Unexpected Strength and Toughness in Chitosan-Fibroin Laminates Inspired by Insect Cuticle

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The unusual strength and toughness of insect cuticles, crustacean shells, mollusk nacre, and other chitin-containing living materials depend on complex structural interactions between chitin polysaccharides and proteins in these materials.[1,2] Because the structural basis for these interactions is not fully understood,[4] it has been difficult to engineer artificial materials that reproduce these novel properties. Here we describe the fabrication of a bioinspired material that reproduces the chemical composition and phase-separated structure of natural insect cuticle. This design-controlled material reproduces the outstanding properties of the natural composites, including strength and toughness similar to aluminum alloys, but obtained at half their density, which are ten times greater than the unstructured component blend and twice those of its strongest constituent. Bioinspired cuticle mimics may prove useful as replacements for plastics in consumer products and for certain medical applications, as chitosan and fibroin are biodegradable, biocompatible, and used in approved clinical products.[5]

Living materials often exhibit novel properties, including high strength, energy absorption, flexibility, and biocompatibility due to unique physical and chemical interactions among biomolecular components on the nanometer scale.[6] This is especially true for the cuticles of insects and crustaceans that are composed of chitin, which is the second most abundant polymer on earth. The simplest arthropod procuticle is a composite of a microfibrous film of chitin embedded in a resilin protein matrix;[7] however, the diversity of proteins found in cuticle is extraordinary as it represents one of the largest multigene families in insects.[8] The other main cuticular structural proteins include fibroin, elastin, abductin, and collagen, which all share a high content of glycine and alanine. Fibroin-like proteins are particularly interesting because they function as structural proteins in diverse species and phyla, ranging from arthropods to mollusks.[9]

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Many researchers have strived to engineer bioinspired materials with synthetic components that reproduce the unique properties of natural structures,[2,3] including insect cuticle,[10] but it is difficult to work with chitin because of its low solubility. In contrast, the more soluble, highly deacetylated form of this polysaccharide, chitosan, has been used for many applications, and it is approved by the U.S. Food and Drug Administration (FDA) for wound dressings. Fibroin from silk is also readily available and used widely in surgical sutures. Chitosan-silk composites have been explored in the past for potential medical applications where both biocompatibility and high material strength are required. Unfortunately, the strength of these blended materials is significantly less than that exhibited by chitosan alone.[11]

One potential explanation for the poor material properties of chitosan-silk composites is that they are disorganized conglomerates, that is, they fail to regenerate the phase-separated laminar arrangement of the closely apposed chitin and protein found in natural cuticle.[12] We therefore explored whether we could recapitulate the novel properties of these living materials by fabricating chitosan-fibroin laminates. The laminar domains of chitin and protein that comprise the arthropod cuticle range from 60 nm (e.g., flea) to 20 μm (e.g., *Astacus*) in thickness and, although they are tightly bonded to each other along their interface, the chemical basis for this is unknown. To reproduce this cuticular structure, we first evaporated a chitosan solution dissolved in acetic acid to cast an ≈12 μm thick layer of chitosan polymer on a planar glass surface. To prevent dissolution during next steps, the free-standing chitosan film was neutralized in sodium hydroxide and rinsed in water. Fibroin extracted from *Bombyx mori* silk and dissolved in water was deposited on the surface of the polysaccharide film by evaporation, and the fibroin was treated with methanol to induce an insoluble β-sheet transition.

This simple fabrication method produced a clear thin film (**Figure 1**a) that exhibited a laminate structure composed of phase separated polysaccharide and protein layers with a distinct linear boundary (Figure 1b,c). This material configuration closely resembles the chemical composition and the laminar form of natural cuticle.[13] We refer to this novel composite material as "shrilk" because chitosan is commonly isolated from shrimp shells and fibroin comes from silk. Interestingly, mechanical testing revealed that the shrilk laminate exhibits an ultimate strength of 119 MPa, which is ten times stronger than that previously described for a chitosan:fibroin blend with similar weight:weight ratios,[11] and it exhibits twice the strength of chitosan, which is its strongest component (**Figure 2**a). This is a significant finding as fibroin on its own is 19 times weaker than chitosan, and the precise ratio of chitosan:fibroin is critical for the observed improvement in material properties.

