Protein-based composite materials

Protein-based composite biomaterials have been actively pursued as they can encompass a range of physical properties to accommodate a broader spectrum of functional requirements, such as elasticity to support diverse tissues. By optimizing molecular interfaces between structural proteins, useful composite materials can be fabricated as films, gels, particles, and fibers, as well as for electrical and optical devices. Such systems provide analogies to more traditional synthetic polymers yet with expanded utility due to the material's tunability, mechanical properties, degradability, biocompatibility, and functionalization, such as for drug delivery, biosensors, and tissue regeneration.

Xiao Hu[a], Peggy Cebe[a], Anthony S. Weiss[b], Fiorenzo Omenetto[a], and David L. Kaplan[a]*

[a]*Departments of Biomedical Engineering & Physics and Astronomy, Tufts University, Medford, MA 02155, USA*

[b]*School of Molecular Bioscience, University of Sydney, NSW 2006, Australia*

**E-mail: david.kaplan@tufts.edu*

Natural structural proteins display critical structural and bioactive properties that have evolved in nature for millions of years. However, depending on the specific protein, there may be useful functions, such as mechanical toughness, while other critical features may be more limiting, such as cell compatibility or a broader range of mechanical properties. Nature has evolved strategies to resolve this problem by generating multifunctional composite materials *in vivo*. For example, collagen and elastin are often found together in the body to provide the combination of strength and toughness required for specific tissue functions[1]. Blending (mixing) proteins is a technological approach to generate protein-based biomaterials with a more complete set of specific properties. Blending can also benefit materials engineering through improved processability and material uniformity. As an alternative to blending, genetic engineering strategies have been exploited to generate combinations or hybrids of structural proteins to achieve control of functional features. However, at present this process remains limited due to the costs of scale up for these biotechnologically driven processes. Therefore, generating multifunctional, biodegradable structural protein composite biomaterials is emerging as a useful direction in the field to tailor properties to specific medical needs *in vitro* and *in vivo*, or as a strategy to generate a broader range of functional properties with which to conduct more systematic studies of the impact of the biomaterials on cell and tissue functions.

Natural structural proteins

Many natural proteins have been studied, with distinguishing mechanical, chemical, electrical, electromagnetic, and optical properties. Elastins, collagens, silks, keratins, and resilins are some of the more common structural proteins considered for protein-based biomaterials (Fig. 1). In

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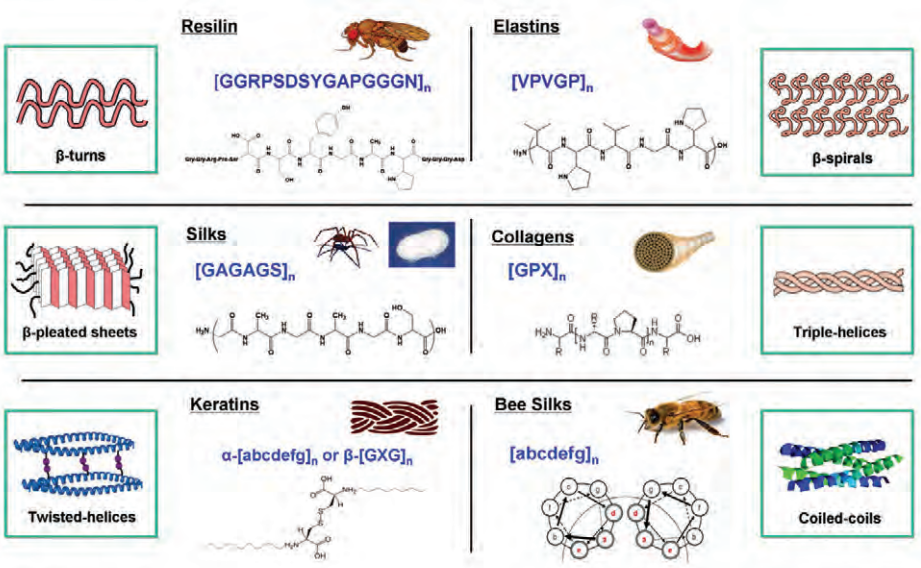

Fig. 1 Natural proteins for composite materials[1-23], including resilins (repeats such as $(GGRPSDSYGAPGGGN)_n$, and possible beta-turn structure), elastins (repeats such as $[GVGVP]_n$, and possible beta-spiral structure), spider and silkworm silks (repeats such as $[GAGAGS]_n$, or $[AAAAA]_n$ and beta-pleased sheet crystal structure), collagens (repeats such as $(GPX)_n$, and triple-helix structure), hair keratins (composed of two protein chains with alpha-helical conformation and cross-linked by cysteines), and bee silks (sequences with a periodicity of $(abcdefg)_n$, where a, d and/or e are hydrophobic, and form tetrameric coiled-coil structures).

general, these families of structural proteins are characterized by long-range ordered molecular secondary structures (*e.g.*, beta-pleated sheets, coiled coils, or triple helices) that arise due to the highly repetitive primary amino acid sequences within the proteins. These features promote self-assembly, the formation of structural hierarchy and thus materials-related functional roles in nature. These long-range ordered features reflect their roles as mechanically important structures with functions at biological interfaces, prompting their utility as a treasured resource of polymers for biomaterials.

