Published in final edited form as:

Acta Biomater. 2014 April ; 10(4): 1532–1541. doi:10.1016/j.actbio.2013.08.003.

Tropoelastin - a versatile, bioactive assembly module

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Abstract

Elastin provides structural integrity, biological cues and persistent elasticity to a range of important tissues including the vasculature and lungs. Its critical importance to normal physiology makes it a desirable component of biomaterials that seek to repair or replace these tissues. The recent availability of large quantities of the highly purified elastin monomer, tropoelastin, have allowed for a thorough characterization of the mechanical and biological mechanisms underpinning the benefits of mature elastin. While tropoelastin is a flexible molecule, a combination of optical and structural analyses has defined key regions of the molecule that directly contribute to the elastomeric properties and control the cell interactions of the protein. Insights into the structure and behavior of tropoelastin have translated into increasingly sophisticated elastin-like biomaterials, evolving from classically manufactured hydrogels and fibers to new forms, stabilized in the absence of incorporated cross-linkers. Tropoelastin is also compatible with synthetic and natural co-polymers, expanding the applications of its potential use beyond traditional elastin-rich tissues and facilitating finer control of biomaterial properties and the design of next-generation tailored bioactive materials.

Keywords

tropoelastin; elastin; structure; elasticity; biomaterials

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The authors declare no conflict of interest in this work.

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Introduction

Elastin is the dominant component of mature elastic fibers, which provide strength and persistent elasticity to a range of important tissues. Its structural and cell signaling roles have classically been recognized within large blood vessels, lungs and the skin [1], making elastin mimicry sought after for biomaterials applications and tissue repair. Elastin-derived materials have shown great promise, but continue to evolve as more is understood about the drivers of elasticity, details of structural features, the elastin assembly process and the capacity for hybrid material formation. These insights, in combination with integrated design approaches [2] promise the ability to tailor constructs, carefully matching both the physical and biological requirements of each elastin-rich tissue to be replaced.

The soluble monomer of elastin is tropoelastin (TE), highly expressed during development, but in adults only in response to injury. The elastin gene encodes a ~72 kDa polypeptide and, depending on alternative splicing and protein maturation yields a mature protein of at least 60 kDa. A dominant feature of the tropoelastin amino acid sequence is its well-characterized alternating hydrophobic and hydrophilic domain structure. Hydrophobic regions of tropoelastin feature non-polar amino acids such as glycine, valine, proline and leucine, [3] often arranged in repetitive motifs, the most common being the repeating sequence VGVAPG [4]. Overall, hydrophobic residues account for 82% of the primary sequence [5]. The hydrophilic domains are rich in lysine and alanine, which participate in cross-linking.

Large quantities of highly purified tropoelastin have only become available relatively recently. Isolation of the monomer directly from *in vivo* sources is difficult and inefficient, due to the speed at which extracellular, expressed tropoelastin is cross-linked and incorporated into the growing elastic fiber [6]. Recombinant expression in an *E. coli* bacterial system [7] and subsequent optimization of a synthetic elastin gene [8] has allowed for the production of a recombinant tropoelastin that is identical to the naturally secreted human form, displaying assembly and coacervation properties expected for native elastin [9].

Access to tropoelastin has facilitated rapid progress in understanding its structure, cell signaling and assembly properties. Techniques previously incompatible with mature insoluble elastin have now been employed to probe the monomer and better understand its relationship to elastin and the elastic fiber. As more is learned, there is a growing appreciation of the relationship between the shape, flexibility and cell interactive regions of the monomer, how these translate in properties of elastin and how these can be manipulated for the production of biomaterials. While this review will focus on TE-based materials, better knowledge of the TE monomer has also enhanced the utility of recombinant elastin-like protein polymers for tissue engineering applications, as recently described [10].

1. Fundamental Understanding of Tropoelastin

1.1 Structural Features

The recent availability of pure, full-length tropoelastin necessitated that the earliest structural insights were from elastin derivatives. Infrared absorption and circular dichroism (CD) studies on α -elastin, a heterogeneous product derived from acid hydrolysis of elastin [11], indicated that solubilized elastin has a predominantly disordered structure. Nuclear magnetic resonance studies reveal that the majority of backbone carbonyl carbon atoms in elastin-based polypeptides are highly mobile [12]. Sequence-based structure prediction algorithms likewise estimated approximately 75% disorder in the structure of the tropoelastin monomer [13]. Subsequent CD and Raman spectroscopy studies carried out

directly on recombinant tropoelastin and elastin-like peptides also confirm a large proportion of highly disordered regions, which are attributed to the hydrophobic domains [13, 14].

The abundance of disordered structures within tropoelastin, defined as a preferential adoption of transient local structural motifs over stably-bonded secondary structures, is manifested in the intrinsic flexibility of the molecule [15]. Elastin-based polypeptides alternate between different conformations in equilibrium, giving rise to a conformational ensemble that includes β -turns, β -strands and polyproline II (PPII) structures [16–19]. PPII structures are defined by an absence of intramolecular and intermolecular hydrogen bonds and can therefore interconvert between conformations [17].