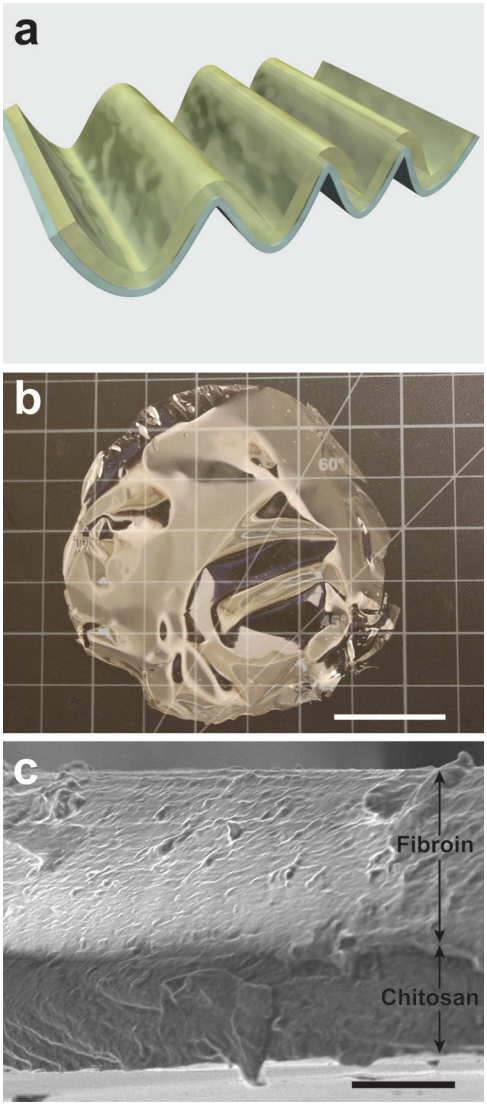

Figure 1. Structure of the shrilk artificial insect cuticle. a) Diagram of a laminar shrilk composite composed of a layer of chitosan film (blue) bonded to a fibroin layer (yellow) that mimics the phase-separated structure of natural insect cuticle. b) Photograph of a shrilk film overlaid on a surface grid (scale bar, 2.3 cm) showing the high optical clarity of this material. c) Scanning electronic microscopy image of a cross section of the shrilk laminate showing the interface between the chitosan and fibroin layers, which appear darker and lighter, respectively, due to the different accumulation of surface charges of the materials (scale bar, 10 μm).

