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Current Opinion in Biotechnology

Synthetic biology for protein-based materials

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Recombinant protein polymers that mimic the structures and functions of natural proteins and those tailor-designed with new properties provide a family of uniquely tunable and functional materials. However, the diversity of genetically engineered protein polymers is still limited. As a powerful engine for the creation of new biological devices and systems, synthetic biology is promising to tackle the challenges that exist in conventional studies on protein polymers. Here we review the advances in design and biosynthesis of advanced protein materials by synthetic biology approaches. In particular, we highlight their roles in expanding the variety of designer protein polymers and creating programmable materials with live cells.

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Current Opinion in Biotechnology 2020, **65**:197–204

This review comes from a themed issue on **Chemical biotechnology**

Edited by **Christoph Wittmann** and **Sang Yup Lee**

https://doi.org/10.1016/j.copbio.2020.04.004

Introduction

As the main components of living organisms, proteins commonly perform their biological roles in the form of supramolecular self-assemblies with fine architectures and versatile functionalities [1]. Inspired by the unique structural and functional beauty of natural protein self-assemblies, diverse protein materials such as spider silk, elastin, collagen and mussel adhesives, have been reproduced through biomimicry in heterologous hosts for mass production [2,3•,4,5]. The applications of these materials have been widely demonstrated in biomedical engineering fields including drug delivery, tissue repair and regeneration [6•,7–9].

With the emergence of synthetic biology, different interdisciplinary efforts are pursued to implement engineering principles to biology [10]. By harnessing the power of synthetic biology, the discovery platforms and fabrication technologies for protein materials have been augmented to expand the materials landscape and define a new material paradigm [11]. In particular, advanced genetic methods are developed to enable the generation of genes encoding protein polymers from biological building blocks with designer architecture. In addition, programmable materials with complex spatial organization through synthetic patterning are created, which have sophisticated functions and show significant advantages over traditional materials as they are able to grow, self-heal and even respond to their environments [12,13••,14,15••]. This enables the delivery of next-generation advanced materials with new and extended functionalities to address a wide range of unmet needs [16,17].

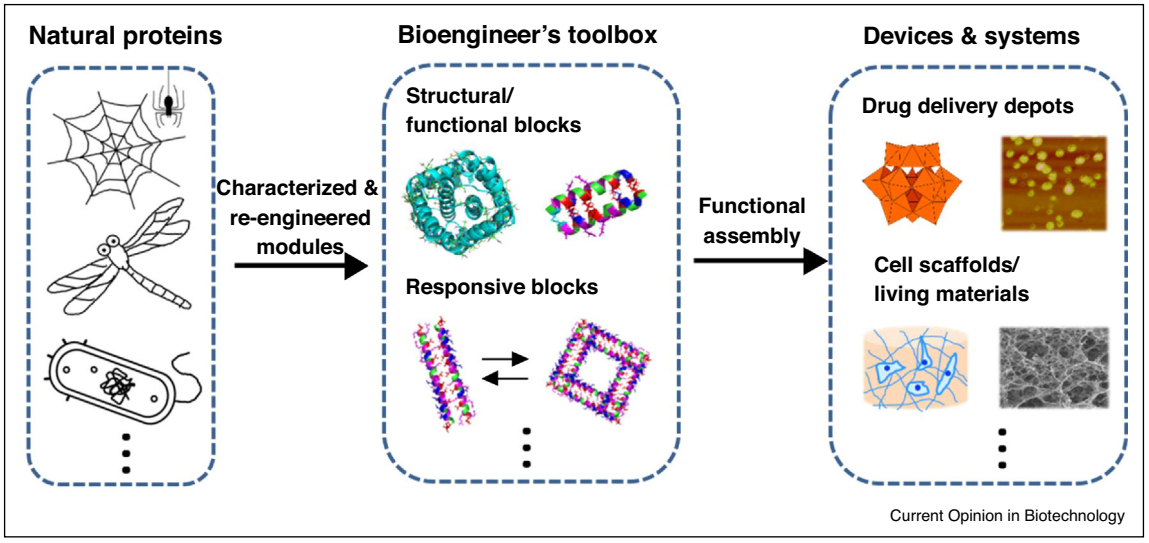
The unification of synthetic biology and material sciences allows rapid expansion of materials diversity, easy fabrication of multifunctional materials, and sustainable routes to bio-based production in next-generation materials manufacture [18]. Here we review how innovations can emerge by combining synthetic biology concepts and enabling tools such as gene assembly with principles from materials sciences, and present strategies and examples for the development of new protein functional materials. We conclude by discussing the challenges and perspectives to realize the huge potential of protein materials for diverse applications.

