Published in final edited form as:

Nanomedicine (Lond). 2012 September ; 7(9): 1409–1423. doi:10.2217/nnm.12.107.

Clues for biomimetics from natural composite materials

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Abstract

Bio-inspired material systems are derived from different living organisms such as plants, arthropods, mammals and marine organisms. These biomaterial systems from nature are always present in the form of composites, with molecular-scale interactions optimized to direct functional features. With interest in replacing synthetic materials with natural materials due to biocompatibility, sustainability and green chemistry issues, it is important to understand the molecular structure and chemistry of the raw component materials to also learn from their natural engineering, interfaces and interactions leading to durable and highly functional material architectures. This review will focus on applications of biomaterials in single material forms, as well as biomimetic composites inspired by natural organizational features. Examples of different natural composite systems will be described, followed by implementation of the principles underlying their composite organization into artificial bio-inspired systems for materials with new functional features for future medicine.

Keywords

bio-inspired; cellulose; collagen; resilin; silk; SP1

As the entwined fields of tissue engineering and regenerative medicine continue to grow and evolve, the search for a 'perfect' scaffold inevitably continues. This ongoing quest to search for new materials and fabrication techniques has led researchers anywhere from arthropods, plants, marine creatures and mammalian kingdoms to precious metals and minerals over the past decade. Researchers are continuously finding new materials and technologies for fabricating scaffolds with superior mechanical features, porosity, biocompatibility and biodegradability.

Natural biomaterials such as cellulose, wool, leather and silks have been used by mankind for thousands of years for daily applications such as construction, furniture and textiles. At the end of World War II, newly developed glass fibers and synthetic polymers, such as polypropylene and aramid, gained popularity due to their excellent mechanical properties, relatively easy and controlled synthesis and relatively low cost, supplanting natural

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Financial & competing interests disclosure The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

materials. Nevertheless, in recent years, synthetic materials are under increasing scrutiny as a result of their dependency on declining oil supplies along with the environmental burdens associated with production and disposal. These factors have reactivated the need for alternative materials derived from renewable resources, leading resurgence in interest in natural polymers and composite systems.

The era of protein scaffolds offers distinct advantages, particularly with the combination of powerful tools of molecular biology. These include, for example, the production of human proteins of uniform quality that are free of infectious agents and the ability to make suitable quantities of proteins that are found in low quantity or are hard to isolate from tissue [1]. A different approach is the generation of scaffolds that provide similar features to native bone matrices. In recent years, biomimetic techniques have been used to reproduce natural bone structure and its chemical composition to make bone scaffolds. Natural polymers such as collagen, gelatin and mineral compounds, similar to those in natural bone (e.g., hydroxyapatite), have been used as biomimetics. For example, Azami *et al.* used the double diffusion method in a physiologically relevant environment to prepare a biomimetic gelatin-amorphous calcium phosphate nanocomposite scaffold [2].

Cellulose composites

Cellulose is the most abundant biopolymer on earth and is traditionally used for clothing, construction, furniture and for making paper. The most complex form of cellulose in nature is in the cell walls of plants where it appears as a composite with other polysaccharides such as hemicellulose and pectin, and with lignins, enzymes and structural polymeric proteins. These polymer composites ordered in unique architectures result in high load transfer when cells are subjected to mechanical stress, and at the same time providing a physical barrier against pathogen attack [3,4]. The best example of the extra ordinary strength of plants cell wall composites is exemplified by their ability to carry the huge mass of some forest trees. Biomimetic applications of cellulose nanocomposites is a large field and was recently extensively reviewed by Teeri *et al.* [4], Hubbe *et al.* [5], Berglund *et al.* [6], Eichhorn *et al.* [7] and Siqueira *et al.* [8]; therefore, in the current review we only highlight some of our own recent experiences with cellulose nanocomposites.

Studies of natural composite structures show that high strength is achieved by the architecture of the polymers' initiated molecular and nanoscale interfacial features. These interactions originate from the single polymers and the interactions propagate to the macroscale as displayed by the organization of a single plant cell to a whole tree. In recent years, efforts have been made to produce nanostructured bio-inspired composites to mimic the features in natural cellulose composites. This led to development of nanoscale cellulosic materials such as acid-hydrolysed nanofibers (nanocrystalline cellulose [NCC]) or micro-/ nano-fibrillated cellulose (MFC/NFC), which are produced by high shear grinding of pulp forming micrometer-long entangled fibers.

Nanocellulose

MFC/NFC is not in the scope of the current work. In short, these are nanocellulosic fibers produced by high shear grinding of cellulose fibers forming 10–20 nm wide and micrometer-long entangled fibers. Nanofibrillated cellulose was widely utilized for manufacturing of nanostructured composites (see reviews [4–8]). Nevertheless, its mechanical performance is limited, since being produced exclusively by mechanical shearing of cellulose it contains the amorphous domains, which prevent the fibers from reaching their theoretical mechanical properties.