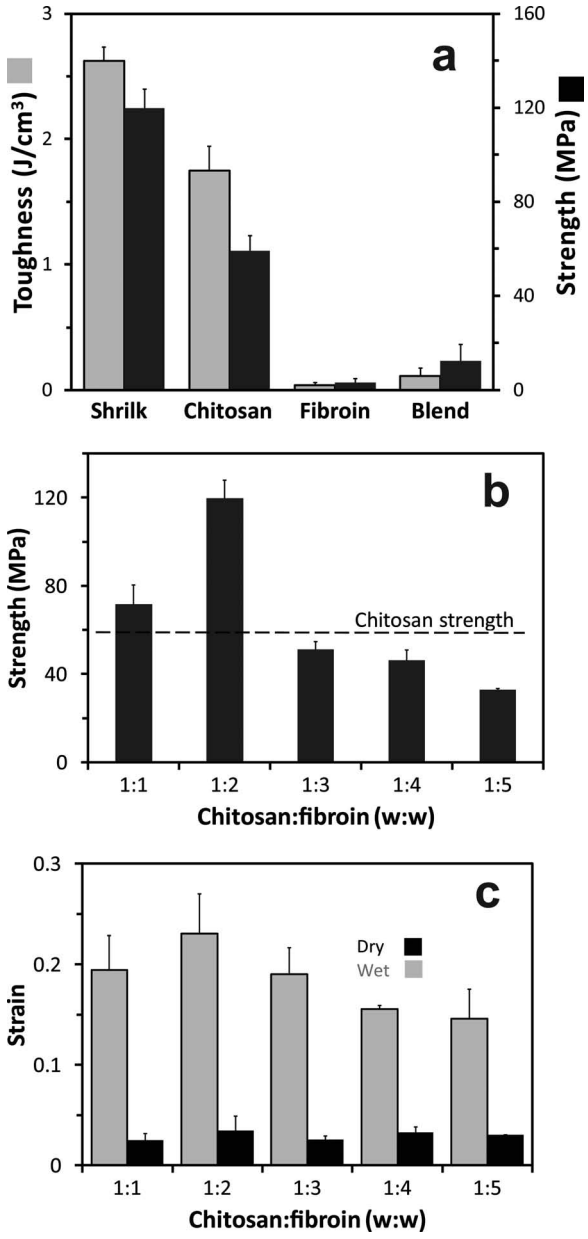

Figure 2. Mechanical characterization. a) Modulus of toughness (grey bars) and breaking strength (black bars) of the shrilk laminate compared with similar shaped and sized layers of chitosan alone, fibroin alone, or a blended composite that has the same 1:2 chitosan:fibroin ratio as shrilk. All error bars indicate standard deviation (SD). b) Variation in breaking strength of shrilk as a function of the chitosan:fibroin ratio in the laminate. Dashed line indicates breaking strength of the chitosan alone. c) Strain at the break point for shrilk when dry (black bars) compared to when saturated with water by immersion for 24 h at 37 °C (grey bars).

The laminate exhibited maximal strength at a 1:2 (w/w) ratio of chitosan:fibroin (Figure 2b), whereas increasing the protein concentration further gave rise to a material mechanically closer to fibroin. Shrilk also exhibited much higher toughness as it absorbed 1.5 times more energy than chitosan per volume before breaking, while the ultimate strain was significantly lower (Supporting Information Figure S1 and Table S1). This unexpected increase in toughness that allows shrilk to resist external tension without deforming or damaging internal structural components is reminiscent of the natural insect cuticle's protective function.

These data show that replicating one layer of the phase separated laminar structure of the cuticle using similar carbohydrate and protein components (chitosan and fibroin, respectively) is sufficient to greatly improve the mechanical characteristics of the material relative to its individual components. Moreover, this shrilk material exhibits a mechanical strength that is similar to non-tanned cuticles[14] and other natural structural composites

(Supporting Information Figure S2), thus illustrating the dramatic importance of 3D design for the mechanical behavior of living materials.[15] Importantly, shrilk even exhibits novel properties compared to other common high-strength materials, as it is twice as strong as nylon or polylactic acid (PLA), and it has similar strength to aluminum alloys but at half their weight due to its lower density (1.46 g cm^{-3}).

One of the most remarkable characteristics of the living cuticle is its wide range of material properties, which can vary from very elastic in joint regions to very hard protective covertures and wings.[16] Interestingly, these variations in physical properties do not correlate with the type of protein present in the cuticle and instead appear to depend on water content.[14] Chitosan absorbs water more than twice as efficiently as fibroin, and quantitation of water absorption by shrilk films by weight comparison of dry and water-saturated samples confirmed that the fibroin and chitosan layers both uptake water independently of their interaction along their interface (Supporting Information Figure S3). Saturation of shrilk with water reduced its strength to ≈3.5 MPa, which is more than thirty times below that exhibited by the dry material; however, the energy absorbed before breakage was only reduced by two. The remaining energy appeared to be stored as an increase in the material's elasticity, which supported up to 23% ultimate strain; this is almost ten times higher than the strain shrilk can bear in a dry state (Figure 2d). This water-content-dependent variation in flexibility is reminiscent of some insect species, such as *Rhodnius*, that dynamically control the hydration of their abdominal cuticle during blood feeding to achieve changes in cuticle stiffness ranging from 10 to 250 MPa.[17] Importantly, shrilk exhibits similar versatility and it can be reversibly transformed between rigid and highly flexible states by altering water content alone. At the same time, the high strength and shape stability of shrilk should be able to be retained by protecting it from absorbing moisture by coating it with a protective layer, much like the outer layer of the natural cuticle (i.e., epicuticle) protects inner levels from moisture by a mixture of proteins and wax. In fact, preliminary studies in which shrilk has been coated with a water resistant material (parylene-C) support this possibility (Supporting Information Figure S7).

