DEVELOPMENT OF A TISSUE SCAFFOLD FOR VASCULAR REGENERATION THROUGH THE USE OF LAMINATED HUMAN AMNIOTIC MEMBRANE ENHANCED BY INTERFACIAL ADHESIVES

By

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To my family
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<td>amniotic fluid</td>
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<td>CABG</td>
<td>coronary artery bypass grafting</td>
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<td>CAD</td>
<td>coronary artery disease</td>
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<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
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<tr>
<td>DAPI</td>
<td>4,6-diamino-2-phenylindole</td>
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<td>DHT</td>
<td>dehydrothermal</td>
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<tr>
<td>EC</td>
<td>endothelial cell(s)</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>ePTFE</td>
<td>expanded polytetrafluoroethylene</td>
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<td>FD</td>
<td>freeze-dried</td>
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<td>FG</td>
<td>fibrin glue</td>
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<td>glycosaminoglycans</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>hAM</td>
<td>human amniotic matrix</td>
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<tr>
<td>HUV</td>
<td>human umbilical vein(s)</td>
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<td>ID</td>
<td>internal diameter</td>
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<tr>
<td>OD</td>
<td>outer diameter</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PCI</td>
<td>percutaneous coronary intervention</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<td>SEM</td>
<td>scanning electron microscopy</td>
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<td>SiNP</td>
<td>silicon dioxide nanoparticles</td>
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<tr>
<td>SIS</td>
<td>small intestine submucosa</td>
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<td>SMC</td>
<td>smooth muscle cell(s)</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>TCP</td>
<td>tissue culture plastic</td>
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<tr>
<td>TEBV</td>
<td>tissue engineered blood vessel(s)</td>
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<tr>
<td>TESA</td>
<td>tissue engineering by self-assembly</td>
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<tr>
<td>TEVG</td>
<td>tissue engineered vascular graft(s)</td>
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<tr>
<td>UBM</td>
<td>urinary bladder matrix</td>
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<td>WJSC</td>
<td>Wharton’s Jelly stem cell(s)</td>
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Despite significant advances in diagnostic and treatment modalities, cardiovascular disease remains a leading cause of death worldwide. Bypass grafting of diseased native vasculature remains an important intervention for reperfusion of critically ischemic tissue. Autologous native vessels, such as the internal thoracic artery, are considered to be the most effective conduits utilized in bypass grafting, but their availability is limited, and harvesting of such native vasculature can be associated with morbidity. Conversely, grafts constructed from synthetic material, such as polytetrafluoroethylene (ePTFE), are no longer used in small diameter applications due to excessively high failure rates. Grafts incorporating biomimetic elements approximating native vascular structure and function confer advantages over conduits composed of inert material. Extra-embryonic tissues hold promise as substrates for development of ex vivo-derived tissue scaffolds, given their ready availability, minimal immunogenicity, and favorable biochemical properties. Human amniotic membrane (hAM) in particular has been increasingly recognized as demonstrating marked
regenerative capacity in diverse clinical applications, from ophthalmic surgery to wound healing.

This dissertation describes the progressive development of a vascular graft derived from “rolling” hAM into a laminated tubular scaffold. Despite initial measures utilized to stabilize the scaffold, handling properties remained suboptimal due to poor mechanical integrity. As such, this work investigated the use of adhesives – fibrin glue and silica nanoparticles (SiNP) – in an effort to increase cohesion of adjacent hAM mural layers. The application of fibrin glue demonstrated increased graft stability \textit{in vitro} and \textit{in vivo}, with promising remodeling of the scaffold noted. While improved, the stability conferred by this adhesive remained inadequate when challenged with the mechanical stresses of surgical implantation and subsequent hemodynamic flow. The application of SiNP ultimately demonstrated the most robust and enduring stabilization of graft layers, with preserved tubular geometry and cohesion of mural layers. Studies additionally revealed SiNP-enhanced scaffolds to exhibit preserved biocompatibility, favorable cell adhesion properties, and suggested hemocompatibility. Provided the appropriate balance of mechanical stabilization with biocompatibility, the results of this work collectively demonstrate that tissue scaffolds derived from rolled hAM represent a promising approach to constructing vascular grafts with customizable geometry.
CHAPTER 1
INTRODUCTION TO VASCULAR TISSUE REGENERATION