In a hydrated environment, tropoelastin presents a highly flexible form [13]. The structural dynamics of the elastin backbone is mainly achieved by the proportion of rigid proline and flexible glycine residues [20]. Steric constraints imposed by proline and the entropic disadvantage of glycine confinement determine the possible backbone conformations [15, 21]. The placement of recurring proline and glycine residues promotes the formation of labile β -type turns, which contribute significantly to flexibility within the protein [22]. Accordingly, skewing the ratio of proline to glycine in elastic-like polypeptides away from the natural sequence promotes the formation of β -structure-rich amyloid-like fibrils, which show a greater degree of conformational restriction than the native elastin structure [21].

In addition to PPII and other labile structures, the hydrophobic repeat units in tropoelastin also form more compact motifs such as short β -sheets or β -turns [14, 23–25]. In contrast, the hydrophilic domains are associated with a low content of α -helices in the free protein [25–28]. The presence of independent clusters of structure in tropoelastin is consistent with the gradual unfolding transition observed upon urea treatment [13].

Obtaining a definitive high resolution tertiary structure of tropoelastin has been hindered by its inability to form resolvable crystals. In addition, the insoluble nature of polymerized elastin precludes the use of classical spectroscopic techniques [29]. Nevertheless, the binding of hydrophobic probes to tropoelastin and the ability of hydrophobic sequences to be cleaved in protease mapping experiments indicate the presence of surface-exposed hydrophobic regions, suggesting that tropoelastin hydrophobic domains are not restricted to the core as is expected for folded globular proteins [13, 30]. Furthermore, a portion of hydrophobic regions remain partially accessible in cross-linked elastin [31].

Small angle X-ray and neutron scattering analyses of recombinant human tropoelastin in solution revealed an extended asymmetrical shape with a long narrow rod from one terminus that branches into a larger region at the other terminus [32]. By comparing the solution structures of full-length tropoelastin and mutant isoforms truncated at various lengths from the N-terminus, specific domains were assigned to distinct spatial locations within the model. An elongated coil region encompasses the N-terminus to domain 18, and leads downstream to a spur containing domains 20–24 that corresponds to a predicted hinge region [33, 34]. The model shows a protrusion around domains 25–26 that is assigned to a bridge region, which connects to an open foot region at the C-terminus (Figure 1A).

The uniqueness of the tropoelastin structure directly accounts for the elastomeric properties of the protein [29, 35, 36]. The flexibility of the polypeptide chains contributes to the high entropy of the relaxed state. When elastin is stretched in an aqueous environment, the hydrophobic sequences, most likely those in the coil region [32], become more exposed to the solvent and constrain the local order of water molecules [14]. This decrease in entropy acts as a driving force for spontaneous elastic recoil back to the native disordered state [37–39].

The N-terminal Region—The solution structure of human tropoelastin comprises a distinct narrow (2–3 nm), elongated segment that persists for ~11 nm before branching into the bridge and foot regions [32]. Structural comparison of the full-length species with constructs truncated at various distances from the N-terminus allowed the assignment of this linear section to the tropoelastin N-terminal region encompassing domains 2–18. The spring-like coil region is proposed to account for tropoelastin elasticity, which is characterized by the ability of the single molecule to reversibly extend to 8 times its resting length [32]. The N-terminal region displays the same structural motif organization as in the full length molecule, comprising alternating hydrophobic and hydrophilic domains [40]. Mechanistically, the elasticity of tropoelastin molecules arises from the substantial entropy associated with the flexibility of hydrophobic domains in the relaxed state [38, 39]. Extending the monomer increases order by restricting the range of accessible conformations [41]. Furthermore, the increased solvent exposure of hydrophobic regions induces local ordering of water molecules and contributes to a decrease in entropy. The spontaneous recoil manifested by tropoelastin after release of the stretching force is driven by the return to a more favorable disordered state [37].

Preceding studies on the functional significance of specific tropoelastin domains in elastogenesis primarily implicated the central and C-terminal regions in the coacervation, microfibrillar deposition and cross-linking stages of elastic fiber assembly [30, 42–45]. However, the N-terminal region may also play important but less well-characterized roles in elastin assembly. For instance, it likely contributes to the overall structure of the coacervate, as evidenced by the conformational transition displayed by the isolated domain 2–7 peptide from β strand to α -helix upon an increase in ambient hydrophobicity [40]. The N-terminal segment may also facilitate tropoelastin integration into the microfibrillar scaffold. While fibrillin-1, the most abundant microfibrillar protein, binds multiple sites within tropoelastin, its primary point of contact is localized within the N-terminal domain 4 of tropoelastin [46]. Several N-terminal domains may also participate in cross-linking. At least three intramolecular lysinonorleucine links have been observed in the region spanning domains 6–15, including contacts between domains 6 and 8, domains 12 and 13, and domains 14 and 15 [9]. Intermolecular cross-links may likewise exist between the N-terminal region of one monomer and the central or C-terminal region(s) of another, supporting an end-to-tail model of the initial phase of assembly (Figure 1B). In particular, interactions are proposed to occur between domains 6 and 36, domains 8 and 23, domains 12 and 21/23, domains 13 and 19, domains 13 and 23, and domains 15 and 23 [43]. A major cross-linking site in elastin is formed through the association of domains 10, 19 and 25, in which the domain 10 sequence of one tropoelastin strand bridges the desmosine-linked domains 19 and 25 of a second molecule via two lysinonorleucine links [47]. The importance of the tropoelastin N-terminal region in elastogenesis is supported by the association of a P211S mutation in domain 12 with an autosomal recessive cutis laxa phenotype [48] that is characterized by deficient dermal elastic fibers [49, 50].