Elastin proteins are critical in forming elastic fibers in most tissues, such as blood vessels and dermis[1,2]. Different elastin protein networks can be isolated from animal tissues such as skin, while recombinant human tropoelastin (full-length 60 kDa), the soluble precursor of elastin, is an alternative source[2]. The hydrophobic domains of elastin are rich in non-polar amino acids, with common repeat motifs such as $[GVGVP]_n$, while hydrophilic domains contain a high content of lysine that are involved in elastin cross-linking[1,2] and help to stabilize the structure.

Resilin proteins are 'super elastic rubbers' found in the flight and jumping organs of insects where cyclic extension and retraction are needed millions of times over the lifetime of the animals[3,4]. Cross-linked resilin (via tyrosines) exhibits high resilience up to 95 % under high-frequency motion[3,4], and has over 300 % elongation before breaking[3], providing options for biomaterial applications during high frequency elastic motions. Full length resilin in *D. melanogaster* contains three domains, with the first exon enriched in repeats of $(GGRPSDSYGAPGGGN)$[4], and the third exon enriched in repeats of $(GYSGGRPGGQDLG)$[4]. The middle exon encodes for a chitin binding domain, putatively involved in anchoring to the chitin component of the exoskeleton[4].

Collagens (Type I ~ XXVIII) are the most abundant proteins found in tissues of animals, including tendons, ligaments and skin[5,6], with up to 25 % ~ 35 % of whole-body protein content consisting of the different collagens. Collagen is composed of three left-handed helices intertwined to form a right-handed triple helix, and generally consists of different chain compositions depending on the specific type of collagen[5,6]. The most common amino acid sequence in collagen is $(GPX)_n$, where X is any amino acid other than glycine, proline or hydroxyproline[5]. If collagen is irreversibly hydrolyzed (e.g., by heat and chemicals), it is termed "gelatin", which is frequently used as a biomaterial[5,6].

Silk proteins are fibrous proteins synthesized by silkworms and spiders. In the salivary gland of *Bombyx mori* silkworms, silk fibroin exists in a water-soluble form and is spun into fibers while being coated with glue-like sericin proteins. The spinning process leads to the rapid transition from the silk solution state to an insoluble form dominated by anti-parallel beta sheet crystals. Silk fibroins combine high tensile strength and toughness[7-9]. The primary structure of *B. mori* silk fibroin consists mainly of the repeat sequence $[GAGAGS]_n$[7-9]. The transition of silk fibroin molecules from the solution state to beta sheet crystalline state can be induced physically with methanol, heat, low pH, vortexing, or sonication[7-12], where energy input or dehydration drives interactions among the hydrophobic domains to form the beta sheet crystals which then serve as physical cross-links. Spider silks are also actively pursued as biomaterials, while the sources must be generated by genetic engineering[9]. Unlike silkworm silk available from the textile world, there is no commodity source of spider silks. With a broader screening of other naturally fibrous proteins, insect silks can be grouped into more than 23 independently evolved lineages with different secondary structures

such as coiled coils, cross-beta sheets, or polyglycine II structures[13]. The silks from the social *Hymenoptera* (bees, ants, hornets), which includes over 144 000 species, are composed of α-helical proteins assembled into tetrameric coiled coil conformations[13-15]. This is a fundamentally different silk structure than the β-sheet crystallites in the silkworm cocoon and spider dragline silks. The coiled-coil structure has a seven-residue periodicity ($(abcdefg)_n$), in which the first and fourth residues (a and d) in each heptad are generally hydrophobic (sometimes also including e), and other positions are generally polar or charged[13-15]. Measurements of the mechanical properties of honeybee silk fibers show excellent toughness and extensibility (> 200 %)[14].

Keratin proteins[16,17] are another broad category of insoluble proteins that form intermediate filaments in the cytoplasmic epithelia and epidermal appendage structures (*e.g.* hairs, nails, wools, hooves). Both "hard" epithelial keratins and "soft" hair keratins are composed of two protein chains with different compositions and molecular weights (types I and II), yet each contains a central alpha-helical domain[16]. Although different keratins are closely related in secondary structures, "soft" hair keratins contain a higher content of cysteines in the non-helical domains and thus form tougher and more durable structures through formation of intermolecular disulfide bonds[16].

In addition, there are other natural proteins currently studied for different biomaterial applications, including reflectins for optical devices[18,19], amyloids for biosensors[20-23], and various plant proteins for tissue regenerations[24]. These examples of natural proteins, together with their genetic variants generated by protein engineering, provide a tool-kit of biopolymer options with different material properties, suggesting blending as an efficient approach to generate biomaterials with a broader range of properties or with properties that capture a combination of features from the components.