Biological building blocks: learning from nature

Nature has evolved many biological modules that form hierarchical structures and endow diverse biological functions [19]. Many of these modules have been directly adapted as building blocks or reduced into minimal consensus amino acid sequences that can be homopolymerized into repetitive proteins to recapitulate the properties of their natural counterparts [2,20]. The diverse building blocks allow researchers to assemble genes encoding protein copolymers with adjustable type, number and order of the building blocks [21–23]. This assembly concept is very powerful as it allows multiple functions to be integrated into one polymer molecule so that complex protein materials can be rationally engineered in a piecewise fashion [24]. According to their functions, the building blocks can be categorized into three groups: structural blocks to endow physiochemical properties, functional blocks to enable specific biological functions, and responsive blocks to impart dynamic properties to the resulting materials upon exposure to environmental stimuli (Figure 1).

The spectrum of structural blocks is quite broad, which can be derived from structurally simple proteins such as

Figure 1



Engineering principles of protein materials through synthetic biology approaches. Diverse building blocks can be adapted from natural protein materials and re-engineered into new materials for various applications.

protein cages and complex ones like spider silk (Table 1). A variety of structural blocks are derived from natural materials with repetitive protein components such as resilin, silk, collagen, and mussel byssus [13••,25–32]. For example, the consensus pentadecapeptide block (GGRPSDSYGAPGGGN) was excavated from the repetitive pro-resilin protein of fruitfly, and recombinant polypeptides with varying numbers of the blocks could be chemically crosslinked into elastomeric hydrogel networks with native resilin-like resilience [13••]. In addition, structural blocks may also be derived from non-repetitive proteins such as viral capsids. These structural blocks have been utilized to form artificial virus-like particles or synthetic organelles via cooperative self-assembly *in vitro*, and recent innovations in these synthetic systems are reviewed in detail elsewhere [33,34].

Functional blocks include large amounts of bioactive peptides with a length of a few to tens of amino acids derived from native proteins [35,36]. For example, RGD and REDV cell adhesion peptides for enhancing mammalian cell attachment [37], proteolytic enzyme recognition sites favoring biomaterials degradation [38], and antimicrobial peptides for preventing infection [39] have been used to improve *in vitro* and/or *in vivo* functions of protein materials by genetic fusion (Table 1). In addition, many interacting polypeptide chains such as Split-inteins, SpyTag-SpyCatcher and heterodimer-forming coiled coils serve as selective binding blocks that can be exploited in *de novo* designed polypeptides for the construction of functional materials [40,41]. Notably, the interacting polypeptide blocks can also be engineered to be sensitive to diverse environmental triggers such as small molecules, temperature and light so that they could be exploited for the construction of 'smart' responsive materials [42].

Many responsive blocks are derived from natural biological switches, and have been utilized to incorporate sensing and actuation functions into materials (Table 1). For example, transcription factor/DNA switches responding to cellular or environmental stimuli, have been incorporated into materials to confer responsive properties [43]. Responsive polypeptide blocks that switch between monomeric and dimeric states have also been identified and used for the functionalization of protein materials. For example, the subunit B of the bacterial gyrase (GyrB) forms dimers upon binding to the antibiotic coumermycin, and can be dissociated with the addition of novobiocin by competitive binding to GyrB [44]. In contrast, the F36M variant of human FK binding protein 12 (FKBP12) naturally exists as dimers, which dissociates by the addition of the small molecule FK506 [45]. In particular, some polypeptide blocks such as Cryptochrome 2 (Cry2) and C-terminal adenosylcobalamin binding domain ($CarH_C$) can be switched between monomeric and different oligomeric states in response to light [46,47••,48]. These responsive blocks have been either genetically fused or non-covalently bound with precursor polymers to form materials, and the use of these blocks as switches have been demonstrated for the controlled release of cargo molecules and delivery of cells [43,48]. As reported in a recent study, an entirely protein-based hydrogel composed of $CarH_C$ polymers was formed through adenosylcobalamin-induced $CarH_C$ tetramerization in the dark, and the physically self-assembled hydrogel disassembled

