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Elastin-like protein matrix reinforced with collagen microfibers for soft tissue repair

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Abstract

Artificial composites designed to mimic the structure and properties of native extracellular matrix may lead to acellular materials for soft tissue repair and replacement, which display mechanical strength, stiffness, and resilience resembling native tissue. We describe the fabrication of thin lamellae consisting of continuous collagen microfiber embedded at controlled orientations and densities in a recombinant elastin-like protein polymer matrix. Multilamellar stacking affords flexible, protein-based composite sheets whose properties are dependent upon both the elastomeric matrix and collagen content and organization. Sheets are produced with properties that range over 13-fold in elongation to break (23 – 314%), six-fold in Young's modulus (5.3 to 33.1 MPa), and more than two-fold in tensile strength (1.85 to 4.08 MPa), exceeding that of a number of native human tissues, including urinary bladder, pulmonary artery, and aorta. A sheet approximating the mechanical response of human abdominal wall fascia is investigated as a fascial substitute for ventral hernia repair. Protein-based composite patches prevent hernia recurrence in Wistar rats over an 8-week period with new tissue formation and sustained structural integrity.

Keywords

elastin; collagen; mechanical properties; fiber reinforced composite; surgical mesh; recombinant protein

INTRODUCTION

The fibrous protein structures of natural tissues demonstrate that variation in collagen and elastin networks generate highly specialized biomechanical responses in numerous tissues and diverse species [1, 2]. In turn, biomechanical properties such as modulus, elasticity, and

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strength dictate tissue-specific performance characteristics that affect function and durability, as well as local cellular behavior. In many instances, including blood vessels [3], heart valve leaflets [4], and dermis [5], the arrangement and structure of elastin and collagen jointly determine biomechanical properties. However, even among soft tissues containing relatively little elastin, collagen fiber organization is critical. All told, if salient features of native protein microstructure can be replicated in bioengineered tissues, the potential exists to design improved materials for soft tissue repair and reconstruction.

In this report, we combine extracellular matrix (ECM) protein analogs to yield multilamellar, elastin-like protein sheets reinforced with collagen microfibers (Figure 1). The process lends itself to varying fiber orientation and volume fraction within and between individual lamellae of a planar sheet, leading to tunable biomechanical properties that resemble those of native tissues. Although textile and fiber composite technology cannot match the complexity of the ECM, the angle-ply format employed here resembles the oriented, lamellar features of a range of tissues, such as the intervertebral disc, arterial media, and abdominal wall fascia [6–8]. Moreover, it is likely that such composites can be incorporated into schemes that seek to integrate cells within the analog, prior to or after implantation in vivo. Significantly, although the composite is based upon structural proteins, it is distinct from decellularized cadaver and animal biologics [9] because the designer may directly specify the proportion and orientation of collagen fiber to meet a range of mechanical specifications. In addition, the constituent protein materials are purified in solution prior to matrix fabrication, avoiding the incubation and rinsing steps required by cadaver and animal tissue scaffolds.

Matching the elastin content and organization of the ECM using existing tissue engineering approaches remains an ongoing challenge [10–12]. In contrast, the scaffolds reported herein are fabricated with a high fraction of elastin-like protein without the necessity for prolonged culture within a bioreactor, as often required for cell-secreted ECM in engineered tissue substitutes [12, 13]. Of several recombinant elastin-like protein polymers synthesized in our laboratory, triblock copolymers have emerged as a promising elastomeric matrix with the capacity to undergo either physical or chemical crosslinking. As detailed elsewhere, an aqueous solution of elastin-mimetic triblock protein polymer forms a physically crosslinked gel above a defined transition temperature, facilitating molding and laminating processes through mild temperature adjustment [14]. These materials exhibit excellent biostability in vivo, even in the absence of covalent crosslinks [15], can be processed as nanofibers with tunable mechanical properties [16], as micelles [17], or used for controlled drug delivery [18]. Combining this elastomeric protein matrix with an organized collagen fiber network necessitated the development of a scalable spinning process for production of continuous fiber composed of D-periodic collagen fibrils [19]. After observing the favorable mechanical properties of similar composites for vascular grafts [20], we sought to investigate the feasibility of these materials in abdominal wall repair.

Decellularized tissues are widely used for abdominal wall repair and display certain advantages over their synthetic counterparts, composed from poly(tetrafluoroethylene) (PTFE), polypropylene, or polyethylene. Nonetheless, both cadaveric and animal derived acellular collagen scaffolds also suffer from a number of limitations, including variable stability, seroma formation, and poor tissue integration [21, 22]. Thus, there exists a need for new design scheme for the creation of biologic tissue repair materials with improved properties. We believe that an initial step in this direction is provided by the engineered collagen-elastin composite biomaterials described here.

