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ENGINEERED TROPOELASTIN AND ELASTIN-BASED BIOMATERIALS

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Abstract

Elastin is a key mammalian extracellular matrix protein that is critical to the elasticity, compliance, and resilience of a range of tissues including the vasculature, skin, and lung. In addition to providing mechanical integrity to tissues, elastin also has critical functions in the regulation of cell behavior and may help to modulate the coagulation cascade. The high insolubility of elastin has limited its use to researchers, while soluble derivatives of elastin including elastin peptides, digested elastins, and tropoelastin have much broader applications. Recombinantly produced tropoelastin, the soluble monomer of elastin, has been shown to exhibit many of the properties intrinsic to the mature biopolymer. As such, recombinant human tropoelastin provides a versatile building block for the manufacture of biomaterials with potential for diverse applications in elastic tissues. One of the major benefits of soluble elastins is that they can be engineered into a range of physical forms. As a dominant example, soluble elastins including tropoelastin can form hydrogels when they are

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chemically cross-linked. These self-organized constructs swell when transferred from a saline to aqueous environment and are highly elastic; these tunable responses are dependent on the types of cross-linker and elastin used. Soluble elastins can also be drawn into fine fibers using electrospinning. The morphology of these fibers can be altered by modifying spinning parameters that include delivery flow rate and the starting protein concentration. The resulting fibers then accumulate to form porous scaffolds, and can be wound around mandrils to create conduits for vascular applications. Electrospun scaffolds retain the elasticity and cell-interactive properties inherent in the tropoelastin precursor. Additionally, soluble elastins serve as versatile biomaterial coatings, enhancing cellular interactions and modulating the blood compatibility of polymer- and metal-based prostheses. Soluble elastins, and in particular tropoelastin, have highly favorable intrinsic physical and cell-interactive properties, warranting their adaption through incorporation into biomaterials and modification of implantable devices. The multiple choices of ways to produce elastin-based biomaterials mean that they are well suited to the tailoring of elastic biomaterials and hybrid constructs.

I. INTRODUCTION

Elastin is responsible for the elasticity of multiple tissues in all vertebrates apart from the cyclostomes, imparting resilience and recoil to elastic fibers. It is one of the most hydrophobic proteins currently known, with over 75% of the sequence made up of just four nonpolar amino acids; glycine, valine, alanine, and proline. Elastin is prevalent in several tissues, constituting up to 57% of the aorta, 50% of elastic ligaments, 32% of major vascular vessels, 7% of lung, and 5% of the dry weight of skin (Vrhovski and Weiss, 1998). Elastin is an extremely durable and insoluble biopolymer that does not turn over appreciably in healthy tissue, with a half-life of ~70 years (Petersen *et al.*, 2002).

Elastin is formed through the lysine-mediated cross-linking of its soluble precursor tropoelastin, which is a 60–72 kDa alternatively spliced extracellular matrix (ECM) protein. *In vivo*, tropoelastin is secreted from diverse elastogenic cell types including smooth muscle cells, fibroblasts, and endothelial cells. The deposited elastin is generically referred to in the elastin literature as the elastic fiber, although it should be appreciated that this is a broad term that is intended to encompass elastin in other forms, including elastin in the lamellar vascular wall. In mammals, it is deposited in the late fetal and early neonatal periods, after which tropoelastin production drops precipitously as there is very little turnover of mature elastin, so at maturity the production of new elastin ceases (Swee *et al.*, 1995). In the event of injury to elastic fibers, the repair production of tropoelastin can be quickly restarted and is influenced by a exogenous factors that include tumor necrosis factor-α (Kahari *et al.*, 1992), interleukin 1β (Kuang and Goldstein, 2003), insulin-like growth factor-1 (Jensen *et al.*, 1995), and particularly by transforming growth factor (Parks, 1997).

An important property of tropoelastin, in terms of elastic fiber formation, is its ability to self-assemble through coacervation. Coacervation has been well studied and is characterized by an inverse temperature transition and phase separation then fusion of approximately micron-sized particles of assembled protein molecules (Cox *et al.*, 1973a; Starcher *et al.*, 1973). Hydrophobic interactions between molecules at 37 °C, physiological salt, and pH 7–8 force tropoelastin out of solution as a viscous aggregate. The process can be monitored by observing the change in turbidity as a function of temperature (Urry and Long, 1977). The key outcome of coacervation is the alignment and concentration of tropoelastin prior to lysyl oxidase-assisted cross-linking (Kagan and Li, 2003).