Table 1

Building blocks for engineering protein materials

Block type	Amino acid sequences/typical examples	References
Structural blocks		
Silk-like	GAGAGS, GAGAGY, GGX (X = L, Y, Q or A), GPGXX (X = G, Q, Y)	[2,22]
Elastin-like	GXGVP (X is any amino acid except proline)	[4,20]
Collagen-like	GXX' (XX' are often proline and hydroxyproline, respectively)	[8,27]
Resilin-like	GGRPSDSYGAPGGGN, GYSGGRPGGQDLG, AQTPSSQYPAG	[13••,22]
Mussel byssus-like	AKPSYPPTYK	[28]
Suckerin	PAAASVSTVHHP, YGYGGLYGGLYGGLGY	[29,63]
Leucine zipper	$(VSSLESK)_2$VSKLESKKSKLESKVSKLESK VSSLESK	[30,35]
βTail	MALKVELEKLKSELVVLHSELHKLKSEL	[31]
Amyloid precursor protein	CsgA protein, FapC protein, Aβ(1-42), Prion, Transthyretin (TTR1)	[32,58]
Functional blocks		
Cellular adhesion	RGD, REDV	[37]
Growth factor	Vascular endothelial growth factor, Keratinocyte growth factor	[35]
Antimicrobial activity	Hepcidin, HNP-2,4, Magainin, Lactoferricin	[39]
Proteolytic degradation	Matrix metalloproteinase sensitive peptides	[38]
Targeting	Antibody fragments, DNA aptamers	[36]
Cross-linking	SpyTag-SpyCatcher, Split-inteins, CutA trimer-forming peptide	[40,41]
Responsive blocks		
Transcription factor/DNA	TetR/*tetO*, LacI/*lacO*	[43]
Regulation proteins/ small molecules	GyrB/coumermycin, FKBP12/FK506 Calmodulin/Ca^{2+}, TetR/ tetracycline	[44,45]
Light-responsive proteins	$PhyB_{FR}$, $CarH_C$, Dronpa145N, Cry2	[46,47••,48]
Other stimuli-responsive proteins	Elastin-like ELPs, RLPs, abductin, CTD of spidroins	[4,23,26]

on light exposure, which enabled the facile release/recovery of cells after 3D cell culturing within the hydrogel matrix [48].

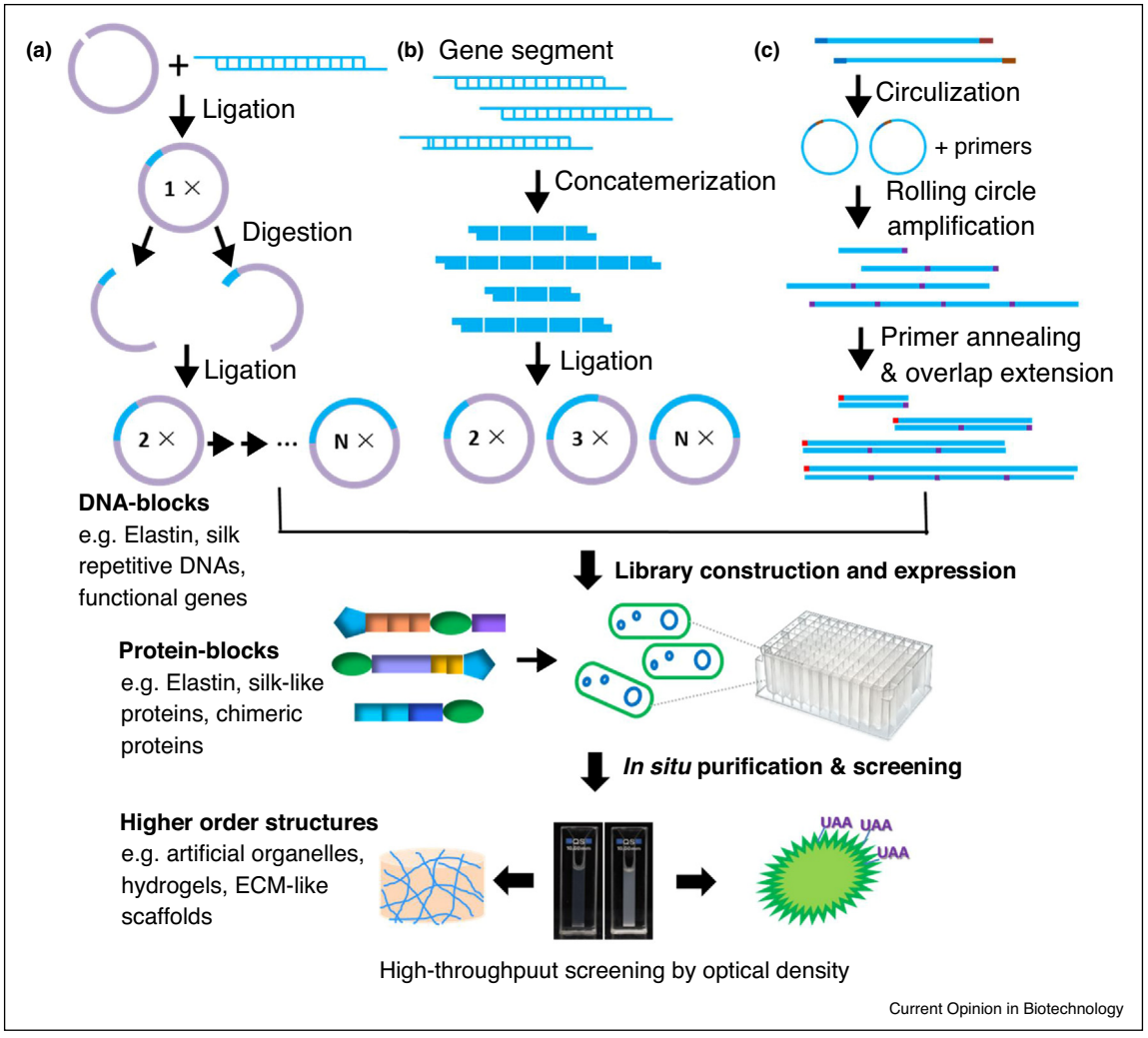
Bottom-up assembly of genes encoding functional protein materials