The Hinge Region—Exon 22 codes for a short hydrophobic sequence, GAAGAGVLGGLVPGAPGAVPG VPGTGGVP, in the central region of tropoelastin [42]. Exclusion of domain 22 interrupts the highly-conserved structure of alternating hydrophobic and hydrophilic domains in tropoelastin [51]. The consequently adjoining hydrophilic domains 21 and 23 contain a central GVGTP peptide, which is computationally predicted to form a flexible turn or hinge region [34]. The presence of a tropoelastin hinge was subsequently empirically confirmed by small angle x-ray scattering [52] and nuclear magnetic resonance analyses [35].

The hinge sequence is unstructured with neither alpha-helical nor beta-sheet content [34, 35, 53] and is therefore postulated to contribute substantially to the structural flexibility of the

molecule [54]. In support of this proposal, molecular dynamic studies have demonstrated that the hinge region can adopt a closed conformation in which the flanking helical segments are in an anti-parallel arrangement, or an open structure where the neighboring regions are parallel and extended [33]. The hinge region is proposed to oscillate between these models before converging into a closed hairpin. This conformational equilibration is consistent with the broad spatial distribution accessed by elastin peptides [55] and the significant motions exhibited by hydrated elastin [56, 57]. Accordingly, an elastin-like peptide corresponding to domains 21/23 displays a more rigid structure and less mobility upon alanine substitution of the central turn sequence [58]. These findings support an involvement of the hinge region in tropoelastin elasticity.

The conformational flexibility of the hinge region appears to be intricately linked with tropoelastin function during elastic fiber assembly. The hinge structure may contribute to the position and dynamics of hydrophobic regions involved in tropoelastin association by coacervation [58]. The hinge also lies within a stretch of domains enriched in cross-linking sites [43, 59]. The geometry adopted by this region is proposed to enable inter- and intra-molecular contacts between lysine residues, allowing spontaneous condensation into cross-links upon modification by lysyl oxidase [54].

The Bridge Region—The hinge region leads to a bridge region that most likely encompasses domains 25 and 26. Unlike the N-terminal elastic spiral and C-terminal cell-interactive foot regions of tropoelastin (*vide infra*), little has been described for the functional role of the bridge region. One model proposes that the bridge region may be involved in elastic fiber assembly, as it includes domain 26 which is essential for coacervation and lies near other large central hydrophobic domains implicated in the process [30, 60]. The bridge region also either partially encompasses or resides proximally to domains 19–25 which dominate intermolecular cross-linking to form elastin [42, 43, 47]. Structurally, the bridge region directly leads from a region of high flexibility [54] and may therefore contribute to the extensive conformational changes associated with monomer alignment and organization during elastin assembly [27].

Preceding work presented a protease-resistant tropoelastin construct in which the bridge region was disrupted by substituting the R515 residue with an alanine (R515A) [61]. When compared to mature, full-length human tropoelastin, the R515A mutant displays less efficient coacervation, impaired hydrogel formation and decreased dermal fibroblast attachment compared to wild-type tropoelastin. R515A mutant tropoelastin additionally shows reduced elastic fiber formation upon addition to human retinal pigmented epithelium cells and dermal fibroblasts. Furthermore, the SAXS nanostructure of the R515A mutant protein reveals increased conformational flexibility around the bridge region and C-terminal regions. These findings point to a role for the tropoelastin R515 residue in stabilizing the structure of the bridge region, which is critical for elastic fiber assembly.

1.2 Cellular Interactions

In addition to innate flexibility and elasticity, tropoelastin has key cell signaling and adhesion properties. Cellular interactions with tropoelastin are wide ranging, influencing normal cellular physiology as well as those associated with the progression of pathologies [62]. Tropoelastin interacts with surrounding cell types, affecting chemotaxis, morphogenesis, proliferation, ion transport and actin fiber formation [63–65]. As is characteristic of ECM proteins, elastin-mediated interactions are predominantly adhesion based, involving elastin-receptor binding interactions. Three main classes of receptors have been implicated in elastin-cell binding: the elastin binding protein (EBP), integrins and glycosaminoglycans (GAGs). These receptors have been reviewed in detail previously [66].

Originally thought to be predominantly an adhesive receptor involved in the binding of tropoelastin to the cell, the EBP is now primarily associated with detection of elastin fragments following elastic fiber damage [67]. This new assignment is consistent with β -galactosidase knockout mice, which lack the gene encoding EBP yet display correctly deposited elastin [68]. Full-length tropoelastin has also repeatedly been shown to support cell attachment even when EBP is inhibited [69]. These findings in turn have increased the emphasis on GAGs and integrins in the study of tropoelastin-cell interactions.