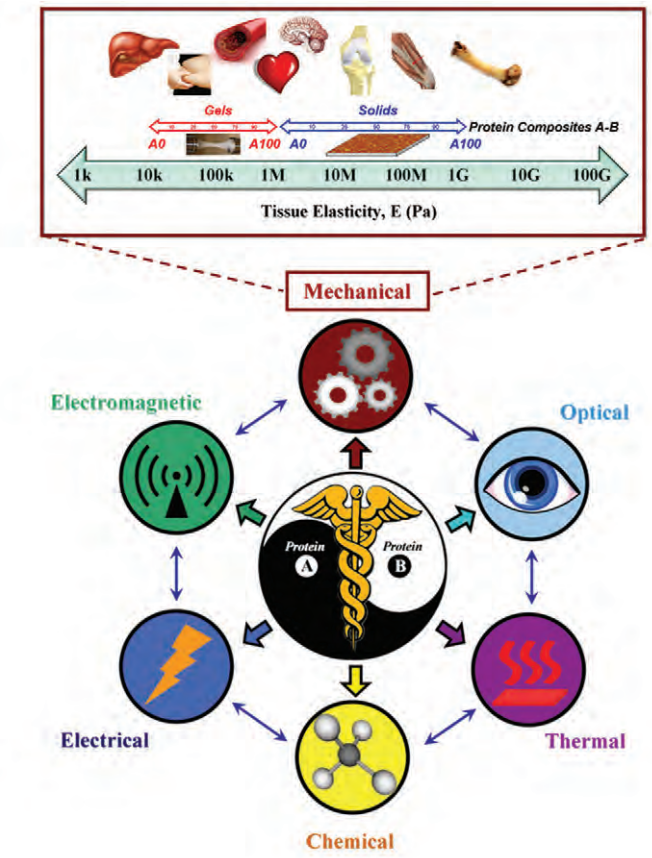

Fig. 2 Combinations of proteins A and B can generate new multifunctional protein composite systems with tailorable properties for specific mechanical, electrical, electromagnetic, optical, chemical, or thermal applications. For example, by mixing rigid protein A (e. g., silk) with a soft protein B (e. g., tropoelastin) in different ratios (e.g., A100 means a blend of 100 % A – 0 % B), a range of mechanical properties can be achieved to mimic the range of elasticities of different human tissues[27].

Mechanisms of interaction

Desirable features from a biomaterials standpoint may include mechanical strength or resiliency, electrical and dielectric response, optical transparency, chemical signaling and processability, or thermal stability (Fig. 2). For example (Fig. 2), combinations of tough, durable proteins A (silk as an example) with the highly elastic structural protein B (tropoelastin as an example) results in new multifunctional protein composite systems with variable mechanical properties to offer a broad platform of utility to the biomaterials field[25,26]. Tropoelastin can provide highly flexible and dynamic structural features with specific human cell signaling due to specific peptide epitopes in the native sequence, while silk provides mechanical toughness, insolubility, and slow degradation *in vitro* and *in vivo*[25,26]. The elastic modulus of protein blends or composites should be intermediate between the moduli of two individual materials, assuming the two components interact in a stabilizing manner. Fig. 2 illustrates the rough range of mechanical rigidity found in different human tissues[27]. By mixing a more rigid protein A (*e.g.* silk) with softer protein B (*e.g.* tropoelastin) in different ratios, a range of mechanical properties can be achieved. The toughest and stiffest tissues, such as bone and cartilage, can be mimicked more by protein A-dominated composites in a solid dense form (*e.g.*, blocks, posts), while the modulus of the softest tissues such as skin and livers can be accommodated by protein B-dominated composites in a hydrated looser format (*e.g.* hydrogels, foams). Therefore, one can select the appropriate combination or blend to match targeted tissue mechanics, to gain an appropriate starting point when examining regeneration of such tissues with a protein biomaterials approach.

Fundamentally, the interaction of protein A with protein B can be viewed as the interaction between a "solvent" (the predominant protein component in the blend) and a "solute" (the minor component), based on the Flory-Huggins's lattice model[28-30]. Typical protein-protein interactions include charge-charge (electrostatic), dipole, hydrogen bonding, hydrophobic-hydrophilic, as well as solvent, counter ion, and entropic effects between the specific domains of the two proteins (Fig. 3)[31]. The key factor that governs the miscibility of the blend is the free energy of mixing[28-30]. Considering two proteins which are mixed,

