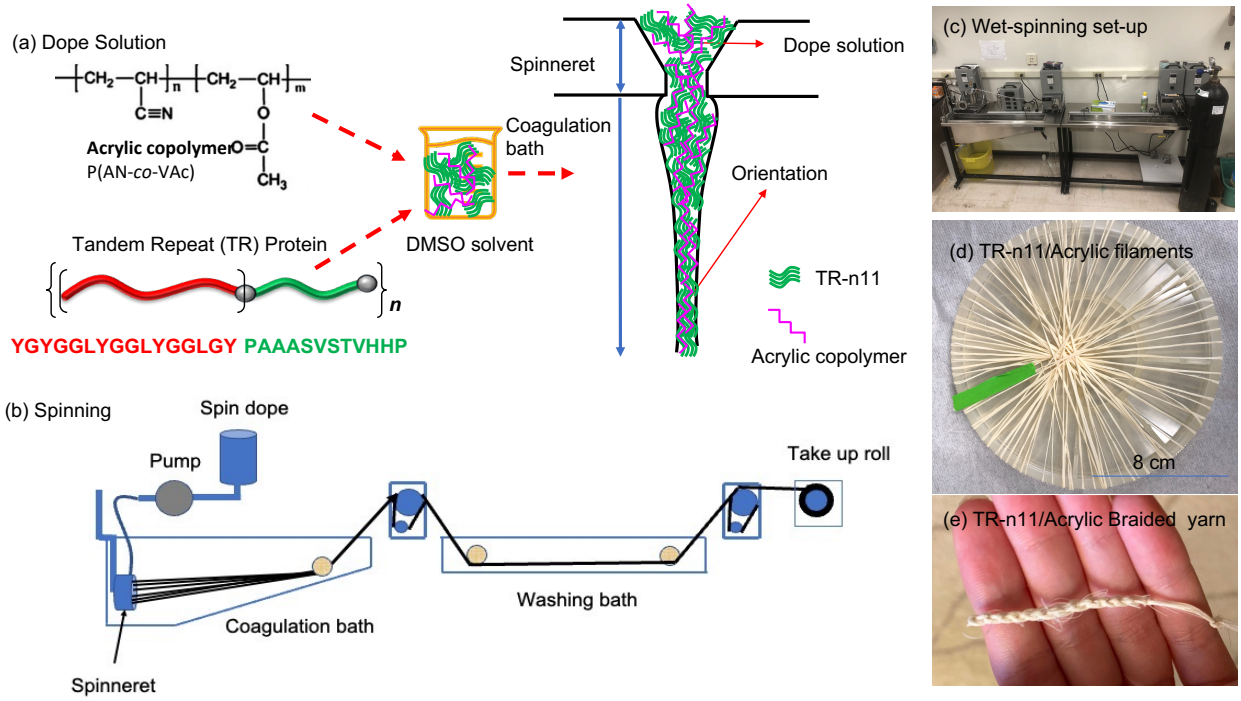


Figure 20. Wet spinning and blend fiber production: Schematic of a) extrusion of acyclic copolymer with TR-n11 protein, and b) wet-spinning process. Optical images of c) wet-spinning equipment, d) filaments and e) brained yarn made by TR-n11 and acrylic blends.

We characterized the uniaxial mechanical responses of blend fibers (**Figures 21a** and **21b**) following ASTM D638. TGA analysis of hydrated and ambient samples showed that blend fibers of 60:40 have very similar protein content but a significantly higher water content for the hydrated (wet) sample (**Figure 21c**). We performed initial calibration of the tensile experiments on a micro-scale with a fine-scale load (800 μN) and displacement (20 nm) resolutions. The modulus, strength, and toughness were measured from the stress-strain responses in **Figure 21d-e**, and their properties are summarized in Table 1. The hydrated SRT-acrylic fiber with a 60:40 weight ratio has an elasticity that is a hundred times that of ambient filaments, but the yield strength drops four times. These mechanical values agree well with our earlier finding of pure SRT films in wet (e.g., blend mechanics is governed by the soft SRT component) and dry (e.g., weighted average of pure acrylic, ~100 MPa, and SRT ~20MPa) conditions. The findings also demonstrate that the tunability of the plasticity (e.g., fracture strain and tensile strength) can be further improved by drawing the samples, a unique characteristic of rubbery transition and strain-induced crystallization in SRT proteins.