The C-terminus of tropoelastin is highly conserved across species, terminates with a charged RKRK sequence and contains the only two cysteine residues in the molecule which are closely spaced and combine to form an intramolecular disulfide bond. These features were early pointers to its functional significance: initially related to assembly, disruption of the disulfide dramatically reduces the ability of tropoelastin to incorporate into fibers, and its removal abolishes fiber incorporation [70]. With respect to cell binding, a GAG mediated interaction was first identified for fetal bovine chondrocytes, which bind to domains 15–36 of TE, but not 15–29, indicating the necessity of the C-terminus. This binding interaction was further narrowed to the last 17 C-terminal amino acids of tropoelastin, with the last 25 residues required for full binding [69].

To narrow down this interaction using human dermal fibroblasts, studies using synthetic peptides of the last 12 amino acids of tropoelastin (ACLGKACGRKRK) and a shorter peptide, without the last four amino acids (ACLGKACG) showed the longer version competitively inhibiting cell adhesion [71]. Direct, dose dependent adhesion was subsequently shown for domain 36, and finally for a GRKRK peptide sequence, representing only the last 5 amino acids of TE (Figure 1C). This interaction was found to be integrin mediated, with the majority of binding through the $\alpha_v\beta_3$ integrin.

This detailed understanding of fibroblast binding to TE has been translated to surface functionality. Tropoelastin immobilized on polystyrene increase human dermal fibroblast cell spreading by 80% relative to uncoated controls [72]. However, similarly treated polytetrafluorethylene (PTFE), which is made substantially more hydrophilic when treated for binding, attracts and buries the C-terminus of TE, and so reduces fibroblast attachment in a targeted manner [73]. The known binding mechanism due to tropoelastin's orientation on these surfaces thus allows for fine control of cell interaction and surface patterning.

Fibroblasts are also induced to produce transient pro-inflammatory molecules when grown on tropoelastin-coated surfaces, indicating an early signaling role in addition to facilitating attachment. Expression of the matrix metalloelastase MMP-12, which is normally expressed by macrophages, is dramatically increased 1056 ± 286 fold by 6 hr, persisting for 24 hr. Chemokines including CXCL8 (interleukin-8), CXCL1 and CXCL5 also increase 11.8 ± 2.6 , 10.2 ± 0.4 and 8593 ± 996 fold by 6–12 hr, before decreasing to baseline expression levels. This response is mediated predominantly by the EBP, via a protein kinase A (PKA) pathway. A parallel, substantial contribution to signaling via the integrin $\alpha_v\beta_3$ was also observed [74].

Endothelial cell attachment and proliferation is also enhanced on tropoelastin-coated surfaces, though the binding site and mechanism are less well elucidated. Human umbilical vein endothelial cell (HUVEC) attachment to TE is comparable to fibronectin (FN). Proliferation of HUVECs on TE coated surfaces is also increased $54.7 \pm 1.1\%$ at day 3 and $22.8 \pm 1.1\%$ at day 5, and again the same as on FN [75]. Translated to stainless steel surfaces, TE coating increases HUVEC attachment $86.3 \pm 10.5\%$ and significantly improves endothelial cell proliferation by $36.3 \pm 12.2\%$ at day 5 and $76.9 \pm 6.4\%$ at day 7 [76]. Human coronary artery endothelial cell (HCAEC) attachment to tropoelastin is in part mediated

through the integrin $\alpha_v\beta_3$, however the site of this interaction and the involvement of other mediators has not been elucidated [77].

1.3 Tropoelastin Self-Assembly

Following secretion from the cell, tropoelastin molecules rapidly self-associate, driven by hydrophobic interactions to form protein-rich aggregates. This self-assembly process of coacervation was recently reviewed in depth [78]. Coacervated tropoelastin is subsequently deposited on the pre-existing microfibrillar network via a mechanism that requires a complete and functional C-terminus as suggested by deletion [79] and targeted antibody studies [69]. The microfibrillar network provides a structural framework for the elastic fibers [80, 81]. In the final stage of elastogenesis, members of the lysyl oxidase family facilitate the formation of cross-links between lysine residues on adjacent proteins to produce an interconnected elastin network [82, 83].

Initially when heated to 37°C, tropoelastin monomers aggregate into evenly dispersed spherical nanoparticles. The size of these particles is concentration dependent, but reach a maximum (~200 nm) when critical tropoelastin concentrations of 2 μ M are achieved. Each 200 nm sphere contains approximately ten thousand tropoelastin molecules. These spheres then coalesce, and in high concentrations of tropoelastin (10mg/mL, 166 μ M) form larger interconnected sphere structures, called droplets (~2–6 μ m). The degree of order for these bridged spheres is again concentration dependent, with higher tropoelastin concentrations (30–40 mg/mL) producing fibers with diameters similar to *in vivo* elastic fibers and adding the additional feature of nascent pores [84]. This assembly process can be rendered irreversible by prolonged incubation above the transition temperature, addition of chemical cross-linkers, modulation of the solution pH or modification of tropoelastin side chain chemistry. Tropoelastin assembled and set in this manner can form biomaterials with diverse mechanical and biological properties, as discussed in detail below (Figure 2). Elastin inspired biomaterials such as these have a range of promising applications, particularly in dermal and vascular settings [66, 85].