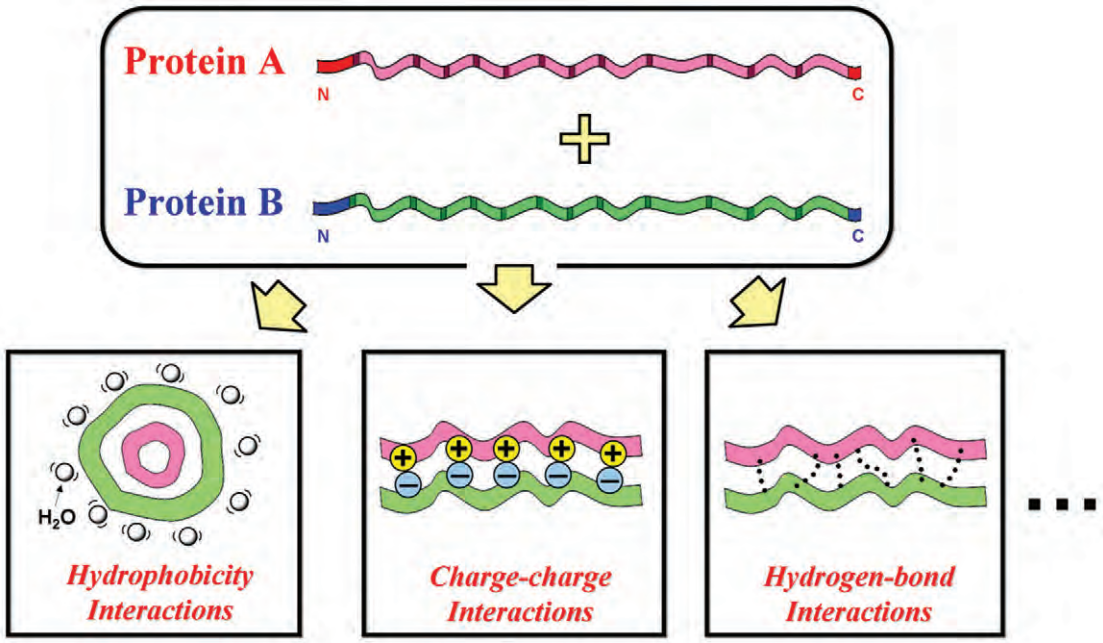

Fig. 3 Types of interactions between chains of protein A and protein B that can generate stabilized structures, such as hydrophobic-hydrophilic interactions, charge-charge (electrostatic) interactions, or hydrogen bonding interactions.

complete miscibility in the mixture requires the following condition to be fulfilled:

$$\Delta G_m = \Delta H_m - T\Delta S_m < 0 \quad (1)$$

where ΔG_m, ΔH_m, and ΔS_m are the Gibb's free energy, the enthalpy, and entropy of mixing at temperature T, respectively. For binary systems the Flory-Huggins equation can be expressed in the following form:

$$\Delta G_{m=} RT[\phi_1 ln\ \phi_1/r_1 + \phi_2 ln\ \phi_2/r_2 + \chi\ \phi_1\ \phi_2] \quad (2)$$

where ϕ_i is the volume fraction of the component i (i = 1, 2) and r_i is the number of protein segments, R is the gas constant, and χ is the Flory-Huggins binary interaction parameter. The first two terms of the right hand side in equation (2) are related to the entropy of mixing and the third term is originally assigned to the enthalpy of mixing. A schematic phase diagram for two protein blends is shown in Fig. 4a. There are three regions with different degrees of miscibility: (a) miscible (one-phase), (b) metastable, and (c) immiscible (two-phase) regions. The binodal (Fig. 4a, solid line) separates miscible and metastable regions, while the spinodal (Fig. 4a, dashed line) separates metastable and immiscible regions[28-30].

Experimentally, the least ambiguous evidence for protein miscibility is the presence of one glass transition temperature (T_g) for a fully miscible protein system by Differential Scanning Calorimetry (DSC), which is normally intermediate between the T_gs of the two individual protein components, T_{g1} and T_{g2} (See Fig. 4b)[32-35]. Full phase separation (immiscible blends) can be determined by the presence of both T_{g1} and T_{g2} at their original positions (Fig. 4b), and with each T_g step height in proportion to the composition. A more complex case arises from partial mixing. In this condition, there might be micro-heterogeneous phase structures formed between the two protein components, where the composition varies from location to location. This type of interaction will result in one very broad glass transition, or still two glass transitions, but each of which have migrated closer to each other [T_g(a) and T_g(b)] (Fig. 4b). Generally, this nearly miscible protein matrix is called a "semi-miscible" system. Based on this theory, we investigated temperature modulated DSC (TMDSC) scans of silk-tropoelastin blends in film form with different mixing fractions[25]. With the increase of tropoelastin content, the glass transition temperatures (T_g) of the blends increased gradually from 178 °C (pure silk) to 190 °C (pure tropoelastin)[25]. A homogeneous single glass transition for the silk-tropoelastin blends indicated that a stable single silk-tropoelastin macrophase was formed for each sample, resulting in a fully miscible protein system from these two protein components[25]. Furthermore, the effects of bound water molecules on the glass transitions of the protein composites need to be considered[35-38]. Bound water can act as a plasticizer and expand the accessible conformational space of the protein molecules, which results in a new "glass transition" for the solid-like protein composite system. This effect has been demonstrated in many natural protein systems such as soy proteins[36], sunflower proteins[37], or wheat proteins[38].