MATERIALS and METHODS

Synthesis of a recombinant elastin-mimetic triblock protein polymer

Genetic engineering, expression, purification, and characterization of the elastin-mimetic protein polymer, designated **LysB10**, has been described elsewhere [23]. Briefly, the flanking 75 kDa endblocks of the protein polymer contained 33 repeats of the hydrophobic pentapeptide sequence [IPAVG]₅, and the central 58 kDa midblock consisted of 28 repeats of the elastic, hydrophilic sequence [(VPGAG)₂VPGE(VPGAG)₂]. Additional sequences between blocks and at the C terminus include the residues [KAAK], which along with the N-terminal amine provide amino groups for chemical crosslinking.

The protein polymer sequence is contained in a single contiguous reading frame within the plasmid pET24-a, which was used to transform the *E. coli* expression strain BL21(DE3). Fermentation was performed at 37°C in Circle Grow (QBIgene) medium supplemented with kanamycin (50 µg/mL) in a 100 L fermentor at the Bioexpression and Fermentation Facility, University of Georgia. Cultures were incubated under antibiotic selection for 24 hr at 37°C. Isolation of the **LysB10** consisted of breaking the cells with freeze/thaw cycles and sonication, a high speed centrifugation (20,000 RCF, 40 min, 4°C) with 0.5% poly(ethyleneimine) to precipitate nucleic acids, and a series of alternating warm/cold centrifugations. Each cold centrifugation (20,000 RCF, 40 min, 4°C) was followed by the addition of NaCl to 2M to precipitate the protein polymer as it incubated for 25 min at 25°C. This was followed by warm centrifugation (9500 RCF, 15 min, 25) and resuspension of the pellet in cold, sterile PBS on ice for 10 to 20 min. After 6 to 10 cycles, when minimal contamination was recovered in the final cold centrifugation, the material was subject to a warm centrifugation, resuspended in cold sterile PBS, dialyzed, and lyophilized. Lyophilized protein was resuspended in sterile molecular grade water at 1 mg/mL and endotoxin levels were assessed according to manufacturer instructions using the Limulus Amoebocyte Lysate (LAL) assay (Cambrex). Levels of 0.1 EU/mg were obtained (1 EU = 100 pg of endotoxin), which corresponds to endotoxin levels for clinically used alginate (Pronova sodium alginate, endotoxin ≤ 100 EU/g).

Isolation and purification of monomeric collagen

Acid-soluble, monomeric rat-tail tendon collagen (MRTC) was obtained from Sprague-Dawley rat tails following Silver and Trelstad [24]. Frozen rat tails (Pel-Freez Biologicals, Rogers, AK) were thawed at room temperature and tendon was extracted with a wire stripper, immersed in 10 mM HCl (pH 2.0; 150 mL per tail) and stirred for 4 hr at room temperature. Soluble collagen was separated by centrifugation at 30,000 g and 4°C for 30 minutes followed by sequential filtration through P8, 0.45 µm, and 0.2 µm membranes. Addition of concentrated NaCl in 10 mM HCl to a net salt concentration of 0.7 M, followed by 1 hr stirring and 1 hr centrifugation at 30,000 g and 4°C, precipitated the collagen. After overnight re-dissolution in 10 mM HCl the material was dialyzed against 20 mM phosphate buffer for at least 8 hr at room temperature. Subsequent dialysis was performed against 20 mM phosphate buffer at 4°C for at least 8 hr and against 10 mM HCl at 4°C overnight. The resulting MRTC solution was stored at 4°C for the short-term or frozen and lyophilized.

Production of a collagen microfiber by continuous co-extrusion

Collagen fibers were produced continuously and in large scale using a lab scale automated fiber spinning system, described elsewhere [19]. Briefly, a collagen solution (5 mg/mL in 10 mM HCl) and wet spinning buffer (WSB: 10 wt% poly (ethylene glycol) Mw = 35000, 4.14 mg/mL monobasic sodium phosphate, 12.1 mg/mL dibasic sodium phosphate, 6.86 mg/mL TES (N-tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid sodium salt), 7.89 mg/mL sodium chloride, pH 8.0) were extruded with a dual syringe pump (Harvard Apparatus,

Holliston, MA). The collagen solution emerged through a 0.4 mm inner diameter spinneret into the center of a vertical tube (1.6 mm inner-diameter $\times$ 1 m long fluoropolymer tubing) at 0.08 mL/min. Wet spinning buffer simultaneously advanced through a bubble trap and down the fluoropolymer tube at a rate of 1.0 mL/min. As it exited the spinneret, the collagen coagulated into a gel-like fiber and traveled downward with the WSB stream.