Research into elastin and tropoelastin was initially restricted by the difficulty in obtaining these materials from animal sources, accentuated by the extreme insolubility of massively cross-linked elastin (Lansing *et al.*, 1952) and the short *in vivo* lifetime of tropoelastin which is quickly incorporated into elastin (Mecham and Foster, 1979). Overcoming these concerns, recombinant tropoelastin (rTE) was first expressed as a fusion protein in an *Escherichia coli* bacterial system after an expression vector containing the human cDNA sequence was constructed (Indik *et al.*, 1990). The high purity of the rTE produced in this system was beneficial; however, persistent degraded fragments and small yields were problematic. Following the optimization of a bacterial expression system using a synthetic elastin (SE) gene construct, a recombinant 60 kDa mature form of tropoelastin was expressed (Martin *et al.*, 1995). Significantly higher yields were achieved following several molecular biology modifications including the substitution of expression-limiting rare codons in the tropoelastin sequence with those more regularly found in the bacteria. This recombinant human tropoelastin (rhTE) is identical to the naturally secreted common form of human tropoelastin, is recognized and can be used by mammalian cells to form elastin (Stone *et al.*, 2001), associates at 37 °C (Toonkool *et al.*, 2001) and can be cross-linked *in vitro* both enzymatically (Mithieux *et al.*, 2005) and chemically (Wise *et al.*, 2005) to form an elastin-like material (Mithieux *et al.*, 2004). Refinement of purification and expression protocols now sees the monomer reproducibly produced in multigram quantities. The implicit advantages of rhTE are its relative availability, high purity and the philosophy of using the full-length molecule that provides for the requisite cell–molecular, intermolecular, and intramolecular interactions.

For biomaterials applications, tropoelastin is a preferred elastic construction component, as it mimics elastin's physical and biological properties and has the potential to replace damaged elastin-rich tissue. Engineered soluble elastins, including rhTE, α-elastin, and recombinantly produced elastin sequence-based variants such as elastin-like polypeptides, elastin-like proteins, and recombinant human elastin polypeptides, have the potential to augment tissues with a requisite elastin content such as skin dermis and vasculature. Potential dermal substitutes seek to take advantage of the natural elasticity, promoted cellular interactions, and opportunities for enhanced tissue regeneration as observed for soluble elastin-based biomaterials. As a vascular substitute, these biomaterials benefit from being able to support new matrix synthesis and to be endothelialized, while imparting some physical strength and particularly bestowing the recoil required of these vessels (Wise and Weiss, 2009).

rhTE provides the greatest versatility for biomaterials applications as it can be engineered to be suitable for a range of applications. Depending on the method of manufacture, rhTE can form elastic hydrogels and fine microfibers, and can be used as a surface coating. In these and other examined forms, rhTE constructs retain the key properties of elastin such as elasticity, enhanced cellular interactions, and blood compatibility which make it conveniently suited to biomaterial development. We describe here some key methods for the bioengineering of soluble elastins with a specific focus on rTE, outlining areas where it is likely to generate the greatest impact in the next generation of biomaterials.

II. HYDROGELS

Highly porous, interconnected, biocompatible scaffolds are particularly desirable for tissue engineering as they allow enhanced nutrient and oxygen transfer, cell migration, and proliferation. Polymer hydrogels are highly attractive for this purpose due to their hydrophilicity and high permeability (Annabi *et al.*, 2009a). Hydrogels are cross-linked, water-swollen polymer networks that have great technological importance as biomedical materials (McMillan and Conticello, 2000). Researchers have long recognized the benefits of incorporating elastin and its derivatives into hydrogels, employing elastin polypeptides, acid-solubilized α-elastin, and ultimately rhTE. These hydrogels can be produced with a range of porosities and mechanical properties, while supporting the growth of a number of diverse cells types.

A. *Elastin Sequence-Based Hydrogels*

Dominating over 75% of its content with just four nonpolar amino acids (Gly, Val, Ala, Pro), the amino acid sequence of tropoelastin contains many repeating motifs. Typical examples include sequences such as VGVPG (Sandberg *et al.*, 1986) and VPGVG or VGGVG (Li and Daggett, 2002). Synthetic polypeptides taking advantage of the intrinsic elasticity provided by these sequences have been widely studied and have strong potential for tissue engineering applications (Chilkoti *et al.*, 2006). Individual domains of tropoelastin have also been produced, which have subsequently formed the basis of a minimalist approach to understanding the assembly of elastin. Specifically the relationship between structure and mechanical properties of elastin can be studied using reassembled tropoelastin domains, given that even very short sequences will self-assemble and can be cross-linked (Bellingham *et al.*, 2001). Soluble elastin sequence-based molecules have been stabilized through the use of chemical cross-linkers including 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS), glutaraldehyde (GA), bis(sulfosuccinimidyl) suberate (BS3), genipin (GP), pyrroloquinoline quinone (PQQ), and 1,6-diisocyanatohexane (HMDI). Cross-linking of these soluble elastins has led to the formation of a variety of hydrogels. (Mithieux *et al.*, 2009).