These concepts of protein-protein miscibility are important in the design and dynamic control of different properties of protein-based composite materials to match function to biomaterial goals. Both miscible and immiscible composite materials could be useful for specific biomedical applications.

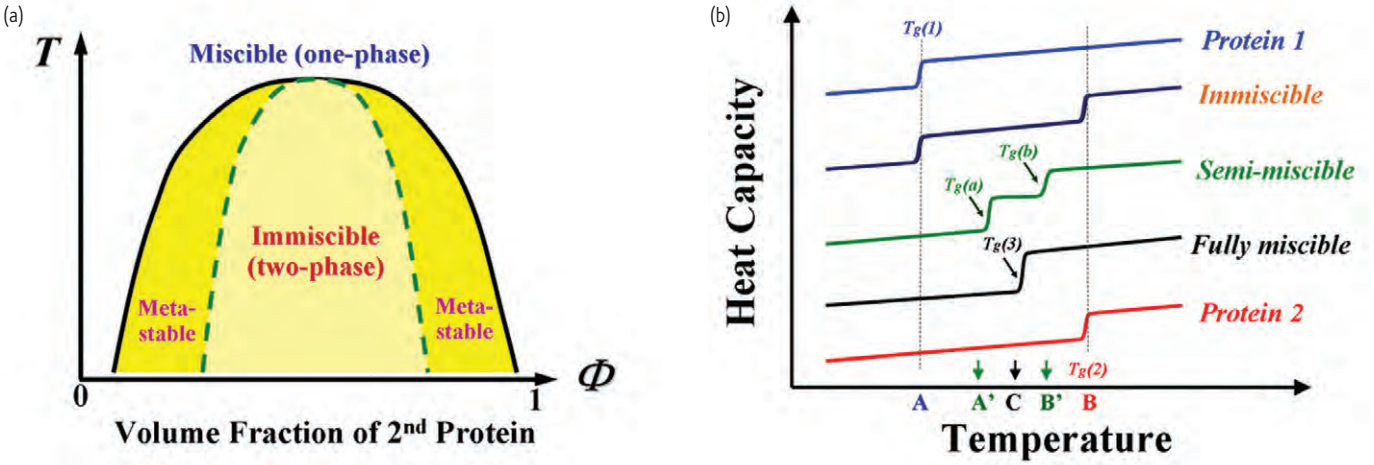

Fig. 4 (a) Schematic phase diagram for two protein blends (temperature T vs. volume fraction Φ of the second protein). The binodal (solid line) separates miscible (one-phase) and metastable regions, and the spinodal (dash line) separates metastable and immiscible regions. (b) Schematic DSC heat capacity traces for two-protein blend system with different miscibilities.

Protein-based composite materials

In the last decade, many protein-based composite materials have been studied for different applications, due to the needs in contemporary biomedical research. Fig. 5 (left side) shows typical protein based composite materials that have been studied recently, including composite films, foams, gels, fibers, grafts, and particles. Skopinska-Wisniewska *et al.*[39] generated collagen-elastin thin films with different mixing ratios. The collagen components supported tensile strength for regenerated tissues, whereas the elastin component provided resilience. The surface properties of collagen/elastin based biomaterials, such as surface polarity, could be modified by the blending ratio. Chen *et al.* discussed the impact on stem cells in knitted silk-collagen scaffolds for tendon tissue engineering[40]. Buttafoco *et al.*[41] generated electrospun collagen-elastin nanofiber meshes from aqueous solutions for artificial blood vessels. By controlling flow rate, applied electric field, collecting distance and composition of the starting solutions, the morphology of the fibers was controllable. Increasing the elastin content resulted in an increase in fiber diameter from 220 nm to 600 nm[42]. Rajkhowa *et al.*[43] investigated silk-silk composite materials to control the mechanical properties. Silk protein particles dispersed in a silk protein network were stiffer than the network itself, even though they are the same proteins. Thus the modulus of the particulate composite could be increased, such as via porogen leached silk scaffolds prepared from solutions and reinforced through the addition of silk