2. Pure Tropoelastin Biomaterials

2.1 Classical Materials

Hydrogels—Hydrogels comprise a class of biomaterials that have wide biomedical applicability ranging from tissue engineering to drug delivery [86]. A hydrogel is a water-saturated polymer structure that consists of hydrophilic polymer networks formed by cross-linking a single hydrophilic monomer (homopolymer) or with other co-monomers (co- and multi-polymers). The properties of the resulting structure are dependent on the concentration and choice of monomer and cross-linker, as well as the method of synthesis. The alteration of these parameters impacts on the charge, chain arrangement, porosity, stiffness, swelling behavior and biodegradability of the hydrogel. Additionally, hydrogels are amenable to functionalization with bioactive molecules in order to tailor their cell interactions to facilitate cell adhesion, proliferation and differentiation, allowing hydrogels to be specifically tuned to model the 3D tissue microenvironment.

During the natural process of elastogenesis, tropoelastin monomers align and are cross-linked by the amine reactive enzyme lysyl oxidase. However, due to this enzyme's low solubility and limited activity [87], alternatives have been investigated for use in bioengineering. Well characterized cross-linking agents used successfully with tropoelastin include bis (sulfosuccinimidyl) suberate (BS3), glutaraldehyde (GA) and 1,6-diisocyanatohexane (HMDI). Cross-linking with BS3 produces fixed cross-link distances between TE molecules, while GA and HMDI can co-polymerize to give a range of cross-

link sizes. The TE hydrogel casting process generates two surfaces: one highly porous and the other a smooth protein layer which is non-porous. The high porosity of the first surface permits higher levels of cellular infiltration, albeit shallow. The second surface forms a non-porous flat surface that creates a barrier resistant to cellular infiltration.

The flexible nature of the casting process for hydrogels allows for the creation of various structures including sheets and tubes. Tropoelastin is the most linearly elastic biosolid material known, with a Young's Modulus of 220–280 kPa, similar to natural elastin which ranges from 300–600 kPa [88]. *In vitro* testing has demonstrated that chemically cross-linked tropoelastin hydrogels support cellular growth and proliferation of multiple attachment cell types including epithelial cells (HEK 293 and HT-1080) and human primary dermal fibroblasts [89, 90], which deposit type I collagen and fibronectin when attached. Cell infiltration within the hydrogel structure is typically shallow, as while gels are highly porous, the porosity is not contiguous [89]. *In vivo* investigations of cellular responses to BS3 and GA cross-linked tropoelastin sponges implanted in the dorsum of male guinea pigs for 13 weeks revealed that implants were well tolerated, eliciting similar *in vivo* reaction to collagen controls [88].

Tailoring pore size to maximize cellular interactions with the structure is an important consideration in hydrogel design. Large pore sizes allow efficient nutrient supply, gas diffusion and metabolic waste removal [91]. Conversely, small pores facilitate elevated attachment, however infiltration is limited by the poor diffusion characteristics [92]. Dense CO₂ gas improves the interconnectivity of pores to facilitate cellular infiltration and allows for improved diffusion of nutrients and gas and waste removal within the structure. Initial studies using glutaraldehyde cross-linked α -elastin and tropoelastin under high pressure CO₂ produce thin walled structures with highly interconnected pores [93]. Increasing pressure from 1 to 60 bar increases the average pore size from 20 μ m to 35 μ m and causes both faces of the hydrogel to form large pores, in contrast to those produced under atmospheric conditions [94]. The increased connectivity of pores in CO₂ hydrogels allows for superior levels of cellular infiltration compared to traditional TE hydrogels. This method is limited for α -elastin due to the low number of free lysines, whereas tropoelastin-based hydrogels have most or all 35 lysines available for reaction, translating to superior structural integrity [94].

The production of TE/ α -elastin hybrid hydrogels demonstrates the value of TE in conveying improved mechanical characteristics to the structure, with 50/50 TE/ α -elastin hydrogels retaining their structure following swelling [94]. Increasing the ratio of TE to α -elastin also greatly increases the compression and tensile moduli of the final product. As the use of TE increases the degree of cross-linking, higher concentrations lead to a reduction in pore size and provide a potential means of controlling pore size as well as mechanical strength in hybrid gels to suit their desired application.

Electrospun Materials—The versatile nature of electrospinning has established it as a favored method for producing tissue-engineered scaffolds. The process produces nano- to micro- diameter fibers using both synthetic and natural polymers, formed into interconnected porous networks. Scaffolds can be tailored to mimic the nanoscale architecture of the extracellular matrix, including surfaces with large void volumes, tightly controlled surface structure, chemistry, degradation and mechanical strength. Typical parameters that determine the shape and size of electrospun fibers include: concentration of polymer solution, flow rate, applied voltage and the distance to target [95].