Elastin-like polypeptides consisting of VPGXG repeats (where X was Lys every 7 or 17 pentapeptides, otherwise V), have been synthesized and chemically cross-linked to produce hydrogels. Gels ranged in stiffness from 0.24 to 3.7 kPa at 7 °C and from 1.6 to 15 kPa at 37 °C depending on protein concentration, lysine content, and molecular weight. Changes in gel properties suggest that at low temperatures, these structures are nearly completely elastic (Trabbic-Carlson *et al.*, 2003). Improvements to the mechanical and cell-interactive properties of these polypeptide materials has been achieved by copolymerizing sequence blocks from multiple parts of the elastin sequence, or combining with elements from other proteins. Constructs containing repeating elastin-derived (VPGIG)*x* repeating sequences, as well as cell-binding domains derived from fibronectin were cross-linked using glutaraldehyde. Tensile properties of cross-linked protein films were found to be inversely related to the molecular weights of the engineered constructs which varied from 14 to 59 kDa. At the highest cross-link density and lowest molecular weight, the elastic modulus was found to be similar to that of native elastin (Welsh and Tirrell, 2000).

The use of recombinant human elastin polypeptides has demonstrated that as few as three tropoelastin hydrophobic domains flanking two cross-linking domains are sufficient to support a self-assembly process that aligns lysines for cross-linking (Keeley *et al.*, 2002). These sequences contain sufficient information to self-organize into fibrillar structures and promote the formation of lysine-derived cross-links. When cross-linked with PQQ, these materials had an extensional elastic modulus of ~250 kPa, approaching the value commonly described for native elastin (300–600 kPa) (Fung, 1993). Sheets could be subjected to extensional strains of ~100% before breaking. The intrinsic ability of such polypeptides to self-organize into polymer structures not only makes them a useful model for understanding the process of assembly of elastin, but also sheds light on the design of self-assembling biomaterials (Bellingham *et al.*, 2003).

Recombinant human elastin polypeptides have also been cross-linked using GP, impacting on their physical and mechanical properties. The micron-scale topography of GP hydrogels revealed the presence of heterogeneity compared with PQQ analogs, which were comparatively uniform. It was also shown that the porosity of the GP hydrogels was much greater. GP-cross-linked sheets also exhibited significantly greater tensile strength, with a modulus greater than fourfold higher than similarly produced PQQ scaffolds. The change in physical characteristics appears to be caused by a higher cross-link density in the case of GP, likely due to its capacity to form both short- and long-range cross-links (Vieth *et al.*, 2007).

B. α-Elastin Hydrogels

As an alternative to tropoelastin or elastin-based peptides, some researchers artificially make "soluble elastin" by chemically treating animal derived elastin samples. The soluble elastins include α-elastin (Cox *et al.*, 1973b), an oxalic acid derivative of elastin, and κ-elastin, solubilized with potassium hydroxide. Using circular dichroism and Raman spectroscopy, the secondary structure of κ-elastin (Debelle *et al.*, 1995) was found to be similar to that for free monomer tropoelastin, supporting its use in a model system (Debelle *et al.*, 1998).

An elastic mimetic scaffold formed from α-elastin, cross-linked with a diepoxy cross-linker was produced in various pH conditions. Reaction pH was shown to modulate the degree of cross-linking, the swelling ratio, enzymatic degradation rate (8–50% per hour in 0.1 u/ml elastase), and elastic moduli (4–120 kPa) of these hydrogels. Cross-linking with a combination alkaline followed by neutral pH process resulted in materials with the highest degree of cross-links, as indicated by a low swelling, slow degradation rate, and high elastic modulus. Cross-linked α-elastin materials were subsequently shown to support vascular smooth muscle cell adhesion and a decreased proliferation rate compared to polystyrene controls (Leach *et al.*, 2005). They were also found to be fragile, as seen previously for other α-elastin-based materials (Bellingham and Keeley, 2004).