As a unique feature of natural protein-based materials, modularity inspires bottom-up construction and hierarchical self-assembly across multiple length-scales, ultimately resulting in desirable materials with advanced mechanical and biological properties [19]. For recombinant production of these natural protein-based materials, robust modular gene assembly strategies are required to make genetic constructs that encode these proteins composed of desirable peptide building blocks as described above. Until now, three main strategies have been reported. The most commonly used one is a step-by-step, directional approach, which allows precise control over the sizes of target genes through consecutive digestion and ligation steps (Figure 2a). This approach provides high accuracy in cloning but the procedures are rather tedious and time consuming. In addition, to realize seamless cloning without extraneous nucleotides at the ligation junctions, the choice of appropriate restriction enzymes such as type IIS enzymes is necessary [49,50].

The second type of gene assembly approach, termed concatemerization (Figure 2b), is quite useful to obtain a library of genes with different sizes in one ligation reaction. As a step-growth polymerization mechanism of DNA, concatemerization uses synthetic DNA encoding one or a few copies of the peptide building blocks to generate variable repetitions of the DNA sequence arranged in tandem. In accordance with this approach, combinatorial libraries have been constructed for high-throughput biosynthesis and screening of silk-elastin-like protein polymers (SELPs) with unique stimuli responsive features and mechanical properties [51]. First, concatemerization ligation of DNA fragments encoding silk-elastin peptide blocks was performed to generate libraries of genes encoding SELPs that differed in molecular weight and composition of the silk-elastin blocks. These blocks could be rationally designed by varying the ratio of silk to elastin units and key residues in the elastin units. Second, plate cultivation, *in situ* expression and purification of the protein polymer libraries were performed for screening SELPs responsive to temperature, pH, redox, and phosphorylation. The relatively huge number of identified protein polymers offered fascinating opportunities for in-depth understanding of the sequence-structure-property relationship, which is vital to provide a foundation for future library designs. In contrast, the newly identified SELPs exhibited specific material properties and functions for diverse applications [51].

The above concatemerization strategy is limited to reach a high degree of concatemerization resulting from the involvement of enzyme-restricted overhangs for ligation. To address this obstacle, an improved method called overlap extension rolling circle amplification (OERCA) was developed for the highly parallel synthesis of genes encoding repetitive protein polymers. OERCA involves a single PCR-type reaction for the rolling circle amplification of a circular DNA template and simultaneous overlap extension by thermal cycling (Figure 2c). Its robustness, high-throughput, and versatility by synthesizing variants of elastin-like polypeptides (ELPs) and protease-responsive polymers of glucagon-like peptide-1 analogues were

Figure 2



Library construction and high throughput screening of functional protein materials. Schematic of snapshots depicting the gene assembly approaches for library construction: **(a)** Recursive Directional Ligation (RDL), **(b)** Concatemerization, **(c)** Overlap Extension Rolling Circle Amplification (OERCA) approaches. The concept of high throughput screening was adapted from Refs. [51,54].

elegantly demonstrated. Notably, the OERCA strategy generated libraries of repetitive genes over a wide and tunable range of molecular weights in a 'one-pot' parallel format [52]. To further improve the OERCA method, a codon-scrambling algorithm was recently developed by exploiting the codon redundancy of amino acids and finding the least-repetitive synonymous gene sequence [53].