Electrospun tropoelastin fibers produce micron diameter fibers 2–3 times wider than α -elastin fibers produced under the same conditions. Unlike collagen, which produces

uniformly round nanometer fibers, tropoelastin micro-fibers tend to be flattened ribbon structures, the diameter of which is strongly influenced by flow rate [96]. Analysis of electrospun TE fibers show them to be stable following electrospinning, remaining free of degradation and maintaining the secondary structure and biological activity of the monomer in solution. For tissue-engineering applications, spun TE microfibers are further processed with a cross-linking step to prevent re-solubilization in aqueous environments. The use of cross-linkers (commonly GA vapors or HMDI) induces additional structural modifications, widening fiber diameters and giving them a wave-like appearance. Once cross-linked, scaffolds are stable in aqueous environments for at least 180 days [96]. Tensile testing of tropoelastin fibers show them to be highly elastic (Young's Modulus ~265 kPa), and demonstrating superior elasticity, ultimate elongation and tensile strength to gelatin and collagen [97]. Uniaxial testing of small-diameter tubular scaffolds made from electrospun TE reveals tensile strength and elastic moduli comparable to that of native elastin, combined with a burst pressure of 485 ± 25 mmHg [98].

Electrospun tropoelastin scaffolds have demonstrated compatibility with a wide range of cell types, including human palatal mesenchyme (HEPM) [97], HUVECs, human coronary artery smooth muscle cells (HCASMC) and primary human dermal fibroblasts [96]. At 24 hr post-seeding, these cell types display attachment and migration, with internal actin filaments aligning towards the fibers. By 72 hr, proliferation is evident, not impacted by the choice of cross-linker (GA vs HMDI). For dermal applications, cellular infiltration is a key parameter and for electrospun TE scaffolds is linked to porosity and ultimately solution flow rate during manufacture.

Electrospun TE delivered at 1, 3 and 5 ml/hr has substantially different structural properties. Higher flow rates result in more than doubling of the mean fiber width which in turn increases the average pore size, from 8.0 ± 0.2 μ m at 1 mL/hr up to 21.5 ± 0.9 μ m at 5 mL/hr. The degree of fibroblast infiltration is proportionally improved. While 1 mL/hr scaffolds support only monolayer growth, 3 and 5 mL/h scaffolds display cellular infiltration throughout the structure and support expression of ECM proteins, including collagen type I and fibronectin [99]. In a rat full-thickness skin excision model, 3 mL/rh TE scaffold-covered wounds heal thoroughly within 3 weeks, complete with regenerated normal skin epidermis accompanied by rete ridges and dermal papillae. The scaffolds remain in the wound bed for sufficient time to promote the migration of monocytes and dermal fibroblasts and support angiogenesis within the scaffold. Scaffolds degrade within 3 weeks and are replaced by native tissue including *de novo* rat collagen.

2.2 New TE-based Materials

Classical tropoelastin-based materials are reliant on chemical cross-linking to stabilize the final scaffold structure and to make it resistant to aqueous dissolution. This necessitates the inclusion of additional reagents; some such as GA have their biocompatibility frequently questioned; lengthy quenching and washing protocols are needed to maximize *in vivo* tolerance. New TE-based materials that do not rely on chemical linkers, are one way to overcome these concerns.

pH Stabilization—Coacervation brings the lysine residues of neighboring TE molecules into close proximity to facilitate cross-linking by lysyl oxidase. In addition, lysyl oxidase oxidizes the lysyl ϵ -amino group to generate an adipic semi-aldehyde, minimizing charge repulsion between molecules, which have a high isoelectric point on account of their high lysine content. One means of generating cross-linker free tropoelastin hydrogels takes advantage of this lysine positioning and mimics the elimination of the repulsive charge effect. Highly alkaline conditions (pH > 10) at 37°C initiate a two-stage sol-gel reaction

resulting in hydrogel formation. Tropoelastin molecules self-associate to form a reversible coacervate and once a critical time point of ~2 hr is achieved, a kinetically stable hydrogel product is formed, which remains stable at neutral pH over a wide temperature range [87].

Subcutaneous injections of pH stabilized tropoelastin hydrogels are well tolerated in female Sprague-Dawley rats over a 2 weeks period, promoting collagen deposition and encapsulation of the material [90].

Methacrylated Tropoelastin—During hydrogel synthesis, the use of chemical cross-linkers, organic solvents and high pressure prohibit the addition of cells until the structure has been fully cast. In response to this, recent advances utilize methacrylation of tropoelastin (MeTro), modifying primary amines to facilitate ultraviolet radiation activated linker-free constructs. MeTro gels form within 35 s compared with ~ 24 hr for standard TE-based hydrogels [100].

Methacrylation of tropoelastin also allows for aqueous solutions to be photo cross-linked following exposure to ultraviolet light. In the absence of chemical cross-linkers, MeTro forms highly porous and elastic three dimensional hydrogel structures. The extent of methacrylation has a significant impact on cross-linking density with high densities of methacrylation reducing the average pore size and swelling ratio of the gels. Higher degrees of methacrylation also result in increased material stiffness and strength, at the expense of decreased elasticity. When compared with native tissues, MeTro gels have similar extensibility and strain characteristics to large arteries and has a similar compressive modulus to native elastin [101].