One means of improving the properties of α-elastin-based hydrogels has been to form the constructs in the presence of high-pressure carbon dioxide (CO_2). The dense gas process facilitates coacervation, improves cross-linking, and dramatically alters the micro- and macrostructures of pores within the sample. A complex network of interconnected pores was observed for GA cross-linked α-elastin hydrogels fabricated using high-pressure CO_2. *In vitro* cell culture studies demonstrated that these channels facilitated fibroblast penetration and proliferation within α-elastin structures (Annabi *et al.*, 2009b). However, the mechanical properties of these scaffolds was lower than anticipated, attributed to the low number of lysine residues (less than 1%) in α-elastin available for cross-linking with GA. Cross-linking of α-elastin with HMDI was performed in the presence of Dimethyl sulfoxide (DMSO) and gave rise to highly porous structurally stable α-elastin hydrogels. The increased solubility of CO_2 in DMSO compared to aqueous solution contributed to the fabrication of large pores and channels within the scaffolds as the dissolved CO_2 escaped on depressurization. This porosity substantially promoted cellular penetration and growth throughout the matrices. The highly porous α-elastin hydrogel structures fabricated in this study have potential for soft tissue engineering applications (Annabi *et al.*, 2009a).

C. *rhTE Hydrogels*

rhTE has been cross-linked with both BS3 (Mithieux *et al.*, 2004) and GA (S. M. Mithieux and A. S. Weiss, unpublished data) to form SE hydrogel constructs. Using this approach, elastic sponges, sheets, and tubes have been formed (Fig. 1). These constructs have physical characteristics similar to those seen for native elastin. BS3-based constructs have

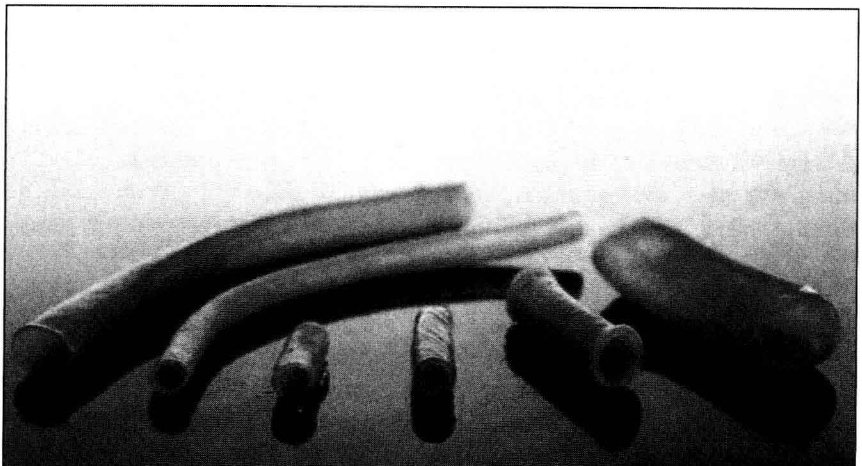

FIG. 1. Tubes manufactured from BS3 cross-linked rhTE. A range of lengths and diameters are readily produced.

a Young's modulus ranging from 220 to 280 kPa and extensibility of 200–370%. GA cross-linked hydrogels had an average Young's modulus of 133 kPa and a mean elongation at failure of 234%. Like natural elastin, these SEs when dry are hard, brittle and inelastic but become elastic on wetting. They act as hydrogels and display stimuli-responsive characteristics toward temperature and salt concentrations, swelling under low salt and temperature conditions, and contracting at physiological salt and temperature. The manufacture of BS3 cross-linked hydrogels results in a smooth lower surface and a porous upper surface. Culture of human and murine adherent cells on the smooth surface leads to monolayer formation, while cells seeded on the open weave surface infiltrate into deeper compartments. Both BS3 and GA cross-linked SE hydrogels persist for at least 13 weeks following implantation into the dorsum of male guinea pigs where it was well tolerated, indicating that the material was innocuous and compatible *in vivo* (Mithieux *et al.*, 2004; S. M. Mithieux and A. S. Weiss, unpublished data).

The lysyl oxidase purified from the yeast strain *Pichia pastoris* (PPLO) can be used to enzymatically cross-link rhTE into hydrogel structures (Mithieux *et al.*, 2005). The process is considerably less efficient than when tropoelastin is oxidized *in vivo* by mammalian lysyl oxidases. Compared to elastin purified from horse ligamentum nuchae, PPLO-treated tropoelastin constructs contain ~10-fold more lysine residues and 25-fold less desmosine residues, indicating a decreased level of lysine oxidation. It is likely that PPLO is unable to sterically access all the lysine residues that would normally be oxidized by a mammalian LO (~30 kDa) due to the large size of this enzyme (~230 kDa). The decreased level of oxidation and subsequent cross-linking are reflected in the physical properties of the cross-linked tropoelastin. The Young's Modulus of the hydrogel is ~10 kPa, which is ~30–60-fold lower than that seen for elastin fibers (Fung, 1993). These values indicate a decreased stiffness in the PPLO-treated tropoelastin material, which is attributed to a decreased number of cross-links. In addition, the constructs swell following aqueous exposure to a much greater extent than normally seen for elastin further indicative of a loosely cross-linked matrix.