NCC (in some cases termed 'cellulose whiskers') was discovered by Rånby [9]. Real technological progress was only achieved in the mid-1990s by Favier *et al.* [10], who produced NCC–latex composites resulting in a more than three orders of magnitude increase in the shear modulus of the latex rubbery state.

NCCs are defined as fibers which have been grown under controlled conditions that lead to the formation of high-purity single crystals. These crystals display extremely high mechanical strength equivalent to the binding forces of adjacent atoms. The NCC modulus is estimated to be approximately 150 GPa [11] and their tensile strength is estimated to be approximately 10 GPa, similar to very strong materials such as aramid fibers (Kevlar®, DuPont, DE, USA) and carbon fibers. The mechanical properties of NCCs encouraged many research groups to attempt to utilize them for nanocomposite development. NCCs produced by H₂SO₄ are particularly interesting. During the hydrolysis, process the cellulose nanoparticles are charged with sulfate groups and form stable honey-like liquid crystal suspensions. The addition of ionic solutions or water soluble solvents such as acetone to these suspensions results in gel formation due to the strong hydrogen bonds between the crystals. Evaporation of the water from the suspension results in strong transparent film formation, and lyophilization of the suspensions results in the formation of highly porous foams (Figure 1). NCCs also display optical properties such as birefringence due to their organized crystalline structure. The increasing number of reports on novel composites from NCCs implies strong potential for new durable environmentally friendly composite materials. An example of recent NCC-based nanostructured composites is the work of Capadona *et al.* [12] inspired by sea cucumber skin, where composites of NCC and rubbery ethylene oxide–epichlorohydrin 1:1 copolymer were generated. These new materials generated stimuli-responsive polymers that harden upon mechanical stress. Other recent examples are cellulose whisker/epoxy resin composites [13] and the production of NCC/polyvinyl acetate nanocomposites by Rusli *et al.* [14]. For broader reviews on MFC and NCC composites see [4–8,15]. Apart from nanoscale features for achieving durable composites, further key issues to be considered are the ability to exploit molecular recognition between components based on chemical crosslinking, electrostatic interactions, hydrogen bonding and van der Waals interactions that are responsible for the stabilization of natural composites such as cellulose.

One of the mechanisms that has evolved in nature with regard to molecular interactions are carbohydrate-binding modules (CBMs). These amino acid sequences bind structural proteins to polysaccharide scaffolds such as cellulose in the plant kingdom and chitin in the invertebrate kingdom. For example, 70% of the proteins isolated from insect cuticles contain the conserved Rebers and Riddiford (R&R) Consensus chitin-binding domain (ChBD) [16], displaying the importance of CBMs in interfacing between the fibrous polysaccharide scaffolds and the matrix proteins in natural composites

Cellulose-binding domains (CBDs) belonging to the CBM family have the ability to bind to cellulose as well as to physically disrupt the crystalline structure of cellulose fibers [3,17]. This provided the foundation for CBM utilization in numerous biotechnological applications aimed towards cellulose fiber modifications immobilization of different proteins to cellulose and for the production of cellulose–CBM fusion protein composites [3,17]. For example, CBMs were successfully used to improve the properties of paper. Cellulose used in paper manufacturing is extracted from plant material while other cell wall natural binders that reinforce the natural composite are lost; therefore, chemical treatments are required to retain sufficient mechanical properties in paper using additives that are relatively expensive and not environmentally friendly [17].

Recombinant proteins composed of a double CBD fusion protein and CBD fused to a starch-binding domain mimicked the function of natural cellulose crosslinkers, and significantly improved tensile strength, energy to break and water repelling features were achieved either by crosslinking the cellulose fibers to themselves or to starch [18,19].

Composites with polymeric proteins

In addition to polysaccharides, biopolymer research has focused in recent years on fibrous proteins such as resilin, silks from silkworm and spiders, elastin and collagen. These protein polymers exhibit unique mechanical properties that are conferred by their highly repetitive secondary and tertiary peptide structures [20]. Biopolymers in plants are often dominated by highly organized cellulose fibers and glue-like polyphenolic lignin components, to provide structural integrity to form stable structures that resist gravitational forces. Biopolymers in invertebrate exoskeletons are often dominated by organized chitin with protein components to form composite structures. Biopolymers in vertebrate tissue and skeletal systems are usually dominated by hierarchically organized collagens with a variety of other proteins and polysaccharides associated with the collagens. In addition, polymeric proteins display varied mechanical properties from high strength to elasticity, which are often superior to their synthetic counterparts. The repetitive structures enable systematic interactions among domains in these proteins, resulting in extensive intra- and inter-chain interactions. Consistent secondary structural features can facilitate the formation of structures with hierarchical features. Such scaling results in robust mechanical properties initiated at the molecular level and propagated to the macroscopic material features. The repetitive peptide domains with the proteins also enable new protein designs based on genetically engineered variants of the native sequences; short segments for the consensus repeats can be used as building blocks for the design of new proteins. Crosslinking in fibrous proteins varies depending on the specific protein; silks utilize physical β -sheet forming regions, while in resilins and elastins, chemical crosslinks provide stabilization.