Of particular interest, an integrative 'one-vector-toolbox-platform' (OVTP) approach that combines concatemerization and directional ligation was developed to construct combinatorial material libraries composed of diverse structural and functional blocks [54]. Major components of the modular OVTP are vectors consisting of an adapted vector backbone and a tailor-designed linker region, within which the localization of the recognition sequences of two type IIS restriction enzymes and one type IIP restriction enzyme as well as the generated overhangs are precisely defined. Hence the OVTP approach allows for the assembly, multimerization, intentional arrangement, and direct translation of defined DNA building block libraries, in combination with the selective functionalization of the resulting protein polymer libraries upon incorporation of genetically encoded unnatural amino acids (UAAs). Notably, the approach enables access to multitudes of biohybrid materials for self-assembled superstructures for diverse applications (Figure 2).

'Living' protein materials with genetically programmable functions

Living systems such as bacteria, yeasts, and mammalian cells can be genetically programmed with synthetic gene circuits

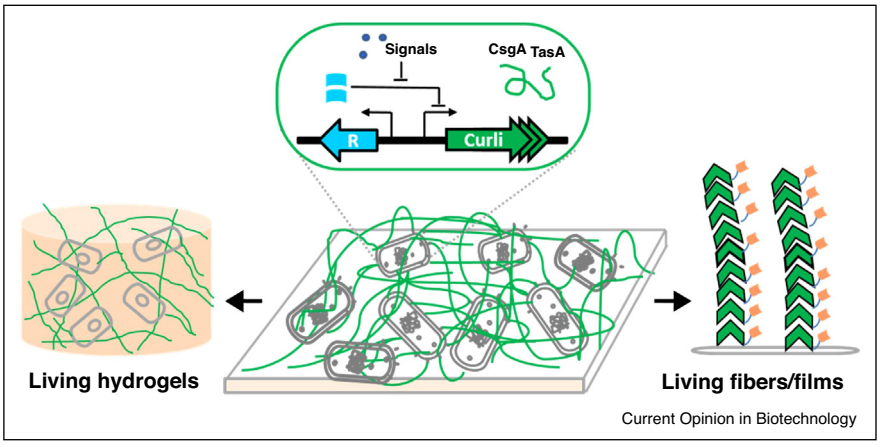
that execute sensing, computing, memory, and response functions. In principle, integrating these living components with preexisting protein materials will enable access to novel 'living' functional materials. Alternatively, living materials can be designed by programming a cellular system to biosynthesize and secrete protein components for self-assembly into surface nanostrucutres capable of entangling *in situ* to form macroscopic cell-laden materials. As a proof-of-concept, synthetic gene circuit-enabled curli amyloid production in bacterium *Escherichia coli* was demonstrated to be both a functional material in its own and a materials synthesis platform [55]. This work lays a foundation for synthesizing, patterning and controlling functional composite materials with engineered live cells. To improve the tunability of the above system, fabrication of three-dimensional (3D) materials was recently reported by printing engineered self-patterning *E. coli* [56]. The resulting dome structure with hybrid organic-inorganic materials acts as a resettable pressure sensor that can process signals in response to varying pressure intensity and duration.

To expand the function of the above curli system, an approach called Biofilm-Integrated Nanofiber Display (BIND) was reported [57]. An *E. coli* biofilm was programmed by genetically appending diverse peptide domains to the amyloid protein CsgA, the major subunit of *E. coli* amyloid curli fibers. These fusion proteins were successfully secreted for extracellular self-assembly into amyloid nanofiber networks that retain the functions of the displayed peptide domains (Figure 3). The BIND system confers diverse artificial functions to the biofilm matrix, such as nanoparticle bio-templating, substrate adhesion, covalent immobilization of proteins, or a combination thereof. To realize smarter control of the amyloid biosynthesis and expand its application, a synthetic gene circuit was designed to detect mercury in the environment (via MerR) and to direct the synthesis of curli nanofibers to sequester mercury ions in an extracellular matrix. This work paves the way for the development of on-demand biofilm materials that can operate autonomously as heavy-metal absorbents [58].