Upon emergence from the fluoropolymer tube, the fiber entered a 2 meter-long rinsing bath of 70% ethanol in water. Continuous fiber was produced and collected by winding it out of the rinsing bath onto segments of a polyvinyl chloride (PVC) carrier cylinder that rotated and translated automatically. After spinning, the fiber was placed in fiber incubation buffer (FIB: 7.89 mg/mL sodium chloride, 4.26 mg/mL dibasic sodium phosphate, 10 mM Tris, pH = 7.4) at 37°C for 48 hr [25]. Fiber was incubated directly on the carrier cylinders, followed by rinsing in ddH₂O for 15 min before drying and transferring the fiber onto a second cylinder under tension using an automated fiber collecting system.

Fabrication of laminated fiber reinforced composites from collagen fibers within an elastin-like protein polymer matrix

Composite sheets were fabricated by winding defined collagen fiber layouts onto rectangular frames and implementing the transition temperature fiber embedding and lamination protocol (Figure 1). To arrange fibers with defined spacing and orientation, the frame translation speed, translation distance, and rotation speed were computed with a MATLAB script. An automated linear actuator (Velmex, Inc, Bloomfield, NY) and a DC gear motor translated and rotated the frames. After winding, each fiber layout was transferred onto a sheet of ultrasoft polyurethane, secured with tape, and photographed. Images from at least three regions of each layout enabled the measurement of average fiber spacing (ω) and angle (θ).

Solutions of protein polymer were prepared at 10-wt% concentration in ice-cold ddH₂O. Argon was bubbled through the solutions, followed by centrifugation at 4°C and 500g for 5 min to remove bubbles. To embed the fiber layouts, precision 50 μ m thick plastic shims (Precision Brand, Inc., Downers Grove IL) were placed around the layouts, and all embedding materials were cooled to 4°C. The protein polymer solution was distributed over the fibers and a sheet of acrylic was pressed on top of the solution. The fibers and the protein polymer solution were located within a 50 μ m space, sandwiched between the acrylic sheet and polyurethane base that were separated by precision shims. The embedding assembly was incubated at 4°C for one hour to allow the protein polymer solution to hydrate the fiber layout, followed by transfer of the assembly to a 37°C incubator for 30 min. When the polyurethane and acrylic sheets were peeled apart, the fiber layout remained embedded in a gel film of protein polymer, adherent to the polyurethane base. After a 5-minute incubation in 37°C ddH₂O, the fiber-reinforced film could be separated from the polyurethane base.

Each composite sheet consisted of a multilamellar stack of ten 40 μ m thick layers. The eight central layers contained embedded fiber while the top and bottom layers contained only protein polymer. Ten-layer stacks were covered with plastic wrap to prevent drying, cooled to 4°C for 12 hr, and transferred to 37°C for 30 min to facilitate interlamellar bonding with formation of a cohesive sheet. The sheet was removed, rinsed in 37°C PBS for 30 min, and crosslinked in 0.5% glutaraldehyde in PBS for 24 hr at 37°C. Vigorous shaking in PBS for 6 hr at 37°C with three buffer changes served to remove excess glutaraldehyde.

Analysis of collagen fiber orientation and volume fraction

Seven different fiber layouts were analyzed. To study the effect of fiber spacing on mechanical response, we prepared samples with fiber orientations of 15° and spacing equal

to 0.15 mm, 0.45 mm, or 1.3 mm, as well as samples without fiber. Fiber volume fraction was calculated from measurements of average fiber spacing taken from digital photographs of the fiber layouts, an estimated fiber diameter of 40 μm , and thickness of the multilamellar sheet (400 μm). Fiber spacings of 1.3 mm, 0.45 mm, or 0.15 mm corresponded to ~3, 7, and 17% fiber volume fractions, respectively. The effect of fiber orientation on mechanical behavior was determined by fixing fiber volume at 17% and varying fiber orientation from 0°, 15°, 25°, to 90°. Primary orientations of fiber layouts were measured from digital photographs using the Fast-Fourier Transform tool from ImageJ software [26].

Digital volumetric imaging

For digital volumetric imaging (DVI), collagen fiber was conjugated to tetramethyl rhodamine isothiocyanate (TRITC) [27]. In brief, fiber was wound about a carrier cylinder and placed inside a larger hollow cylinder. This arrangement created a 100 mL annular volume in which 20 to 40 m of fiber could be reacted without tangles or breaks. A 1 mg/mL solution of TRITC in DMSO was added to a 0.1 M sodium carbonate solution to a concentration of 0.05 mg/mL. This solution was added between the pipe segments and stirred for 12 hr at 4°C, after which the fiber was rinsed four times with ddH₂O for 2 hr and for 5 min with 70% ethanol, and then dried as it was transferred to a second carrier cylinder. Composite sheets containing TRITC-conjugated fiber were prepared for DVI by serial dehydration in ethanol and xylene. Samples were embedded in Spurr's epoxy modified with an optical opacifier, Sudan Black B, and imaged with a DVI Microimager (Microscience Group, Inc. Redwood City, CD).