When compared with other synthetic biodegradable elastomers such as poly(glycerol-sebacate) (PGS) [102], MeTro gels benefit from increased mechanical stability [88], the presence of integrin attachment sites on TE, while avoiding cytotoxicity issues associated with polyurethanes [103]. MeTro is of particular interest as it is compatible with current 3D printing and cell encapsulation technology, giving it many potential applications.

HUVECs and fibroblasts demonstrate adhesion, spreading and proliferation when seeded on pre-formed MeTro gels. In addition, experiments encapsulating fibroblasts within the gel structure during synthesis demonstrate that cells remain viable for at least a week in culture [100].

3. Hybrid Tropoelastin Containing Materials

While TE provides a number of mechanical and biological benefits, being highly elastic and signaling multiple cell types, its extracellular matrix environment contains a range of additional important biomolecules. These augment tissue strength, provide additional cell adhesion locations and signaling cues. Evolving materials engineering increasingly seeks to combine multiple components within the same scaffold in order to derive the benefits of each. Hybrid materials containing TE allow for its expanded use in a greater number of tissue replacement areas, but each time leveraging the intrinsic benefits TE provides.

3.1 Synthetic Co-polymers: Polycaprolactone

Biomaterials used in vascular replacement and repair require a combination of strength and elasticity. Current synthetic grafts are strong, but completely non-compliant, a property postulated to contribute to their clinical failure [104]. Human vessels used clinically for bypass grafting, such as the internal mammary artery (IMA), provide benchmarks for these mechanical demands. The IMA has an elastic modulus of 268 ± 46 kPa, combined with a burst pressure of 2267 ± 215 mmHg. Saphenous veins (SV), also dominant in clinical

applications are stiffer in the arterial pressure range (683 ± 147 kPa) but have similar burst pressure (2295 ± 272 mmHg). Electrospun TE alone is highly elastic, but has insufficient strength to match the requirements of vessel replacement or bypass.

Polycaprolactone (PCL) is a well-known biodegradable polymer used clinically for meniscus and bone reconstruction, as well as in biomaterial development for vascular reconstruction [105]. Blending of TE with PCL increases scaffold tensile strength almost 4-fold, while the Young's Modulus increases 12-fold, reflecting a substantially stronger and stiffer material. Vascular conduits electrospun from TE/PCL blends provide the best combination of mechanical and biological properties using a multi-layer approach. An outer TE/PCL layer facilitates fine control of mechanical properties, while a luminal layer of 100% TE can be used to modulate biocompatibility. The elastic modulus can be tuned to match the IMA, in contrast to highly inelastic ePTFE (IMA 268 ± 46 kPa, SE-graft 320 ± 24 , ePTFE 3639 ± 373). TE/PCL grafts combine this elasticity with a burst pressure of 1922 ± 197 mmHg, again appropriately resembling *in vivo* benchmarks [75].

It is important that the inclusion of PCL to enhance the mechanical strength of these constructs does not adversely affect the favorable vascular biological properties known for tropoelastin. Consistent with the enhanced HUVEC attachment and proliferation previously observed for TE coatings, HUVEC proliferation on electrospun TE and TE/PCL hybrids also increase $167 \pm 21\%$ ($p < 0.05$) and $152 \pm 31\%$ ($p < 0.01$) respectively from day 1–3 and are indistinguishable. Electrospun TE materials also emulate the known low thrombogenicity of elastin. 111 indium-labeled platelet attachment to TE hybrid scaffolds reduces $88 \pm 1.6\%$ compared to ePTFE ($p < 0.001$) and $80 \pm 8.4\%$ compared to PCL alone [75].

Following promising *in vitro* performance, TE/PCL hybrid conduits were implanted as rabbit carotid interposition grafts. During implantation, hybrid conduits were found to handle and suture well, with no leakage following clamp release. At explant, following 1 month *in vivo*, graft diameter (3.46 ± 0.14 vs 3.37 ± 0.17 mm) and elasticity (414.9 ± 99.5 vs 313 ± 61.47 kPa) were maintained. Burst pressure was also not significantly different (1688 ± 124 vs 1250 ± 180 mmHg), but did show a downward trend, perhaps reflective of the biodegradation of the PCL component of the graft.

3.2 Natural Co-polymers

Natural polymers possess superior cell interactive and immunological properties compared to synthetic materials, but usually require post-production modifications such as cross-linking to maintain their structural and mechanical properties in aqueous environments. In particular, electrospun natural fibers are morphologically similar to native ECM fibers, which promote cellular interactions and new tissue formation. Together with TE, natural polymers promise to enhance the biomimicry of next generation biomaterials by employing the proteins abundant in the native tissue.

Collagen—Elastin is an essential structural and cell signaling molecule in the skin dermis and an important component of any biomaterial aimed at dermal replacement. Electrospun TE shows great potential as a highly porous elastic dermal replacement, promoting primary dermal fibroblast attachment, infiltration and proliferation [89, 99]. TE based scaffolds containing collagen better mimic the mechanical and biological properties of the skin, given the high collagen content in the native dermis.