Table 1. Mechanical properties of blend fiber

Material Type	*Elastic modulus, E,(GPa)*	*Tensile Strength (MPa)*	*Fracture Strain (%)*	*Toughness, T ($MJ{\cdot}m^{-3}$)*
Wet Fiber	10.2±0.7	9.2±0.3	348.0±22.0	20.5±1.5
Dry Fiber	2420±170	42.8±6.3	2.4±0.6	0.6±0.3
Drawn Fiber	137.0±9.8	13.0±2.7	29.9±0.5	2.8±0.5

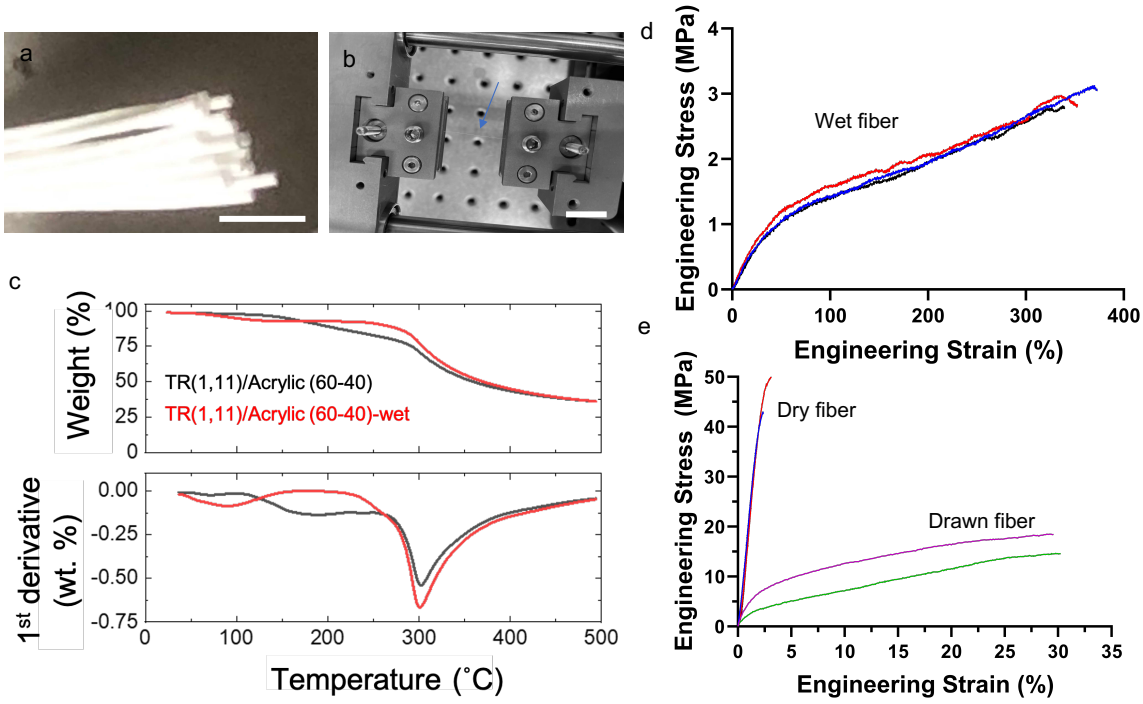


Figure 21. Mechanical Characterization: Optical image of a) TR-n11 and acrylic blend fibers with the weight ratio of (60:40), and corresponding b) mono-filament testing under the mechanical stage. c) Thermogravimetric analysis shows additional water content on wet blend fibers compared to dry fibers. Engineering stress-strain data for d) wet and e) dry and drawn TR-n11/acrylic blended fibers.

We studied the dynamics of SRT by elastic and quasielastic neutron scattering (QENS) to understand the connection between nanostructure, chain dynamics, and mechanical properties. SRT is plasticized by water above its glass transition temperature at 35 °C. Elastic scans (**Figure 22**) revealed an increased protein chain mobility upon hydration, superimposed dynamic processes, and a decrease in dynamic transition temperatures. Further analysis by QENS revealed that while dry protein dynamics are dominated by localized methyl group rotations, hydrated dynamics are dominated by the confined diffusion of flexible chains within a β-sheet nanocrystalline network (8 Å of confinement radius). Our findings establish a relationship between the segment block architecture of SRT, the diffusive motions within the protein structure, and the mechanical properties of recombinant proteins.