Transmission electron microscopy

Transmission electron microscopy was used to investigate the ultrastructure of the composite. Samples were rinsed twice in 0.1 M cacodylate buffer (pH 7.4), fixed (2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4) for 90 minutes, washed in 0.1 M cacodylate water and then dH₂O, postfixed with 1% osmium tetroxide for one hour, and stained en bloc with filtered 2% uranyl acetate in 50% ethanol. Samples were then dehydrated in a graded ethanol series, pre-infiltrated with propylene oxide, and embedded in Spurr's epoxy. Using an RMC MT-7000 ultramicrotome and a diamond knife, ultrathin sections (60–80 nm) were cut to display planes perpendicular to the x, y, and z directions, referred to as the x, y, and z sections. Sections were post-stained with 3% uranyl acetate and 2% lead citrate, and examined and photographed using a JOEL JEM-1210 TEM at 90 kV.

Scanning electron microscopy

Samples for scanning electron microscopy were cut with a razor blade to expose x and y sections of the sheet. Samples were critical point dried (E3000, Energy Beam Sciences, Inc., East Granby, CT), sputter coated with gold (Emscope SC-500, Emitech, Kent, England), and examined and photographed with a DS-150 F scanning electron microscope (Topcon Co., Tokyo, Japan) operated at 15 kV.

Uniaxial tensile tests

The effect of fiber orientation and volume fraction on mechanical behavior was evaluated under uniaxial tension. Conditioning and tensile analysis were conducted on a dynamic mechanical thermal analyzer DMTA V (Rheometric Scientific, Inc., Newcastle, DE) with a 15 N load cell in the inverted orientation, so that samples could be immersed in a jacketed beaker of 37 °C PBS. For each fiber layout, 5 to 7 replicates were tested. Samples 4 mm in width and 0.4 mm in thickness were mounted with gauge lengths of 12 to 13 mm. Force was applied in the reference direction, *f*. Samples were extended to 8% strain for 16 cycles and then to 30% strain. Samples that did not fail when stretched to 30% were remounted on a

miniature materials tester, the Minimat 2000 (Rheometric Scientific Inc., Newcastle, DE), and tested to failure. All tests were performed at a rate of 5 mm/min. For each design, resilience was calculated from the 8% strain data by dividing the area beneath the unloading curve by the area beneath the loading curve and multiplying by 100%, and reported as the mean and standard deviation. To characterize fiber failure modes, samples were treated with Van Gieson's stain and photographed after failure.

Suture retention strength

Test samples had a fiber orientation of 15° and volume fraction of 17%. Sutures (Prolene 4-0) were passed through 4 mm square samples, 2 mm from the sheet edge and the suture fastened to the actuating arm of the DMTA and pulled at a rate of 1 mm/sec. The maximum force measured before the suture tore out of the sheet was recorded as the suture retention strength, reported in grams-force (g-f). For seven samples, the suture was pulled perpendicular to the reference direction, and for four samples the suture was pulled in the reference direction.

Ventral hernia repair model

Full-thickness abdominal wall defects in 8 wk old male Wistar rats were repaired with a protein polymer composite patch (n=5) or a commercially available biologic implant (Permacol™, crosslinked porcine dermal collagen, n=3) as approved by the Emory University Institutional Animal Care and Use Committee. Anesthesia was induced and maintained with isoflurane (2.5% and 1.5%, respectively) inhalation. A 3-cm vertical midline incision centered between the xiphoid and the pubis was created and skin was decollated from the muscular and fascial layers. A 1.5 × 2.0 cm rectangular full-thickness ventral abdominal wall defect consisting of muscles, fascia, and peritoneum was created and repaired with the implant materials using an onlay technique. Animals were monitored continuously for 1 to 2 hours postoperatively and then daily for the course of the study for signs of hernia recurrence or infection.