Using a similar approach, strong and multi-functional underwater adhesives were developed by fusing mussel foot proteins (Mfps) of *Mytilus galloprovincialis* with CsgA in *E. coli* biofilms [59]. To diversify material forms for wider applications, a flexible and tunable functional materials platform was more recently established based on the TasA amyloid machinery of the Gram-positive bacterium *Bacillus subtilis* (Figure 3). The engineered biofilms have the viscoelastic behaviors of hydrogels and can be precisely fabricated into microstructures having a diversity of 3D shapes using 3D printing and microencapsulation techniques [60••].

It has been a great challenge to maintain the viability, functionality, and safety of living cells in freestanding materials and devices. To overcome the obstacle, one approach was reported by using stretchable, robust, and biocompatible hydrogel-elastomer hybrids to host various types of bacterial cells, each harboring a distinct synthetic gene circuit [61]. Under the above scenario, the polyacrylamide-alginate hydrogel provides sustainable supplies of water and nutrients, whereas the silicone elastomer is air-permeable and maintains long-term viability and functionality of the encapsulated cells. In contrast, communications between the environment and the different bacterial strains are achieved via diffusion of triggering molecules in the hydrogel. Furthermore, functions and applications of these stretchable sensors with living cells were well demonstrated to be responsive to multiple chemicals in a variety of forms, including skin patches and

Figure 3



Genetic programming and modularity of the living biofilm platform. The formation of biofilm composed of CsgA or TasA curli fibers is controlled by diverse genetic circuits responsive to various environmental triggers [57,58,60••].

gloves-based sensors. As another intriguing approach to solve the aforementioned challenge, *B. subtilis* spores, as opposed to vegetative cells, were printed within an agarose hydrogel for storage [62••]. The spore-containing material could be desiccated and stored at room temperature indefinitely. The cells germinated following rehydration and performed their genetically programmed functions, including responding to chemicals and sensing or killing of microbial pathogens. Notably, the recalcitrant nature of spores makes this approach potentially compatible with diverse 3D printing technologies under harsh conditions. It is envisioned that the above encapsulating hydrogels may be replaced with protein polymer hydrogels, which resemble native extracellular matrices of human tissues with tunable mechanical properties and biological functions [13••,30].

Conclusion and future perspectives

In summary, synthetic biology tools, such as high throughput gene assembly, artificial gene circuits design and regulation, can be used to construct multifunctional protein materials in a bottom-up, autonomously assembled, and environmentally sustainable manner. These tools can also be employed to engineer the spatiotemporal characteristics of living systems to interface with materials, thus enabling programmable material properties.

Predictive design, as well as rapid and high throughput evaluation is at the core of any synthetic biology approach, along with parallelized assembly of advanced functional materials through laboratory automation and post-biosynthesis processing [63]. However, the predictive design of protein materials remains a challenge. Detailed knowledge on the structures of protein polymers is often unavailable, and these proteins often exhibit low solubility and poor processing characteristics. In such circumstances, it would be rather difficult to predict the amino acid targets for engineering to improve the physiochemical properties of the protein polymers and the ultimate materials. Fortunately, the ability to understand the sequence-structural assemblies-property relationship of protein polymers has been greatly improved by combined experimental approaches and computational simulations at multiple length scales [64]. We anticipate that the rapid creation of protein polymer libraries and high-throughput evaluation of material properties will generate big data for deep learning algorithms, which will support rational and predictive design of protein materials in the discovery pipeline.

Another great challenge is to elevate the production of recombinant protein polymers and materials at a scale and cost that allow them to impact additional markets. To achieve this goal, it is crucial to understand the cellular and metabolic regulatory mechanisms of microbial hosts upon biosynthesis of the protein polymers, which are usually large in molecular weight, highly repetitive, and rich in specific amino acids. With the above information in hand, systems metabolic engineering of the hosts and bioprocess engineering shall be performed to improve the production performances [65••]. Furthermore, continued research shall look at discovering new materials forms for applications that can bring high value to the existing markets of protein materials. Overall, targeting high-value applications such as regenerative medicine, implantable sensors and actuators, in which proteins have inherent advantages, are anticipated to achieve commercial success for protein materials.

Conflict of interest statement

Nothing declared.

Acknowledgements

The work has been supported by the National Natural Science Foundation of China (21674061 and 31470216), MOST of China (2016YFE0204400), and the Program for Professor of Special Appointment (Eastern Scholar) at Shanghai Institutions of Higher Learning.

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- •• of outstanding interest

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