Many cuticles exhibit novel specialized functions, such as the ability to condense humidity from the atmosphere on the surface of insects that live in arid climates,[18] to repel water in moist environments,[19] or to control light polarization for communication.[20] These unique properties often arise from local variations in microtopography at the surface of the cuticle material. We were able to microfabricate shrilk films with defined microsurface architecture by casting the protein between a flat chitosan film and a microstructured polydimethylsiloxane (PDMS) mold and then evaporating the solvent. Microstructured chitosan structures have been fabricated in the past,[21] but because chitin is not present in the epicuticle layer that normally exhibits microtopographical specializations,[22] we chose to micromold the fibroin layer. The micromolded shrilk material displayed a finely structured surface topography that was a precise negative imprint of the mold (**Figure 3**a,b).

Additionally, because mechanical characteristics of shrilk films can be controlled through hydration, the microstructured shrilk film can be formed into more complex shapes, such as tubes (Figure 3c), as previously demonstrated with chitosan films.[23] For instance, we fabricated a hollow cylindrial structure by hydrating a previously deposited and micromolded shrilk film to increase its flexibility, rolling this flexible film around a glass capillary tube, and then drying the film to return it to a rigid state. The dry film retained its cylindrical shape after it was removed from the surface of the capillary tube. In this configuration, shrilk could potentially serve as a biocompatible and biodegradable scaffold for small vessel repair or as a nerve conduit given its high biocompatibility.

The strong affinity between the chitosan and fibroin layers, and the stability of the composite against water also make it possible to join together multiple shrilk laminates by "gluing" them with fibroin, thereby producing thicker, stronger, and more complex structures that can be tailored for specialized applications. Using this approach, we created an artificial procuticle laminate comprised of three shrilk layers (Figure 3e and Supporting Information Figure S4) that retained high mechanical strength (116.7 ± 12.3 MPa).

Because of the complexity of living cuticle, the structural and chemical basis of its novel mechanical properties currently remains unknown. Results of past studies analyzing chitin–protein cross-linking have led to contradictory models of bond formation.[24,25] The availability of the engineered artificial shrilk cuticle therefore offered a new approach to examine this fundamental mechanism of interfacial adhesion. Chitin is usually described as cellulose with one hydroxyl group on each monomer substituted with an acetylamine group (Supporting Information Figure S5).[26] Fourier transform infrared (FTIR) analysis of our chitosan films revealed the characteristic absorption due to C=O stretching of the amide (amide I, 1658 cm^{-1}), consistent with incomplete deacetylation of chitin (**Figure 4**a and Supporting Information Figure S6). Shrilk (chitosan:fibroin at 1:2 ratio) displayed an IR absorption spectrum (Figure 4b) very similar to the sum of chitosan (Figure 4a, black) and fibroin (Figure 4c, black); however, we observed a shift in the amide II band signal (from 1555 to 1536 cm^{-1}) that also has contributions from amine I (N–H bending from the amine and acetylamine) and C–N stretching modes similar to the shift previously reported for chitosan-fibroin blends.[27]

Electron microscopy and X-ray studies suggest that chitin is structured in the form of pure crystalline microfibrils surrounded by a protein matrix in a two-phase system within insect cuticle. As a result, only those chitin chains situated at the interface with the protein phase of the cuticle will be sterically available to interact with other components, and this should be true in the shrilk laminate as well, if it accurately mimics the living cuticle. Because these interfacial bonds are rare, absorption bands of the bulk material should dominate when measured with FTIR. Our ability to engineer shrilk in two separate phases that can be analyzed alone, or when combined in an organized laminate, allowed us to explore their molecular bonding characteristics in detail.

When the spectrum of the bulk fibroin phase in shrilk (Figure 4c, grey) was analyzed independently of the background signal from the chitosan phase, we found that the shift in the amide II band resulted from altered vibration and absorption of the amine I (i.e., nitrogen atom in the number 2 position) in chitin and chitosan at 1548 cm^{-1}. This variation is similar to that produced when we protonated amine groups in chitosan by treatment with acid solution (Figure 4a and Supporting Information