Specimens were retrieved after 8 weeks for histological analysis, measurement of implant dimensions, evaluation of adhesions, and strength of the host-implant interface. Adhesions were characterized by documenting the visceral tissues involved, and grading tenacity graded on a 0 to III scale (0 = represents no adhesions; I = adhesions that can be easily separated; II = mild adhesions that are more difficult to separate; and III = dense adhesions which can only be surgically divided [28]). Changes to implant size were determined by comparing area measurements obtained from in situ photographs acquired immediately after implant and before retrieving the material. Histology specimens were fixed overnight in 10% neutral buffered formalin and processed for paraffin embedding. Five-micron sections were stained with hematoxylin and eosin to distinguish tissue morphology. Strength of the host-patch interface was recorded by mounting strips of the bioprosthetic and neighboring tissue (4 × 20 mm) in the grips of a mechanical tester (DMTA V, Rheometric Scientific) so that one grip contained the implant and the other contained abdominal wall tissue. The strips were pulled to failure and the failure tension (force divided by sample width) was recorded.

Results

Structural analysis of a protein polymer composite

The fabrication scheme outlined in Figure 1 resulted in consistent production of flat sheets of recombinant elastin matrix reinforced with collagen microfibers oriented in an angle-ply format. Digital volumetric imaging (DVI) revealed the multilamellar fiber structure while scanning electron microscopy (SEM) demonstrated that composites were uniformly bonded without evidence of voids or delamination between individual 40 μm lamellae within each

sheet (Figure 2a,b). Transmission electron microscopy revealed that fibers were comprised of axially aligned, D-periodic fibrils resembling native collagen (Figure 2c,d). Observed fiber orientations, approximately 0° , $\pm 13^\circ$, and $\pm 22.5^\circ$ relative to a reference direction, were close to expected values (Table 1). Collagen fiber volume fraction ranged from 0 to 18%, calculated from spacing measurements by assuming an average fiber diameter of $40\text{ }\mu\text{m}$ a sheet thickness of $400\text{ }\mu\text{m}$, and the presence of eight fiber-reinforced layers in the composite sheet.

Uniaxial mechanical responses of composite sheets

Fiber orientation and volume fraction had a dramatic impact on mechanical responses (Figure 3), including Young's modulus, ultimate tensile stress, resilience, and stress at fiber damage (Figure 4, Table 1). Significantly, composite sheets were produced with properties that ranged over 13-fold in elongation to break (23 – 314%), six-fold in Young's modulus (5.3 – 33.1 MPa), and more than two-fold in tensile strength (1.85 – 4.08 MPa). Both fiber fraction and fiber orientation dictated the mode of tensile failure (Figure S1). The ability to tailor structure, such that observed mechanical responses approximated those of native tissues was demonstrated by the design of sheets whose mechanical behavior matched that of human linea alba (Figure 3c) [29]. Notably, the capacity to achieve substantial suture retention strengths, comparable to that of native tissues, was observed. As an example, suture retention strengths, parallel and perpendicular to the predominant fiber axis, were $170 \pm 36\text{ gf}$ and $124 \pm 8\text{ gf}$, respectively, for a composite containing fibers oriented at 15° and a fiber volume fraction of 18%.

Abdominal wall repair with engineered protein fiber composites

Wistar rats underwent repair of a $1.5 \times 2.0\text{ cm}$ full thickness abdominal wall defect with either an engineered implant or decellularized, hexamethylene diisocyanate (HMDI)-crosslinked porcine dermis (PermaCol™). During the 8-week implant period, evidence of a recurrent abdominal wall defect, overlying skin ulceration, or bowel obstruction was not observed. Grossly, both patch materials appeared to have supported native tissue in-growth and firmly integrated with the abdominal wall (Figure 5). Although no outward bulging was observed, perimeter measurements revealed area increases of $78 \pm 53\%$ ($n=5$) and $72 \pm 53\%$ ($n=3$) for engineered composites and porcine dermal patches, respectively ($p=0.89$). Omental adhesions requiring sharp separation (grade III) were noted for all implants, although no other tissues or organs were involved. Respective tensile strengths of integration were similar for engineered composites ($0.62 \pm 0.23\text{ N/mm}$, $n=8$) and porcine tissue-derived patches ($0.88 \pm 0.17\text{ N/mm}$, $n=4$), respectively ($p=0.07$). Upon tensile testing of explanted specimens, samples failed either within the patch itself or the abdominal muscle rather than at the tissue-material interface. Histological analysis of the harvested porcine dermis revealed collagen fragmentation, with cells and host repair tissue infiltrating between and surrounding the fragments. In many fields, fragments of the dermal collagen implant could not be discerned (Figure 6). Histological examination of the engineered composite revealed substantial replacement of the elastin-like protein polymer by infiltrating tissue, while collagen fibers remained apparent throughout all specimens (Figure 6).