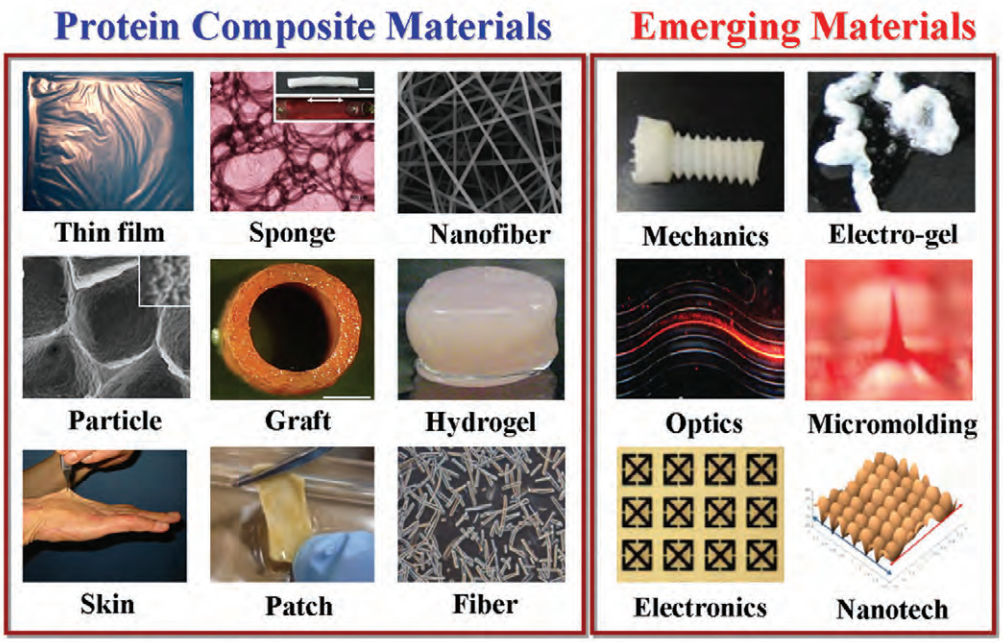

Fig. 5 Novel protein-based composite materials developed over the last decade or so (left), and new generations of protein composite materials recently developed or anticipated in the coming years (right). Reprinted with permission from[9,39-52]. © AAAS, © Elsevier Ltd., © Wiley, © American Chemical Society.

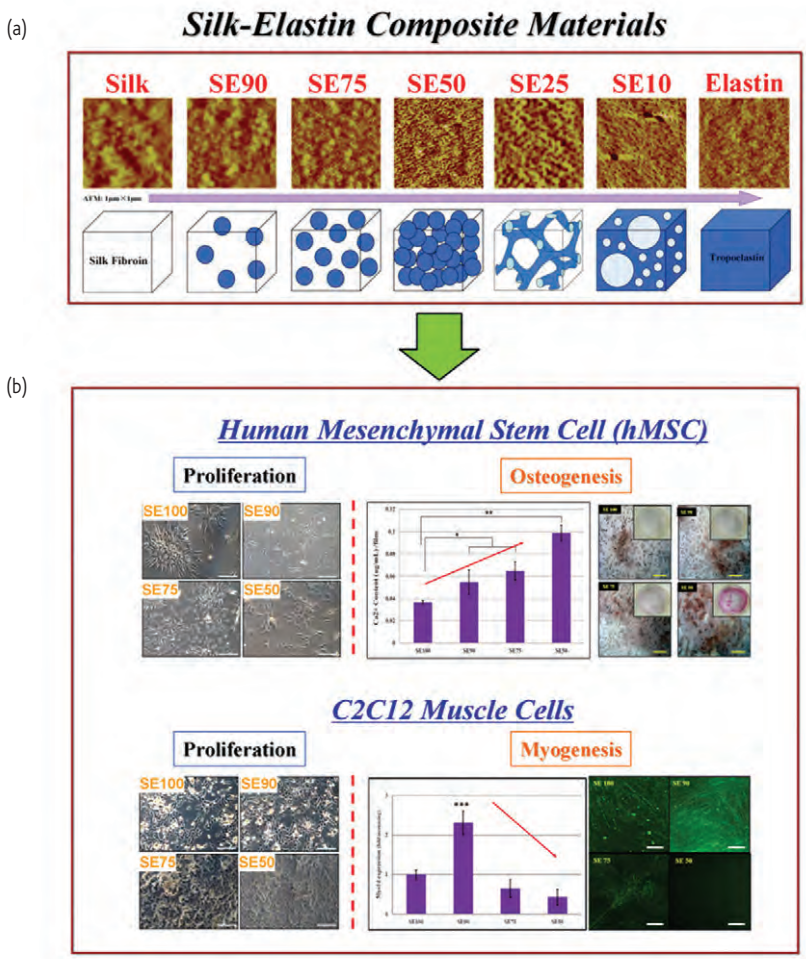

Fig. 6 Cell responses to silk-tropoelastin composite materials. (a) AFM topological morphology of silk-tropoelastin composites with different mixing ratios. Reprinted with permission from[25]. © Elsevier Ltd.) (b) Interactions of human mesenchymal stem cell (hMSCs) and C2C12 muscle cells with silk-tropoelastin composites. Proliferation and differentiation trends for these cells are different, since they respond differently to physical properties such as material elasticity, surface roughness, surface charge and cell signal channel related protein sequences. Reprinted with permission from[26]. © Elsevier Ltd.