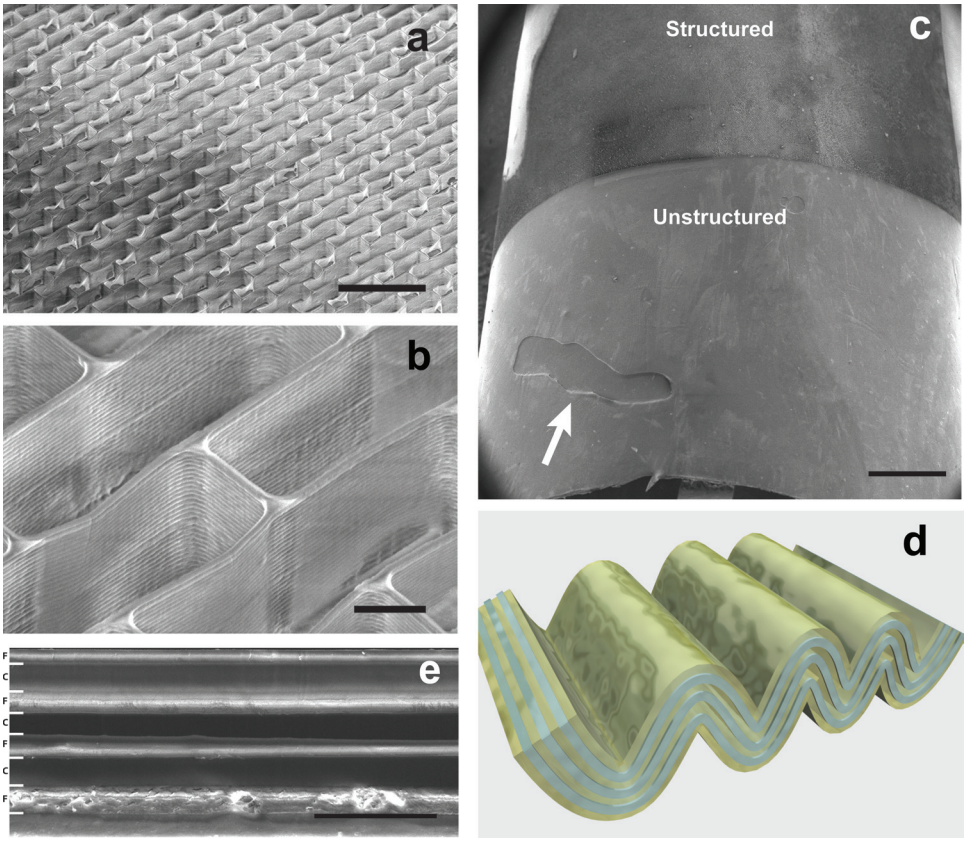

Figure 3. Fabrication of more complex shrilk materials. a) Micropatterned surface topography of the fibroin covering layer of shrilk containing tightly packed rectangular lacunae created using a micromolding method (scale bar, 50 µm). b) Higher magnification view of (a). Horizontal bands on the walls correspond to marks on the original mold used for imprinting that resulted from deep reactive-ion etching (scale bar, 5 µm). c) Shrilk formed into a cylindrical shape that contains a structured region containing the micromolded topography shown in a and a smooth unstructured region. The white arrow indicates a defect in the fibroin protein film that reveals the underlying chitosan layer. (scale bar, 1 mm). d) Schematic of a multi-laminate design composed of three tightly bonded shrilk bilayers. e) A scanning electron microscopy image of a cross section of a microfabricated multi-laminate material with the design shown in (d) (F, fibroin layer; C, chitosan layer; scale bar, 50 µm).

Figure S6). These data indicate that the amine linkage at the number 2 position of the polysaccharide ring of chitosan mediates its bonding to fibroin where these materials interface in this insect cuticle mimetic. This also explains the poor mechanical properties of chitosan-fibroin blends described in past studies and as we confirmed here. This failure results because the nitrogen atom in the number 2 position of the polysaccharide ring mediates interchain bonding within the crystal forms of chitin[28,29] and chitosan.[30,31] Apparently, in the disorganized composite materials, the addition of fibroin interferes with this crystallization process and thereby decreases the strength of chitin or chitosan layer.

Chitin-protein-based composites are the most abundant natural structural materials outside the plant kingdom and, being found in fungi to mollusks, they are also one of the most important examples of convergent evolution. The ease of microfabricating the shrilk material with a defined phase organization and composition provides a new experimental approach to study the structural basis of these natural composites in a reliable controlled system. The mechanical properties of insect cuticle are commonly thought to be due to the presence of chitin, whereas the protein phase has been assumed to play a more functional role. By contrast, our results show that the mechanical properties of these systems cannot be described without considering the interaction between the components, while the unexpected strength and toughness of the new material provide a potential explanation for why these composites have been selected for during evolution in so many unrelated natural systems.