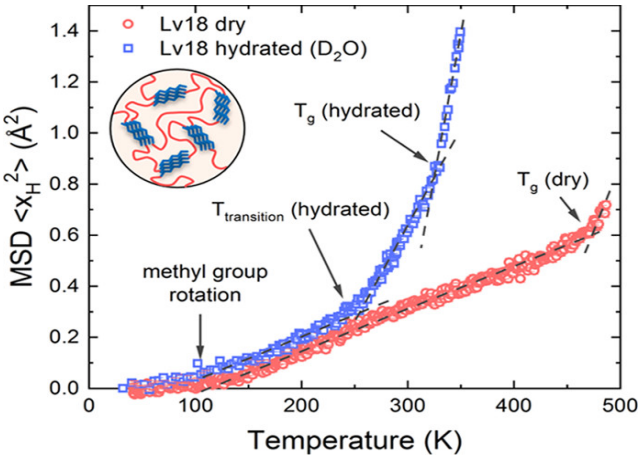


Figure 22. Mean-square displacement (MSD) $\langle x_H^2(T) \rangle$ of dry and D_2O-hydrated recombinant SRT protein as a function of temperature. Error bars are within the size of the symbols.

We report on the ability of tandem repeat proteins to enhance the triboelectric properties of natural fibers. The electrostatic charge that is generated when two materials are rubbed is a well-known physical process known as triboelectricity. Animal fur or human hairs are known to have high triboelectric charges. This is also an important phenomenon in the industry for problems in electrification such as damage to electronic equipment and tanker explosions as well as a solution in the design of industrial processes such as separation of materials in the recycling industry and operations of copiers and laser printers. Natural or bioengineered proteins demonstrate triboelectric response based on the charged amino acids (e.g., histidine, aspartic acid, arginine, lysine, and glutamic acid). The triboelectric value of SRT is over two times the current state-of-the-art of other natural and synthetic materials as shown in **Figure 23**, which compares the normalized voltages and currents reported from the literature. The max achievable output by our fibers is about twice the best reported in the literature.

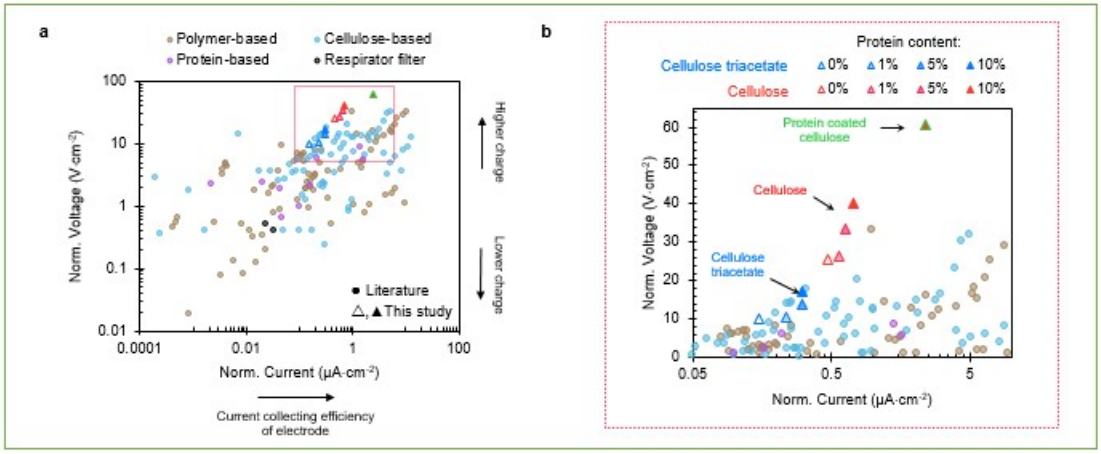


Figure 23. The figure of merit for triboelectric properties: Max norm. voltage and current in various fiber-based materials. The results are segregated into categories, namely, polymer, cellulose, and protein-based materials. Cellulose with 10% protein fiber outperforms all other materials.