Invertebrate skeletal composites as sources of inspiration

Invertebrates rely on exoskeletons for mechanical support and locomotion. These creatures on land and sea have evolved composite architectures that surpass synthetic materials in terms of form and function. Invertebrate skeletons are sophisticated multifunctional structures providing structural and mechanical support, shape, protection and complex locomotion. This function is achieved by the unique architecture of the skeletons as composites of highly crystalline chitin scaffolds embedded in polymeric protein matrices, polyphenols, lipids and water in multilayered formats with plywood-like (helicoidal) structures [21]. Many of the skeleton proteins are bound to the chitin scaffold via a ChBD, a CBM described earlier [16,21–23]. As discussed above, 70% of the proteins isolated from insect cuticles contain the conserved R&R ChBD [16]. It is also interesting to relate the presence of helicoidal structures as a key feature in structural hierarchy throughout biological systems, as a strategy that has evolved to provide mechanical support. Even in soft tissues, such as in mammals, helicoids in the form of liquid crystalline displays permeate most tissues in terms of underlying structure via collagen fibrils and networks.

Invertebrates have evolved different strategies for hardening and strengthening their composite skeletons probably in perhaps the most diverse and fascinating way. These structures commonly involve stiff chitin scaffolds embedded in matrix polymeric proteins and strengthening is achieved through different strategies, including biomineralization, metal coordination or the formation of 3,4-dihydroxy-phenylalanine (DOPA) crosslinks.

Biomineralization is the ability of living organisms to synthesize solid inorganic materials by using nutrient resources in their natural environment, and is a strategy utilized by

diatoms, mussels and porous matrices to achieve mechanical support. Specific peptides generally termed silaffins mediate mineralization in the cell walls of different marine creatures. Silica is an important mineral in the fields of biotechnology, nanotechnology and medicine and therefore the mechanisms involved in the inorganic–organic interactions are well investigated. These insights have enabled a variety of biotechnological developments of silica-based nanostructures. The identification of silaffin proteins, which mineralize silica in aqueous solutions, enabled the development of biomimetic silica-based technologies utilizing both natural silica-binding peptides such as the R5 silaffin-1 precursor polypeptide from *Cerithiopsis fusiformis* [24], as well as new silaffin-like peptides identified from combinatorial phage display libraries that precipitated or bound silica *in vitro*. These findings enabled the production of new silicon-containing biomimetic materials [24–29]. For example, Wong Po Foo *et al.* produced recombinant spider silk protein fused to the R5 peptide, which enabled the precipitation of silica nanoparticles on silk films and electrospun fibers, forming silk–silica composites with potential applications for bone regeneration (Figure 2) [30].

In another example, a silica-binding peptide (TBP-1) developed by recombinant phage display technology [25] was fused to SP1 (Figure 3), a ring-shaped highly stable homododecamer protein complex. SP1 protein displays 12 N-terminal arms, six on each side of the protein ring, and therefore any N-terminal fusion to SP1 forms a multivalent nanoparticle. SP1 has a unique structure and is stable, which allowed the development of self-assembling molecular scaffolds for nanobiotechnology and biomimetic materials [29,31–33]. TBP-1 was fused to the N-termini of the monomers forming multisilica-binding nanoparticles enabling solvent controlled selective binding to silicon surfaces [29]. Furthermore, it was shown that TBP-1–SP1 can form nano-memory units (these memory units relying on the finite capacity of a silicon nanoparticle embedded in an insulating SP1 protein pore, and the ability of atomic force microscopy to locally address a dot) [34].

Another interesting approach for silica biomineralization was presented by Gautier *et al.* and inspired by the skeletal structure of diatoms [35]. Silica precipitation in the presence of protein–polysaccharide hybrid scaffolds resulted in improved templating and ordering of the mineral phase. Multicomponent systems may be better mimics of *in vivo* conditions of silica formation for future developments [35]. Additional approaches for silica biomineralization, design and preparation of biomimetic organic–inorganic composites were recently presented by Li *et al.* [36]. These hybrid beads, containing amidated alginate, displayed superior swelling resistance and mechanical stability when compared with pure alginate beads.

Apart of biomineralization, some creatures have evolved other strategies related to composite materials. The fang-like jaws of the marine polychaete *Nereis virens* possess remarkable mechanical properties. The jaws are the worm's primary tool for feeding and defense and, as such, must be optimized for grasping, piercing and tearing prey in an abrasive environment. Hardness and stiffness properties in the jaw are comparable to human dentin and superior to synthetic polymers. Amino acid analysis indicates that the jaws are composed of approximately 90% (w/w) protein, with a strong compositional bias toward glycine and histidine. The remainder of the jaw is comprised of halogens (~8%) and zinc ions (~2%) [37]. In undertaking this investigation, Broomell *et al.* hypothesized the cutting edge of the *Nereis virens* jaw (i.e., the tip and serrated distal end) to be composed mainly of histidine-rich zinc-binding proteins [37]. Insects such as termites, grasshoppers, cutting leaf ants and others possess similar mechanisms in their mandibles, mouth hooks, claws and ovipositors [38]. Although this mechanism has not been exploited from a biomimetics point of view, the strategy offers options for novel biocomposites.