particles. This approach resulted in about a 40× increase in the compressive modulus and the yield strength. The presence of the particles in the silk scaffolds also slowed degradation rate via enzymatic digestion, providing an additional control point in protein-protein composite design. Caves *et al.*[44] describe a process for the fabrication of multilamellar vascular grafts from a recombinant elastin-like protein reinforced with collagen microfibers. The process provided control over collagen microfiber orientation and density. In turn, fiber architecture and processing of the elastin-like protein modulated suture retention strength, burst strength, and compliance. The results indicated that reinforcement with collagen microfibers helps generate recombinant elastomeric protein-based biomaterials for load bearing tissue needs. Xiao *et al.*[45] synthesized gelatin methacrylate (GelMA)–Silk fibroin (SF) interpenetrating polymer network (IPN) hydrogels. The gelatin methacrylate was mixed with SF solution and photo-cross-linked using UV irradiation to generate the GelMA–SF IPN hydrogel, following methanol treatment to induce silk fibroin crystallization. These mechanically robust and tunable IPN hydrogels could be useful for various microscale tissue engineering applications. Haslik *et al.*[46] also clinically evaluated collagen-elastin matrices (Matriderm®) as a dermal substitute for the treatment of severe burns. These 1 mm thick protein three-dimensional matrices are composed of native, structurally intact collagen fibrils, coated with elastin for supporting dermal regeneration. Successful clinical studies demonstrated utility for these systems, including full range of motion achieved in all patients after recovery, without any infections or allergic reactions[47]. Serban *et al.*[48] chemically cross-linked three components, soluble elastin, hyaluronic acid, and silk fibroin, to control properties of modular elastic patches. The materials obtained by cross-linking the three components had Young's moduli ranging from ~100 kPa to 230 kPa, resulting in different biological effects and enzymatic degradation rates.

In the next few years, new generations of protein composite materials with controllable optical, electrical, chemical, and mechanical properties for a range of medical purposes are anticipated (Fig. 5 right side). Protein-composite based medical devices (*e.g.*, sutures, plates, screws, injectable gels)[9,49,50] can be envisioned for different tissue repair needs,

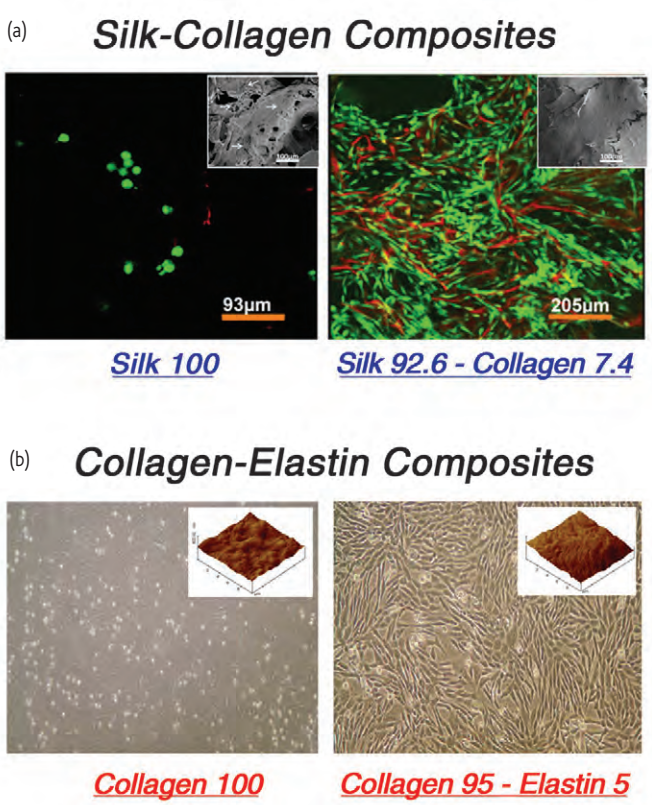

Fig. 7 (a) Cell responses to silk-collagen sponge scaffolds with 7.4 % collagen content, the viability of fibroblasts was higher than the pure silk samples (Silk100) at day 11. Reprinted with permission from[54]. © Elsevier Ltd. (b) Cell responses to collagen-elastin films with 5 % elastin and 1 h of UV light irradiation, the number of 3T3 cells grown on the composite films (elastin5/95collagen) was higher than that on the non-irradiated collagen film (collagen100). Reprinted with permission from[39]. © Elsevier Ltd.

such as bone, ligament, or muscle. Protein-composite based microneedles[51] or other micromolded devices can be designed to control local drug delivery in wound healing or to treat chronic diseases. Follow-on applications for protein composites could include degradable and flexible electronic devices such as meta-materials[52] or flexible LEDs[53], while implantable optical materials from protein composites could be utilized for diagnosis and medical treatment *in vivo*[9,10]. These protein composite-based biomaterials would offer a new generation of medical devices with selectivity and tunable functions, while providing control of biological interfaces, tunable mechanical properties, and programmable lifetime *in vivo*, while also matching tissue compliance and related needs. Such devices could provide important options for implantable devices where post repair surgical retrieval is avoided.