DISCUSSION

Structural features of collagen-elastin composites

Aqueous solutions of the triblock elastin-mimetic protein polymer are capable of a sol-gel transition, which permits incorporation of a fiber layout into a single $40\text{ }\mu\text{m}$ thick membrane and subsequent bonding of a multilamellar stack. The fiber layouts consisted of closely spaced fibers with two predominate orientation angles, resembling several native tissues including the linea alba of the anterior abdominal wall [30], small intestinal submucosa [31,

32], and the annulus fibrosus of intervertebral discs [6]. The laminated geometry offers the potential to incorporate living cells at controlled spatial intervals throughout a stacked sheet, akin to cell sheet tissue engineering methods [33, 34]. Significantly, the use of a recombinant protein matrix affords the engineering of protein-based composites with a large volume fraction of elastomeric protein. In contrast, the generation of tissue-engineered constructs containing significant fractions of elastin produced by cultured cells has presented a challenge, although recent improvements have been reported [12, 35]. Other biomaterials incorporating elastin include devitalized extracellular matrices of collagen and elastin, or elastin alone, from tissues such as dermis, vessel wall, or heart valve leaflet. Collagen-elastin composites are distinguished from these biologics by the opportunity to dictate fiber orientation and volume fraction, as well as to select from several of elastin-like protein polymers with a range of mechanical properties [16]. Moreover, the variability in thickness, fiber organization, and mechanical performance reported for some donor tissues [36] may be avoided in engineered collagen-elastin composites. Indeed, control is achieved over a wide range of protein-based scaffold properties, including compliance, resilience, strength, and anisotropy, not typically provided by the assembly of cultured cell sheets. Moreover, the need for weeks of cell culture to generate a robust collagen layer, which is necessary for the manipulation of cell sheets, is avoided.

High-resolution photographs of collagen fiber layouts indicated that the rotational and translational velocities of the fiber winding apparatus dictated fiber spacing and orientation. Fast-Fourier transform analysis revealed close correlation between nominal fiber angles of 15° and 25° and measured angles of $13.4 \pm 0.9^\circ$ and $22.4 \pm 1.3^\circ$ (mean $\pm$ standard deviation), consistent with limited displacement of fibers as they were tensioned onto the frame. Preservation of uniform fiber orientation and density throughout the stacked sheet was demonstrated by analysis of stained composite structures after fiber winding, embedding, and lamination. Moreover, a cohesive multilamellar structure was obtained following a mild temperature transition process, suggesting that growth factors or other bioactive agents could be easily accommodated in distinctly tailored hierarchically organized laminated structures. Microscopic analysis also displayed preservation of the aligned, D-periodic banded fibrillar collagen ultrastructure following composite fabrication. Assembly of collagen monomers into fibrils is a critical factor in optimization of fiber strength, biostability, and cell signaling responses [37–40].

Mechanics and microfiber arrangement

An angle-ply composite design was selected because flexible fiber-reinforced composites display advanced mechanical properties, such as an extensive usable deformation range and the opportunity to engineer mechanical anisotropy, Young's modulus, and nonlinear mechanical responses [41, 42]. In the present study, adjustment of fiber orientation and volume fraction exerted several mechanical effects with the capacity to design sheets with a broad range of elongation to break, Young's modulus, and tensile strength (Figure 4, Table 1). In particular, the ultimate tensile strengths of several designs exceeded those observed for several native human tissues (urinary bladder, 0.27 ± 0.14 MPa; pulmonary artery, 0.39 ± 0.05 MPa; and aorta, 1.72 ± 0.89 MPa; mean $\pm$ SD) [43–45]. In addition, a fiber reinforcement pattern could be designed so as to match the tensile response reported for human linea alba (Figure 3c). Repair materials that match abdominal wall compliance may reduce patient discomfort, often attributed to restricted abdominal wall mobility following mesh implantation [46].

Mechanical responses for composite sheets of similar collagen content but designed with varying fiber orientation demonstrate the effect of adjusting fiber direction. As expected, stiffness increased with decreasing fiber angle relative to the applied load (Figure 3a). In addition, damage to the collagen fiber network, visible as an abrupt decrease in measured

stress, occurred at stresses and strains related to the fiber orientation. Specifically, damage occurred at stresses of ~ 3 MPa and strains of $12 \pm 1\%$ and $14 \pm 1\%$ for composites comprised of fibers at 0° and 15° , respectively. In contrast, sheets designed with a fiber orientation of 25° displayed damage at higher strain ($19 \pm 2\%$) in response to a lower imposed stress of ~ 2 MPa, while sheets containing fibers at 90° and those sheets without fiber did not display signs of failure or damage in response to 30% strain. Resilience was high for all fiber orientations except for those sheets in which fibers were oriented perpendicular to the loading direction (Figure 4). In summary, designs in which fibers were more closely aligned to the loading direction tended to display a greater degree of stiffness and tensile strength, but with fiber damage evident at lower levels of strain. Collagen fibers contributed to elastic energy storage, even when oriented at 25° with respect to the applied load. Likewise, at a fixed fiber orientation of 15° , resilience, Young's modulus, and stress at fiber damage, all increased with additional fiber content. Increasing fiber volume fraction enhanced ultimate tensile strength, however, these differences were not substantial.