Another strategy involves DOPA as a cross-linker, particularly for adhesives and sealants. The most studied system in invertebrates is the DOPA-protein adhesive used by mussels, which are known for their strong adhesive capabilities to solid surfaces in wet environments. DOPA residues are in equilibrium between a catecholic state, which strongly binds to surfaces and an α -quinone state, which has weak binding ability to surfaces but acts as a strong crosslinker with ionic amine groups. Conversion of catechol to α -quinone occurs under oxidative conditions, alkali pH or by enzymes such as catechol oxidase [39]. Other organisms utilize DOPA in other ways. For example, this crosslinking property is utilized by animals such as the giant squid. Squid beaks are as hard as the jaws of *Nereis virens*. The beaks are composed of a similar composite of chitin and polyhistidine-glycine rich proteins but without the mineral phase, metals or halogens. Instead, the hardening of these composites is achieved by formation of DOPA and histidine crosslinks in a gradual manner across the beak surface from the tip to the base [40,41]. This mechanism was recently used for the production of bio-inspired protein composites. For example, Lee *et al.* conjugated trypsin to a variety of surfaces such as copper, titanium oxide, polycarbonate and cellulose by coating the surfaces with dopamine-HCl at pH 8.5 and formed an active polydopamine coating on surfaces that were further conjugated with trypsin by incubation at pH 7.5 [42]. A different approach, presented by Lewandowski *et al.* [43], involved tyrosinase-catalyzed conjugation of proteins, such as gelatin and green fluorescent protein modified with polytyrosine at the C-terminus, to chitosan. In these biomimetic systems the tyrosinase converts the tyrosine residues in the protein chains into reactive α -quinones that form covalent linkages to the nucleophilic amine groups of the chitosan scaffold. The efficiency of these crosslinking methods should inspire new composite systems. Recently, Brubaker *et al.* generated a synthetic adhesive biomaterial inspired by the protein glues of marine mussels for islet transplantation at extrahepatic sites [44]. The adhesive precursor polymer consisted of a branched poly(ethylene glycol) core, where the ends were derivatized with catechol. Under oxidizing conditions, adhesive hydrogels formed. This approach is new to the islet transplantation paradigm, involving direct immobilization of islets onto intra-abdominal tissue surfaces, using a thin, tissue-adherent conformal hydrogel membrane. This 'islet sealant' approach offers the potential advantage of convenient, rapid and minimally invasive islet transplantation by direct apposition of the islet bolus onto tissue surfaces.

Resilin

In the arthropod kingdom, insects have developed the most complex locomotion skills, including flying and jumping, due to the development of specialized organs consisting of composites of chitin and resilin. Resilin is a rubber-like protein that displays stretching ability of up to 100% and is accompanied with a very high fatigue lifetime and low modulus of elasticity. Resilin is found especially in areas where high resilience and low stiffness are required, resulting in tough and durable structures [45,46]. Resilin binds to the cuticle chitin via the conserved cuticular ChBD type R&R and is further polymerized through oxidation of tyrosine residues resulting in the formation of dityrosine bridges leading to the assembly of high-performance protein-polysaccharide composites.

In nature, resilin must last the entire lifetime of the animal, extending and retracting millions of times. The unique elastic properties and the abundance of resilin in the jumping organs of arthropods such as the frog hopper and cat fleas led many researchers to the conclusion that resilin is the major constituent in the jumping mechanism of insects. This assumption was recently questioned by Burrows *et al.* [47], where it was shown that resilin by itself is not sufficient to store enough energy and must act as a constituent of a composite with the chitin to achieve jumping. In the work of Burrows *et al.*, a composite consisting of approximately 80% chitinous cuticle and 20% resilin was found in the pleural arch of the jumping organ of the frog hopper insect *Aphrophor* [47]. The high elasticity of resilin combined with the

strength of the chitin in the composite enables insects to rapidly restore body shape as well as most of the energy required for the next mechanical movement, either flying or jumping. Inspired by the remarkable mechanical properties of the insect cuticle and plant cell walls, nanobiocomposites of resilin and chitin should display useful mechanical properties, combining strength and elasticity. Ardell and Andersen first provided the full sequence of the *Drosophila melanogaster* proresilin gene, which is comprised of three exons and contains two significant elastic repeat motifs [48]; an N-terminus domain comprising 17 pentadecapeptide repeats (GGRPSDSYGAPGGGN) and a C-terminus domain comprising 11 tridecapeptide repeats (GYSGGRPQGDLG). Each repeat is found entirely in the first and third exons, respectively. In addition, the exon 2 gene comprises the typical cuticular R&R ChBD type 2, which allows direct binding and strong interaction between the resilin and chitin during resilin deposition and construction of the cuticle composite (Figure 4). We have previously produced *D. melanogaster* recombinant resilin in *Escherichia coli* and demonstrated its ability to bind chitin [46]. The ChBD displayed high affinity to chitin and precipitated with chitin beads both in the purified protein form and in crude bacterial lysates. In addition, we have shown that resilin monomers and in particular exon 1, have high intrinsic elasticity and resilience. Furthermore, we have developed novel crosslinking methods, such as citrate-modified photo-Fenton, leading to highly elastic polymers as shown in Figure 5 [49].