Shrilk represents the simplest model of an insect cuticle that contains only two types of molecular components. Other relevant components (e.g., resilin, collagen, and minerals) and structural modifications (e.g., protein tanning mediated through enzymatic reactions and calcium deposition) also can be integrated in the system to provide deeper insight into the structural basis of living materials and to engineer composites based in this and other biological models (e.g., mollusk and crustacean shells).

Based on its outstanding strength and versatility, as well as its low cost and density, shrilk is an excellent candidate as a biodegradable plastic that could have great value as a replacement for existing non-degradable plastics in a wide range of consumer product application areas, including disposable bottles, trash bags, packing materials, and diapers that currently

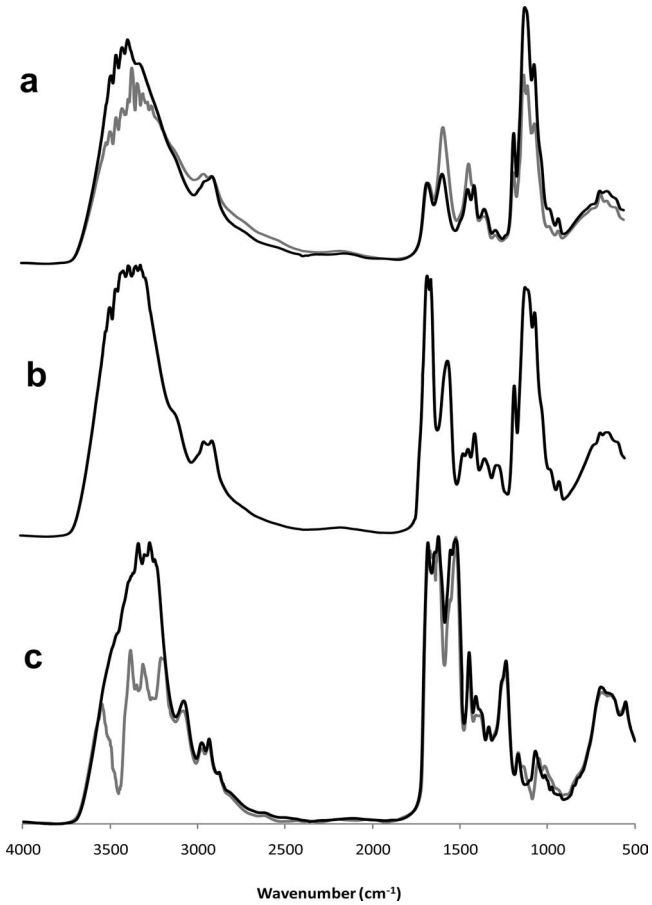

Figure 4. Molecular analysis of shrilk and it components using FTIR. a) FTIR spectra of chitosan (black) compared with protonated chitosan (grey). b) FTIR spectrum of shrilk demonstrating the presence of both the strong polysaccharide structure of the chitosan (950–1185 cm^{-1}) and the amide I and II bands (at 1640 and 1536 cm^{-1}, respectively) from fibroin. c) Spectra for fibroin alone (black) and the fibroin phase within shrilk (grey). A more detailed spectrum of the 2000–500 cm^{-1} region is available in the Supporting Information Figure S6.

pile up in waste sites around our planet. Because chitosan and fibroin are both biocompatible, shrilk on its own or in combination with other materials or crosslinking agents may be valuable for certain medical applications, such as wound dressings and scaffolds for regenerative medicine. Finally, due to the biological origin, wide availability, and low cost of its components, shrilk represents an abundant and sustainable material that can be seamlessly integrated into the environment within several ecological cycles.

Experimental Section

The laminated films were produced by the successive casting of 2% (w/v) chitosan in 1% (v/v) acetic acid and 4% (w/v) *Bombyx mori* silk fibroin. To prevent dissolution, the chitosan phase was treated with 4% (w/v) solution of sodium hydroxide, while the fibroin phase was treated with methanol. Microstructures were produced by casting the appropriate phase over a microstructured PDMS mould. More details about the experiments can be found in the Supporting Information.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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