Abdominal wall repair

Recombinant proteins derived from elastin sequences have been investigated for potential applications in drug delivery [47–49], tissue engineering [50, 51], or as constituents of implanted medical devices [52, 53]. The addition of reinforcing collagen fibers extends the range of mechanical properties of elastomeric protein polymer composites with broader suitability as scaffolds engineered as load bearing soft tissues, such as the abdominal wall or arteries [20]. As noted, fiber reinforced composites could be designed with properties that matched the linea alba and were readily suturable. Significantly, these composites provided excellent mechanical support for a full thickness abdominal wall defect, even though they were 60% thinner than a commercially available patch derived from decellularized porcine dermis (0.4 vs. 1 mm). Moreover, in contrast to the collagen component of the engineered composite, substantial degradation of the HMDI-crosslinked porcine dermal collagen was observed over the same implant period; as reported by others [54]. Greater persistence of the collagen fibers in the engineered composites may be attributed to the dense, aligned fibrillar substructure [19], along with more extensive glutaraldehyde crosslinking in comparison to the dermal collagen. A limitation of the composites was the known cytotoxicity of glutaraldehyde, employed here as a model crosslinking method. A detailed investigation of alternative crosslinkers and degrees of crosslinking applied to protein polymer implants was beyond the reach of this study. Feasible strategies include alternative non-cytotoxic crosslinkers, limiting crosslinking to the collagen fiber array alone, or to a select subset of lamellae. Other modifications to these materials are also likely to improve performance. For example, the protein polymer was not expected to display healing effects such as progenitor cell recruitment, attributed to bioactive components innate to decellularized tissue products [55]. However, the fabrication scheme presents opportunities for the introduction of bioactive compounds in future studies.

A range of degradation behavior has also been reported for elastin-like polymers. Protein polymers based on (GVGVP) and (GEGVP) sequences have persisted for up to 6 months in vivo, depending upon the type of chemical modification [56, 57]. Likewise, sheets of radiation crosslinked poly(GVGVP) were reported stable for 6 months when implanted in the abdominal cavity of rats [58]. We have previously observed that the same protein polymer used in this study elicited minimal inflammatory activity over a three week period when implanted in murine subcutaneous and intraperitoneal spaces [23]. Moreover, when a similar triblock copolymer was implanted subcutaneously in mice, a high degree of stability was observed for periods of up to 1 year, despite the absence of chemical crosslinking [15].

Conclusions

The development of ECM protein analogs and related technologies for fabricating three-dimensional matrices with ordered structure represent important steps for enhanced the design of both non-living and tissue-engineered implants. Towards this goal, we applied an angle-ply composite design scheme for the fabrication of fiber-reinforced flexible protein composites for soft tissue repair. Collagen microfibers were embedded at controlled orientations and densities in 40 μm thick elastin-like protein membranes that were effectively laminated by a temperature transition process. As a result, composite sheets with tailored mechanical behavior, including Young's modulus, yield strength, resilience, and suture retention strength were created. As a case in point, a composite displaying mechanical responses similar to abdominal wall fascia was designed and investigated in a model of abdominal wall repair. Tissue repair patches were well tolerated, prevented recurrent defect formation, and permitted cell and tissue infiltration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Fabrication of a collagen microfiber reinforced elastin-like protein sheet. (a) Collagen microfiber is wound about rectangular frames to obtain the desired orientation and average spacing. (b) A cooled protein polymer solution is distributed over the microfiber layout and molded into a thin membrane through a temperature-drive sol gel process. (c) Stacked membranes are laminated by a temperature transition to yield multilamellar, angle-ply composites (d, e).

Figure 2.

Structural analysis of a multilamellar sheet. As illustrated in (a), the fiber cross-section will appear circular or elliptical when examined by SEM after sectioning along the x- or y-plane (c and d, respectively). Sheets did not display evidence of delamination (b, c, d). Sectioning artifacts appeared in (c) as vertical microgrooves and in (d) as feathered horizontal ridges in the protein polymer, indicated by arrows. Three-dimensional reconstructions using the DVI technique display the fiber component in (e). Transmission electron microscopy sections taken in the plane of the collagen fibers demonstrated a D-periodic banded fibrillar structure, aligned with the overall fiber axis (f), while sections perpendicular to the collagen fiber revealed densely packed fibril cross sections (g). Analysis of collagen fiber by TEM has demonstrated a mean diameter of 33 ± 4 nm and a fibril density of $73 \pm 6\%$ (v/v) [19].