Spider silk

Spider dragline silk, used as the safety line and as part of the frame thread of spider webs, is the strongest natural fiber known, reaching tensile strengths of 740 MPa. Some spiders spin as many as seven different kinds of silks, each optimized for specific biological functions. Dragline silk, with a unique combination of tensile strength and elasticity, provides toughness to silk materials that require a higher energy to break than any other common material, natural or artificial.

The origin of these mechanical properties is in the block copolymer protein designs exploited in silks, wherein large hydrophobic domains dominate the structure to generate crystalline regions that exclude water and stabilize the structure via physical crosslinks, yet these domains are interlinked with less crystalline domains to provide the elasticity to balance the otherwise stiff properties. In contrast to silkworm silk, the isolation of large amounts of silk from spiders is not feasible. Spiders produce silk in small quantities and often in mixtures due to the multitude of silks mentioned above. Furthermore, the territorial behavior of spiders prevents the maintenance of dense cultures of spiders to generate large amounts of silks, in contrast to silkworms which are raised under such conditions via sericulture. Therefore, production of silk protein through recombinant DNA techniques has been pursued in a wide variety of organisms (Figure 6) [50]. Laboratory-scale amounts of silk-like proteins are available. However, appropriate spinning technology capable of converting these recombinant proteins into high performance fiber remains a major challenge. The assembly of the proteins in a liquid crystalline form into solid silk fibers will require mimicking of the functions of spinning apparatus and glands in spiders [50]. Silks are protein-protein composites in nature. In silkworms, fibroin core proteins are interfaced directly with a family of glue-like proteins termed sericins. In spiders, the web exists as a highly engineered composite system where the many different silks act in an orchestrated set of interactions to provide the mechanical functions of the web – including catching flying prey, having the prey stick to the webs, holding web components together, anchoring to environmental surfaces, and providing both strong mechanical functions in the web and flexible features at the same time. Silks are not known to be assembled as composite structures with polysaccharides in nature, as there is little evidence for the involvement of polysaccharides in silk fiber assemblies [51,52]. Recombinant spider silks can be generated

in many variants for biomaterial and tissue engineering. For example, hydrogels can be formed [53] using the connectivity of either physical or chemical crosslinking (e.g., the recombinant construct eADF4[C16], with 16 copies of a 35 amino acid repeating module, self assembled into stable hydrogels via nucleation aggregation followed by concentration-dependent gelation [54]). Gels with reproducible properties can be prepared by dialysis of low concentration protein solutions in 6 M guanidinium isothiocyanate solution into 10 mM Tris/HCl, pH 7.5, followed by further dialysis against high molecular weight (20 kDa polyethylene glycol [53]).

Silks are attractive, protein-based materials for designs of silk–cellulose composites, to combine the impressive mechanical properties of both components. Early attempts towards the preparation of cellulose–silk composites were by blending and regeneration of solubilized cellulose and silkworm silk [55]. Impressive results were reported by Noishiki *et al.* who prepared composite cellulose–silk films from NCC and regenerated silkworm silk [56]. These mixtures improved mechanical properties approximately fivefold over the separate components, including breaking strength and ultimate strain. The authors relate the drastic improvement in the strength of the composite to characteristics of the flat and ordered surfaces of the NCC, which served as a template for the assembly of silk β -sheets that usually require shearing and elongation stress for proper molecular arrangement. These results illustrate the potential of cellulose as a template to control silk assembly and thus the ensuing mechanical properties of the composites. The mechanical properties of both cellulose and fibrous proteins such as silk are dictated through the high molecular order of the repetitive units in the main chain, interacting via intra- and inter-chain mechanisms, that add up to high strength materials. Alignment of these materials when mixed together, as displayed by Noishiki *et al.*, can generate new materials with improved mechanical properties [56].

Despite recent advances in the production of recombinant spider silks, commercialization of such silks is limited owing to the lack of systems for the production of bulk amounts of spider proteins at the high molecular weights found in the native proteins and at reasonable yields and costs. Plant expression presents an efficient and relatively low-cost method for the production of silk proteins. Several studies for the production of recombinant silks in tobacco, potato and *Arabidopsis* have been reported [57–60]. An interesting purification approach was presented by Scheller *et al.*, who produced recombinant silk-elastin fusion proteins that were purified by heat denaturation based on the 'inverse transition cycling' characteristic of elastin [59].