Biological interactions with protein composites

Biophysical properties of protein composites, such as strength, elasticity, surface roughness, surface charge, and chemical signaling, ultimately impact tissue function and durability as well as local cellular behavior. These properties of biomaterials play an important role in biomedicine as the majority of biological reactions occur at the interface of implants. Silk-tropoelastin composite materials have been investigated to understand the influence of these composites on different cells. Atomic force microscopy (AFM) topological assessments were used to demonstrate how the two types of protein chains interacted and formed different structures (Fig. 6a)[25,26]. By adding a small fraction of tropoelastin into silk (SE90), the tropoelastin microphase-separated and formed mono-domains or discontinuous small aggregates in the silk "solvent". With the addition of more tropoelastin into the silk "solvent" (SE75, SE50), tropoelastin "drops" started to connect (SE75, SE50) and interacted with the silk chains. The system transformed to a bi-continuous system (SE25) in which different protein chains interpenetrated to form networks. When tropoelastin dominated the structure (SE10), the polymer network gradually became pure tropoelastin (SE0)[25]. Interactions of human mesenchymal stems and C2C12 muscle cells showed different responses on these silk-tropoelastin composite systems (Fig. 6b:A, B)[26]. hMSC proliferation on silk-tropoelastin films SE50 was significantly higher than on SE100, as was osteogenesis measured via staining with Alizarin red and based on calcium deposition[26]. However, C2C12 muscle cell proliferation showed different trends, with the expression of Myosin-1d (Myo1d) gene by immunocytochemical staining indicating that SE90 exhibited 3× higher myogenic transcript level when the cells were propagated on SE75 and SE50[26].

Cell responses to silk-collagen sponge scaffolds was also investigated. Lu *et al.* found that (Fig. 7a), with a low content of collagen (7.4 %) in silk scaffolds (Silk92.6/Collagen7.4), the viability of fibroblasts was significantly higher than it was on pure silk sponge scaffolds (Silk100) at day 11[54]. The surface roughness and structural confinements at the nano-scale contributed to this significant increase in cell response[54]. Similarly, gelatin was added to aqueous silk fibroin solution to change the conformation of silk fibroin sponges[55], with 30 % gelatin resulting in significantly better L929 fibroblast proliferation than on silk scaffolds alone[55].

Collagen-elastin blend films (Fig. 7b), with only 5 % elastin added into collagen, and with 1 hour of UV light irradiation, resulted in higher 3T3 mouse fibroblast cell growth (elastin5/95collagen) than on non-irradiated pure collagen films (collagen100)[39]. The higher content of elastin promoted specific cell adhesion and viability on the surface, while a suitable dose of UV light can further improve the utility of the materials[39]. These interactions change in the protein-composite materials at the surface so different cell types may respond differently, thereby providing options to control such biological interactions at the interfaces as a route to improved impact of protein-protein composites on medical materials and devices in general.

Conclusions

Protein-based composite biomaterials can be formed into a wide range of biomaterials with tunable properties, including control of cell responses. The ability to tailor or tune the mechanical, surface and regeneration features provides a major advance for biomaterials in general, to address the growing range of medical material and device

needs. These composites have demonstrated promise for providing the next generation of functionalized biomaterials due to their intrinsic biocompatibility, biodegradability, and natural abundance, as well as their unique natural properties (*e.g.* mechanical, optical, electrical, chemical, thermal). Understanding the molecular-scale interactions between these proteins, will help generate the next generation of multifunctional protein composite biomaterials with enhanced properties and functions matched specifically to a regenerative medicine need. Assessment of cell interactions for the range of composite materials generated can be used to understand relationships between the protein composites and biological responses, as well serving as *in vivo* screens of material degradation profiles and inflammatory responses. The outcome of these studies will provide a new biomaterials platform to fill an important need in the field of biomedical science, with direct relevance to tissue regeneration, nano medicine, and disease treatments. The robust mechanical features of such protein systems described here, point further to future implantable devices whereby diagnostic, therapeutic and related features can be envisioned, with full regeneration of native tissue over time. While we have emphasized more protein-protein composites in this paper, many protein-related composite materials, such as protein-inorganic ceramic materials[56-58], protein-synthetic polymer materials[59,60], or protein-polysaccharide materials[61,62], have also played important roles in the recent development of biomaterials for biomedical research. We have also focused mainly on some of the more compelling fibrous proteins that have been intensively investigated, necessitating the omission of many studies on other protein systems[63] in the biomaterials field. For example, most studies on engineered multi-component/multi-block protein materials[64,65] have been omitted. mt

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