Figure 3. Mechanical responses. (a) Composites with fiber angles of 0° (●), 15° (●●), 25° (---), 90° (—), and without fiber (○) demonstrate microfiber alignment with loading enhances stiffness and decreased strain at fiber damage. (b) Designs with 15° orientation and average microfiber fractions of 18% (●), 7% (---), and 3% (○), and without fiber (—) indicate that increasing microfiber content elevates stiffness and strength without changing strain at fiber damage. (c) The mechanical response of the 25° orientation (—) resembled human linea alba. (Symbols represent tissue strips from the infraumbilical or supraumbilical region tested in the oblique or transverse orientations, averages of responses reported by Gräfel and

coworkers [29]. (d, e) The 0° and 15° composites after microfiber staining, images oriented with the loading direction horizontal (scale 2 mm).

Figure 4.

Dependence of mechanical properties on microfiber layout. Increased fiber fraction and alignment to the loading direction increased modulus (a, e). Increased fiber fraction and alignment also tended to increase the stress at which specimens displayed fiber network damage (b, f). Increased fiber fraction did not significantly enhance UTS, but alignment of fibers in the loading direction enhanced UTS compared to higher fiber angles (c, g). Resilience increased with increasing fiber fraction (d), and was also elevated when fibers were aligned or angled to the loading direction compared to the perpendicular layout (h). Significance indicated at the $p < 0.05$ level, ns indicates non-significant differences, error bars represent standard deviations.

Figure 5. Abdominal defect repair. Appearance of the multilamellar elastin-composite immediately following implant (a) and at 8 weeks (b). None of the repaired defects demonstrated hernia formation for the duration of the study (c), as compared to unrepaired defects (d). Scale 10 mm.

Figure 6.

Histology of abdominal repair materials. The appearance of non-implanted multilamellar protein composite sheets and the porcine dermis product is shown in (a) and (d), respectively (100 $\times$). After 8 weeks, the elastin-like protein component of the engineered composites appeared largely absent, except in rare areas (b, c). In contrast, collagen microfibrils were observable throughout the specimens (b, 40 $\times$, elastin-like protein and collagen fiber indicated with solid and dashed arrows, respectively). In regions where cells and fibrous tissue replaced the elastin-like protein, the spacing between collagen fibers increased (c, 100 $\times$). The dense collagen of the porcine dermis product appeared to have separated, with cell and tissue ingrowth between implant fragments (right side of e, 40 $\times$, and in g, 100 $\times$). The arrows in (g) indicate implant fragments. In (e) the host-implant interface is apparent, with the abdominal wall at left in and the porcine dermis at right. Identifiable fragments of the porcine dermal product were absent from many regions of the harvested patches (f, 200 $\times$). Scale bars 200 μ m.

Table 1

Structural and Mechanical Properties of Collagen-Elastin Composite Designs

Design	Volume Fraction (%)	Fiber Orientation (°)	Young's Modulus (MPa)	Stress at Fiber Damage (MPa) [a]	Strain at Fiber Damage (%) [a]	UTS (MPa)	Resilience (%)	Strain to failure (%)	n
1	16.3 ± 0.6	0.8 ± 3.7	33.1 ± 3.9	2.87 ± 0.56	12 ± 1	3.62 ± 1.07	76.1 ± 2.9	31 ± 10	5
2	16.7 ± 0.5	90.8 ± 1.4	5.3 ± 0.6	-	-	1.99 ± 0.28	50.8 ± 0.6	184 ± 38	5
3	17.7 ± 0.8	13.4 ± 0.9	26.0 ± 4.1	2.58 ± 0.37	14 ± 1	2.66 ± 0.72	75.8 ± 2.0	23 ± 3	6
4	6.8 ± 0.3	13.2 ± 0.8	20.9 ± 2.0	1.87 ± 0.34	13 ± 2	2.41 ± 0.17	71.9 ± 2.1	47 ± 7	6
5	3.1 ± 0.1	13.0 ± 0.9	13.9 ± 1.36	1.31 ± 0.19	12 ± 1	1.85 ± 0.66	66.8 ± 1.4	182 ± 135	7
6	0	-	6.1 ± 0.7	-	-	4.08 ± 0.80	53.1 ± 1.4	314 ± 26	5
7	16.6 ± 1.5	22.4 ± 1.3	13.5 ± 1.5	1.90 ± 0.24	19 ± 2	1.98 ± 0.27	78.8 ± 2.7	21 ± 1	5

[a] Designs without fiber (6) or with fibers oriented perpendicular to loading (2) did not display abrupt fiber damage points.