The potential for transgenic plants to further develop CBM technology was recently explored in our laboratory. Transgenic tobacco plants that expressed a synthetic CBD-spider silk fusion protein in the cell wall were produced. The protein accumulated in the plant cell wall and following homogenization of the plant, material could be recovered from tissue debris by biochemical separation. The proteins displayed typical silk biochemical properties, such as a wide range of thermal and pH stability. Large-scale purification of the recombinant CBD silk along with physical analysis of the proteins is currently underway. Thus CBD fusions to polymeric proteins allows for easier purification as well as close connection between the protein and the cellulose during the composite assembly.

Although the main interest in recombinant spider silk production has been dragline silk, other kinds of silks that spiders produce provide additional opportunities that could also be utilized in the future [61]. New silk sequences like those of the spider web glue [62] and the silk that composes the attachment discs to dragline silk of the black widow spider [63] have recently been published and will enable the production of new interesting materials in the

future. Combination of plant expression along with the development of efficient purification methods should allow industrial production of these proteins at a reasonable cost.

Vertebrate composites

Connective tissues (CTs) and extracellular matrices (ECMs) maintain and define the shape of tissues and organs, as well as functioning under tensile and compressive mechanical stresses. The mammalian ECM is mainly composed of collagens and proteoglycans (PGs). Collagens are a family of >27 different types of chemistries and along with other ECM components are involved in cell adhesion, tissue remodeling, cell differentiation, morphogenesis, wound healing and many pathologic states.

Fibrillar collagens are synthesized as soluble procollagen precursors, which consist of three polypeptide chains in a triple-helical array forming a rod with short nonhelical ends. The procollagen molecules are secreted into the ECM and then processed by proteolytic cleavage of the N- and C-nonhelical termini. This cleavage leads to spontaneous specific aggregation of long (300 nm), thin (1.5 nm) triple-helical collagen assemblies [64]. This aggregation process results in fibrils, subsequently stabilized by enzymatically induced covalent crosslinks.

Collagen architecture plays an important role in optimal functioning of various ECM-rich tissues, such as skin, tendons, muscle and cornea. During collagen fibrillogenesis, PGs play a major role in the control of basic size and shape of collagen fibrils and their stabilization. PGs are soluble polymers, which consist of a polypeptide core attached to glycan chains (anionic glycosaminoglycans [AGAGs]). More than one type of PG can be found in the ECM, ranging from small (e.g., decorin, 90–140 kD) to large (e.g., aggrecan, 2000–3000 kD). The glycan chains consist of repeating disaccharide units, one residue of which is always hexosamine, usually with sulfate ester groups attached at the four or six positions. The PGs bind collagen fibrils through their protein cores at specific sites in the gap zones of the collagen fibrils [65], while the bulky, hydrated AGAGs appear in electron micrographs as filaments, regularly and orthogonally bridging between and across the collagen fibrils [66]. AGAG chain lengths determine interfibrillar gap widths and vary from tissue to tissue [66]. Short AGAG bridges appear in tendon collagen fibrils, which are packed close together, whereas corneal collagen fibrils require longer AGAG bridges in order to maintain the regular collagen fibril distribution, without which the cornea would not be transparent [67]. In addition to its important role in collagen architecture in tissue, in *in vitro* collagen fibril-forming assays, some of the PGs inhibit the lateral growth of spontaneously forming fibrils, and are thus implicated in regulating certain aspects of collagen fibril structure [68].

The mechanical behavior of CTs is primarily determined by the composition and organization of collagen. The tension employed by the tissue is transmitted and resisted by collagen protein fibers, while compression is opposed by the water-soluble PGs [69]. The PGs act as a medium to transfer shear stresses between fibrils. The mechanical properties of the tissue correlate with the concentrations and proportions of collagen and AGAGs in the tissue, for example, the weak gel in the vitreous humor of the eye contains <1% by weight of fibers and AGAGs, while the toughest tendons contain over 70% fiber. Harder tissues such as cartilage and corneal stroma contain much more expansible AGAGs, which inflate to a high pressure. Such systems, which combine polysaccharides with specific structural protein-binding domains, may provide useful designs for biomimetic composites.

Biomimetic composite scaffolds for medical applications

A significant development in the use of biomaterial-based biopolymers is in the field of regenerative medicine. In recent years, the focus of this field has turned from the search for

inert metal and plastic-based materials for implantation to the development of biopolymer-based biomaterials systems that interact with tissues and promote regeneration. Furthermore, synthetic implants often require replacement during the lifetime of a patient which is a major drawback to these systems, a feature that can be obviated by the use of degradable polymer systems based on biopolymers and their corresponding composites.

Scaffolds are essential for promoting orderly tissue regeneration. Therefore, in recent years, a major effort has been made in the development of biopolymer-based materials that interact with tissues and promote regeneration. Biocompatibility is required for all engineered tissues but additional characteristics, such as the rate of biodegradation and mechanical properties, vary extensively between different tissue types. While soft tissues and nonload-bearing tissues require limited mechanical support and more rapid biodegradability, CTs like cartilage, tendons and bone require scaffolds with high mechanical strength, resilience and durability [20].