Due to its prevalence in native dermis, ease of isolation, favorable cell interactive properties and low antigenicity, soluble collagen type I dominates the electrospun dermal substitute field [106]. Collagen is typically co-electrospun with other natural or synthetic polymers to increase its mechanical strength and reduce contraction and degradation rates. The addition

of collagen to electrospun biodegradable polymer scaffolds such as poly(L-lactic acid) (PLLA), PCL and blended poly(L-lactic acid)-co-poly(epsilon-caprolactone) (PLLA-CL) has been shown to enhance cell adhesion, spreading, migration, proliferation, morphology and differentiation [107, 108]. Moreover, addition of insoluble elastin to collagen-based scaffolds increases elasticity, reduces collagen degradation, scaffold contraction and increases elastic fiber formation [109].

TE and collagen were co-electrospun and the physical, mechanical and biological properties of the composite scaffolds were compared. In a series of scaffolds containing increasing amounts of collagen (all TE, 20%, 40% and 50% collagen) only the highest collagen content significantly stiffens the scaffolds, raising the Young's Modulus from 139.9 ± 29.0 kPa (all TE) to 841.0 ± 122.1 kPa (50% Collagen). Formation of actin fibers within spread fibroblasts is minimal on all TE constructs, but substantial in the presence of 20 and 40% collagen. Cell proliferation is strong on each these scaffolds, but the rate is proportional to collagen content, with 40% collagen scaffolds highest. Cell infiltration into collagen containing scaffolds is also increased by 50% relative to all TE controls [110].

Co-electrospinning of collagen and elastin is an effective means of combining the beneficial properties of both proteins to better mimic the physical, mechanical and biological cues offered by the skin dermis. If elastin is the major component of composite scaffolds, they maintain an elasticity that was similar to that of TE-only scaffolds. The addition of collagen improves scaffold handling and manipulation, without comprising the pore size or porosity. 40% collagen scaffolds presents the additional benefit of increased scaffold porosity, improved cell migration and increased cell proliferation rate [110].

Silk Fibroin—Silkworm silk fibroin is a remarkable, fibrous protein combining high strength and stiffness with versatility of manufacture. Silk-based biomaterials have been produced in diverse forms including: films, sponges, particles, hydrogels, and fibers, all with tunable biodegradation characteristics [111]. These properties are complementary to the elasticity and cell interactive motifs described above for TE and represent a new class of blended biomaterials. The stabilization of silk through the induction of beta-sheets also raises the possibility that silk-TE hybrids can be stabilized without chemical cross-linking.

Silk fibroin and tropoelastin blends are in the early stages of manufacture and characterization. Films of silk:TE are stable with various component ratios in a full range between completely silk and all TE. Calorimetry experiments indicated that the blends are miscible, while observation of the increasing β -sheet content following methanol treatment suggests linker-free stabilization of these constructs. The mechanical properties of these blends is also tunable, with increasing TE resulting in increasing elastic modulus, from 2 MPa for silk alone up to 9 MPa for all TE. The blending and drying process result in the formation of micro- and nano-scale surface features, which promote human mesenchymal stem cell (hMSCs) attachment and proliferation [112].

Further developing these silk:TE blends, ratios of each component can be used to direct surface roughness, topological patterns and mechanical properties. Construct stabilization is by using water vapor to induce β -sheet crystal formation, as previously shown for silk. A combination of low surface roughness and high stiffness in the silk:TE materials is optimal for proliferation and differentiation of C2C12 myoblasts. In contrast, high surface roughness with micro/nano-scale surface patterns promotes hMSC growth. Increasing TE content in the silk:TE materials also enhances the proliferation and osteogenic-differentiation of hMSCs [113].

Conclusions—The important structural and biological signaling roles played by elastin in a diverse range of tissues make its mimicry high sought after in biomaterial development. The availability of large quantities of highly purified, full length tropoelastin has facilitated an increased understanding of the monomers shape, structural features, assembly, and cell signaling properties. These insights have translated into increasingly sophisticated elastic biomaterials, tailored to match the elastin-rich tissues they seek to replace. Combination of tropoelastin with both synthetic and natural polymers further expands the potential uses of this versatile biomolecule.

Acknowledgments

We acknowledge funding from the Australian Research Council (A.S.W.), Australian Defense Health Foundation (A.S.W.), National Health and Medical Research Council (APP1033079 and APP1039072; A.S.W. and M.K.C.N.) and National Institutes of Health (EB014283; A.S.W and D.L.K). A.S.W. is the Scientific Founder of Elastagen Pty Ltd. G.C.Y. is supported by an IPRS/IPA PhD scholarship.

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Figure 1.

A) Structural features of the tropoelastin monomer; B) Cell interactive C-terminal sequence –RKRK and; C) assembly model showing the N- to C- terminal interaction. Adapted from [32].

Figure 2.

The assembly of a single tropoelastin monomer through defined stages, resulting in increasingly sophisticated intermediates. The in-depth understanding of this process has facilitated the development of diverse tropoelastin-based biomaterials including: A) classical hydrogels; B) electrospun fibers; C) pH stabilized materials [90] and; D) Methacrylated tropoelastin hydrogels, adapted from [100].