The ultimate objective of this dissertation is to develop a scaffold for vascular tissue regeneration from a rolled, tubular human amniotic membrane. This approach to vascular engineering draws upon multiple disciplines, necessitating a broad background to understand the motivation for the work. For the sake of organization, there are three main sections to the introduction. The first section focuses on: 1) the clinical need for, and functional requirements of, small diameter graft replacements, 2) a brief review of rolled approaches to tissue-engineered scaffolds so as to provide context for this project in the current research landscape, and 3) a detailed literature review on the human amniotic membrane. The second section of this introduction delves into bioadhesives, providing background information on medical adhesives, including those used currently in the clinical context as well as those yet under investigation. Detailed literature review of silica nanoparticles, an adhesive investigated in this thesis, will be provided. The third section summarizes the aims and objectives of this work.

While the first two sections of the introduction may seem unrelated, it is important to note that a “rolled” approach to constructing a successful vascular graft necessitates layers of the rolled scaffold to be bound together to form a stabilized construct. Thus, it is of value to 1) provide a review of the clinical problems in vascular grafting to fully understand the design requirements of a rolled vascular scaffold, 2) review the base material of the scaffold (the human amnion), and 3) explore the role of bioadhesives in the context of a rolled vascular graft functioning in the clinical setting.
Clinical Need for Vascular Grafts

Cardiovascular disease (CVD) is the leading cause of death in developed countries despite intensive research efforts to prevent and treat this condition. Each year nearly 1.4 million vascular grafts are utilized to replace diseased native arteries in the United States alone. Of these grafts, more than 600,000 grafts are needed for coronary artery bypass graft procedures (CABG). The type of graft used is a significant determinant of success; autologous arteries, specifically the internal thoracic artery, have the highest success rates in CABG procedures. However, autologous arteries or veins, the ideal small diameter vascular grafts, are not available in nearly 30% of patients due to previous harvesting or due to involvement by systemic vascular disease, thus requiring alternative vascular prostheses. Furthermore, it is likely that CABG procedures will become more frequent, as research investigating the comparative success of CABG procedures versus less-invasive percutaneous coronary interventions (PCI), such as stent placement, provide increasing evidence demonstrating the improved outcomes in those who undergo CABG procedures in the treatment of atherosclerotic coronary arterial disease (CAD) and myocardial infarction.

Synthetic grafts such as expanded polytetrafluoroethylene (ePTFE) or polyethylene terephthalate (Dacron) are successfully used to replace large diameter arteries (internal diameters greater than 8 mm). However, with small diameter vascular graft applications (such as those utilized in peripheral or central bypass arterial grafting) the five-year patency rate is unacceptably low at 50%, due to the graft material’s intrinsic thrombogenicity, and the compliance mismatch between graft and native vasculature. Another disadvantage of vascular grafts made from these synthetic
materials is that they are inert, and thus non-biodegradable. This makes them unsuitable for vascular reconstruction in pediatric patients, as the vessels will not grow with the patients, requiring repeat surgeries.\(^7\) Lack of autologous vessels and shortcomings of the synthetic graft materials have motivated research to pursue the development of biological or bioactive grafts that would fully integrate with the host vasculature and have the capacity to adapt to the hemodynamic environment along with the native vasculature to improve long-term outcomes. Properties of an ideal vascular graft are summarized in Table 1-1.

**Engineering a Graft for Vascular Tissue Regeneration**

In the effort to create a conduit to repair vascular defects, the anatomy, physiology and adaptive nature of native arteries must be appreciated. Then, previously explored approaches should be reviewed to gain further insights into what traits are essential to successful design of a graft to guide vascular tissue regeneration.