Collagen is the major component of the ECM and therefore it is a natural choice for applications in tissue engineering and regenerative medicine. Initial attempts at regenerating CTs have usually been performed using single component scaffolds, mostly type I collagen, from animal sources. However, these scaffolds were not able to achieve sufficient mechanical properties and correct tissue architecture in some studies, and often degrade too rapidly *in vivo* [70–73]. Furthermore, using type I collagen from animal sources involves several risks including allergenicity and potential contamination with pathogens. Recently, two human genes encoding recombinant heterotrimeric collagen type I were successfully coexpressed in tobacco plants with the human prolyl-4-hydroxylase (P4H) and lysyl hydroxylase 3 (LH3) enzymes, responsible for key post-translational modifications of collagen [74]. Plants coexpressing all five vacuole-targeted proteins generated intact procollagen yields of approximately 2% of the extracted total soluble proteins. Plant-extracted recombinant heterotrimeric collagen type I formed thermally stable triple helical structures and demonstrated functionality similar to human tissue-derived collagen, supporting binding and proliferation of adult peripheral blood-derived endothelial progenitor-like cells.

Furthermore, efforts have been made to produce bio-inspired composite scaffolds from biological polymers, mostly polysaccharides such as chitosan, chondroitin sulfate, alginate, cellulose, hyaluronic acid and proteins such as collagen, silk and elastin. These materials can be processed into different forms such as gels, sponges, fibers and mats [20]. These materials combine the biological and mechanical properties of the different constituents in synergy to enable adjustments to a variety of applications. The overall performance of these composites is advantageous compared with scaffolds prepared from each constituent separately, due to the improved tensile strength and support for cell proliferation [70–73, 75–78].

A promising approach presented by Panseri *et al.* involved magnetism in tissue engineering [79]. Magnetic scaffolds are able to support tissue regeneration and can be activated and work like a magnet attracting functionalized magnetic nanoparticles injected close to the scaffold to enhance tissue regeneration. *In vivo* biocompatibility and osteointegrative properties of these magnetic scaffolds have been studied [79].

Conclusion

Early biomaterial research tended to focus mainly on single types of polymer systems, while in nature a single polymer is rarely used for specific functions. Materials in nature are mostly arranged in composite systems that combine the properties of the different

constituents in a synergistic manner. In the biomedical field, composite scaffolds are advantageous compared with single component materials. Nevertheless, production of high performance scaffolds requires further study. In the body, the ECM as composites are hierarchically organized in a nanostructured manner that spans the micro- and macro-scales. In addition, the remarkable ability of nature to use a single dominating building block, like collagen, as a major component in different tissues and organs that require different properties, by applying different chemistries and assemblies, is important to consider when designing new scaffolds. Consequently, the focus for new scaffolds should be on the selection of appropriate materials as well as on the correct fabrication techniques and final architectures that will allow optimal interactions and synergy between the different components to match the needs of the target tissue. Moutos and Guilak have recently reviewed different fabrication techniques for the production of composite scaffolds for articular cartilage repair following fabrication techniques adopted from the textile and composite industries, which include embedded solid structures, multilayered designs or 3D woven composite materials [71]. They concluded that 3D woven fiber-reinforced scaffolds are promising for supporting cartilage regeneration.

Natural composites such as plant cell walls, vertebrate skeletons and mammalian ECM present versatile mechanical properties ranging from high strength to high elasticity. Remarkably, a limited reservoir of building blocks is used for production of this large variety of materials with different mechanical properties. This is achieved by utilization of different fabrication techniques and complex architectures. Current man-made composites do not yet capture this complexity and sophistication, or the performance of natural composites.

Future perspective

The production of new bio-inspired composites requires interdisciplinary and collaborative efforts between biologists, doctors, materials scientist engineers, textile and composite manufacturers to enable new fabrication techniques resulting in new high-end biomaterial composite systems for a range of applications. To capture the benefit of bio-inspired composites, scientists will have to study in detail the self-assembly mechanisms of the different building blocks, and engineer different binding domains to enable combined self-assembly of composite biomaterials that will perform in the challenging environments of medical implants.

Acknowledgments

Parts of the work discussed in this paper were funded by NIH grant No. EB002520.

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Executive summary

Cellulose composites

- Cellulose is the most abundant biopolymer on earth and has traditionally been used for clothing, construction, furniture and for paper making for thousands of years.
- In recent years, efforts have been made to produce nanostructured bio-inspired composites to mimic the features in natural cellulose composites.

Nanocellulose

- Nanocrystalline cellulose (NCC) is defined as fibers which have been grown under controlled conditions that lead to the formation of high-purity single crystals.
- The modulus of NCC is estimated to be approximately 150 GPa and its tensile strength is estimated to be approximately 10 GPa, similar to very strong materials such as aramid fibers (Kevlar®) and carbon fibers.