In a novel development, rhTE can also be assembled into an elastic biomaterial without the requirement for either enzyme- or chemical-mediated cross-linking. Under highly alkaline conditions, tropoelastin proceeds through a sol–gel transition leading to the formation of an irreversible hydrogel. The rhTE self-associates to give stable protein spheres that coalesce to generate solid integral hydrogels. This material supports human fibroblast proliferation *in vitro*, with cells colonizing the surface. Injection of the pH-modified rhTE intradermally into female Sprague–Dawley rats results in rapid localized gelation to form a persistent mass. The resulting elastic deposit persists for at least 2 weeks, encouragingly eliciting just a mild foreign body response and promoting collagen deposition and encapsulation of the material (Mithieux *et al.*, 2009).

Hydrogels made from soluble elastins can be produced with a wide range of physical properties, depending on the elastin starting material and choice of cross-linker. Material stiffness, strength, and porosity can be tailored by using varying sequence lengths and types, creating hydrogels in high-pressure environments and modulating reaction pH. Independent of these parameters, soluble elastin hydrogels importantly maintain the ability to support a range of cell types, and exhibit persistent elasticity. Biomaterials applications for elastin-based hydrogels include replacement of soft tissues, skin repair, and tissue bulking.

III. ELECTROSPUN MATERIALS

Electrospinning has recently emerged as a favored technique for generating biomimetic scaffolds made of synthetic and natural polymers for tissue engineering applications. It allows for the fabrication of multilayered polymer-based scaffolds inspired by the natural architecture of the ECM (Han and Gouma, 2006). As a result, electrospinning of key ECM proteins such as collagen and elastin has become particularly prevalent.

Electrospinning of ECM proteins can generate fibers with diameters in the range from several micrometers down to less than 100 nm, which have a very high surface area to mass ratio. Accumulated fibers can be collected *n*-modifiable orientations to create three-dimensional (3-D) scaffolds with adjustable porosity that potentially present proteins in a form that allows their natural biological cues to be recognized by cells. In addition, electrospun fibers can retain the mechanical properties inherent in the source protein as has been demonstrated for both collagen and elastin (Boland *et al.*, 2004). In this way, biomaterial scaffolds are fabricated by electrospinning to exhibit favorable mechanical properties, facilitating cell attachment, cell growth, and regulating cell differentiation.

In a typical electrospinning setup, high-voltage fields cause polymers in volatile solvents to elongate and splay into small fibers. The fibers are drawn to a grounded surface and/or adhere to surfaces placed between the solution source and ground (Stitzel *et al.*, 2006). The morphology of the fibers collected at the plate as a nonwoven mesh is influenced by several parameters, particularly the solvent used, the potential difference of the applied electric field, the flow rate, and the collecting distance (Buttafoco *et al.*, 2006).

A. Elastin Sequence-Based Electrospinning

Elastin sequence-based fibers were first produced using an 81-kDa elastin-like protein based upon the repeat sequence VGPVG (Huang *et al.*, 2000). Electrospinning of solutions above 10% (w/v) resulted in long uniform fibers. An average fiber diameter of 450 nm was observed for this protein construct with generally a flat ribbon-like morphology. Optimal fiber formation was observed with use of an 18-kV electric field and a 15-cm distance between the spinneret and plate collector. Concentration of the polymer solution and the delivery flow rate were found to have the most effect on final fiber morphology. These early findings established many of the basic principles for the successful electrospinning of elastin-based polymers, investigated further by subsequent researchers.

High-molecular-weight elastin-like polypeptides have also been electrospun. These constructs were based on hydrophobic IPAVG end blocks, separated by VPGVG-repeat mid-block elements. When dissolved in 2,2,2-trifluoroethanol (TFE), electrospinning proved to be a feasible strategy for creating protein fibers with diameters ranging from 100 to 400 nm (Nagapudi *et al.*, 2005). Spinning of the same polypeptides from water results in fibers with diameters ranging from 800 to 3 mm. At identical concentrations polypeptide solutions in TFE displays a lower viscosity at 23 °C than aqueous protein solutions at 5 °C, which may contribute to the formation of smaller diameter fibers.