Composites with polymeric proteins

- Polymeric proteins display diverse mechanical properties from high strength to elasticity, which are often superior to their synthetic counterparts.

Invertebrate skeletal composites as sources of inspiration

- Invertebrate skeletons are sophisticated multifunctional structures providing structural and mechanical support, shape, protection and complex locomotion.
- Many of the skeleton proteins are bound to the chitin scaffold via a chitin-binding domain.

Resilin

- Resilin is a rubber-like protein that displays stretching ability up to 100% and is accompanied by a very high fatigue lifetime and low modulus of elasticity.
- The high elasticity of resilin combined with the strength of the chitin in the composite enables insects to rapidly restore body shape as well as most of the energy required for the next mechanical movement (either flying or jumping).

Spider silk

- Spider dragline silk, used as the safety line and as part of the frame thread of spider webs, is the strongest natural fiber known, reaching tensile strengths of 740 MPa.
- Recombinant spider silks can be generated in many variants for biomaterials and tissue engineering.

Vertebrate composites

- Collagen architecture plays an important role in optimal functioning of various extracellular matrix-rich tissues, such as skin, tendons, muscle and cornea.

Biomimetic composite scaffolds for medical applications

- A significant development in the use of biomaterial-based biopolymers is in the field of regenerative medicine.
- Collagen is the major component of the extracellular matrix and therefore it is a natural choice for applications in tissue engineering and regenerative medicine.

Conclusion

- Natural composites such as plant cell walls, invertebrate skeletons and mammalian extracellular matrix present versatile mechanical properties ranging from high strength to high elasticity.

Figure 1. Nanostructured foams produced from nanocrystalline cellulose liquid crystal suspensions

(A) A lightweight porous foam produced by freezing and lyophilization of the suspension.

(B) Scanning electron microscope image of the foam's internal structure showing its arrangement in a 'puffed pastry' laminated sheets nanostructure.

Figure 2. New silica-based nanocomposites formed from bioengineered fusion proteins
 Consists of two components: the R5 peptide, derived from the silaffin protein of *Cylindrotheca fusiformis*, and a self-assembling domain based on the consensus repeat in the major ampullate spidroin protein 1 (MaSp1) of *Nephila clavipes* spider dragline silk. A schematic representation of the design of fusion proteins and their use in controlled silica nanocomposite formation is shown. (A) Chimeric design with two functional domains: silk and R5. (B) Model of spider silk protein processing into films and fibers and silicification reactions on the assembled materials. Reproduced with permission from [30] © National Academy of Sciences USA (2006).

Figure 3.
Schematic illustration of a 5-nm silicon nanoparticle attached to the SP1 protein inner cavity.

Figure 4. Chitin binding of 6H-resChBD

(A) SDS-PAGE ana lysis of chitin binding of 6H-resChBD crude lysate. T: Total; B: 6H-resChBD affinity purified by chitin beads. Protein eluted by boiling in sample application buffer. (B) SDS-PAGE ana lysis of chitin binding of pure 6H-res (left) and 6H-resChBD (right).

6H-res: Recombinant resilin exon 1 sequence with His tag N-terminal fusion;

6H-resChBD: Recombinant resilin exons 1,2 (with the chitin binding domain) with His tag N-terminal fusion; B: Bound fraction eluted from chitin beads by boiling with sample application buffer; T: Total protein post ion exchange chromatography purification; UB: Unbound fraction.

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Figure 5. Photo-Fenton cross-linked exon 1 resilins

(A) Photo-Fenton cross-linked exon 1 resilins are (B) squashed and (C) extended over 50 cycles via tweezers, without any plastic deformation, showing high resilience. The polymer color is Fe content dependent.

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Figure 6. Generation of chimeric silk proteins as a tool to expand their functional features
(A) The consensus spider silk sequence that constitutes the core repeats of chimera. **(B)** Examples of protein regions used for the generation of chimeric silk proteins. The R5 component of silaffin (sequence shown) was fused to spider dragline silk and was able to foster the polymerization of silica precursors to form glassified materials. The C-terminal domains from DMP 1, a protein found in the mineralized tissue of teeth and responsible for the nucleation and growth of hydroxyapatite, could also be fused to the spider silk and was shown to nucleate and form hydroxyapatite-containing spider silk in film form. The third example shows the addition of a cell-binding domain, RGD from fibronectin, which is responsible for cell binding via membrane integrin receptors. This resulted in cell adhesion on silk films. The last example shows the formation of block copolymers formed from silkworm silk and elastin for biomaterials used as drug delivery systems. **(C)** The basic cloning procedure involving synthetic oligonucleotides is illustrated; this procedure leads to the purification of silk proteins derived from recombinant DNA that have new functions. These novel proteins can be used to generate silk materials with improved functionalities, such as improved cell binding features, glassified silk fibers with increased stiffness and hydroxyapatite mineralized biomaterials potentially useful for bone-related repairs.

DMP: Dentin matrix protein.

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