**Gross anatomy and function of muscular arteries**

The cardiovascular system provides the body with a system of arteries to deliver oxygenated blood to all tissues in the body and veins to return deoxygenated blood back to the lungs. As such, vascular tissue is complex and highly organized. The arteries most relevant to CABG procedures are small diameter muscular arteries (i.e. internal thoracic arteries, the gold standard CABG graft, and the coronary arteries themselves) and are comprised of three distinct layers. From the lumen to the albumen, these vascular layers are termed: the tunica intima, tunica media and the outermost layer, the tunica externa (also referred to as the adventitia), Figure 1-1.\(^8\)

The intima is comprised of endothelium, which lines the vascular lumen and its underlying basement membrane. Given that the endothelium makes contact with the
blood, it is responsible for providing a non-thrombogenic surface. Furthermore, the endothelial cells (EC) of the intima also regulate the smooth muscle cells (SMC) of the subjacent medial layer. The media consists of circumferentially aligned smooth muscle cells and a highly organized ECM that are responsible for modulating vascular contractility. Importantly, collagen and elastin confer the mechanical strength and elasticity characteristic of muscular arteries. The adventitia is comprised of loose connective tissue, sparsely populated with fibroblasts and contains a capillary network that feeds the wall of the blood vessel called the vasa vasorum.

The vascular system is more structurally and physiologically complex than a simple network of static conduits. There are layers of regulation that allow for the efficient delivery of oxygenated blood to meet the demand of each tissue. Within the vascular tissue itself exists paracrine regulation between the different cell types; endothelial cells (EC) assist in the maintenance of the contractile phenotype in vascular smooth muscle cells (SMC). Vascular cells, both EC and SMC, are further regulated by environmental cues including mechanical stimuli (i.e. cyclic radial distension and shear stress, etc.), as well as physical and chemical cues such as the ECM composition and local oxygen tension.

**Approaches toward the development of vascular grafts**

Due to the great clinical demand for an enduring vascular graft, there has been a massive research effort toward their development. There has been vast variety in the explored approaches, and despite much progress and increase in knowledge, research efforts remain ongoing, as there has not been an approach that successfully meets all of the requirements of an ideal graft. Broadly, approaches can be categorized into two general categories based on the intended use: 1) pre-populated tissue-engineered
constructs, or 2) scaffolds for guiding tissue regeneration *in situ*. However, it is worth noting that certain approaches can be explored for use in both categories. For example, an acellular scaffold could be explored as both a tissue-engineered construct by first pre-populating the scaffold with vascular cells prior to implantation while simultaneously exploring the possibility of implantation of the graft as an acellular scaffold for guided tissue regeneration.

Another general categorization of approaches can be based on whether the base material is synthetic or derived from a biological source. Again, the classification of an approach into one of these two categories can often be difficult as there are synthetically produced natural polymeric grafts (i.e. collagen electrospun scaffolds) and composite organic/inorganic scaffolds. Among these approaches, *ex vivo*-derived scaffolds comprise a broad category of approaches that take advantage of native polymeric scaffolds and are described in the following section.

**Ex vivo-derived biomaterial scaffolds.** “Top-down” approach to tissue engineering describes the process to create biological tissues by seeding cells on a polymeric scaffold and providing environmental stimuli (often chemical or mechanical) to guide the cells to populate the scaffold and appropriately produce ECM and tissue microarchitecture.14 The use of this approach in vascular tissue engineering was pioneered by Weinberg and Bell.15 With the goal of creating functional vascular tissue rather than an inert conduit, the tissue must be engineered *in vitro* (commonly referred to as a tissue engineered vascular graft, TEVG) or guided to regenerate *in vivo*. Scaffolds can be synthetic degradable polymers, biological polymers, or decellularized allo- or xenogeneic vessels (complexes of natural polymers).16-19 When this polymeric
scaffold is derived from natural sources by decellularizing allogeneic or xenogeneic vessels, these scaffolds can be referred to as being ex vivo-derived. Examples of ex vivo-derived scaffolds include cadaveric allogeneic arteries, the human umbilical vein, and xenogeneic arteries. One disadvantage of using muscular arteries as a source material is that they often have many branches that must be closed prior to use.

The goal in using decellularized tissue scaffolds is to create a porous, biodegradable mechanical support that already has an ECM architecture able to help guide repopulation by vascular or immature cells.\textsuperscript{20-24} There are several advantages to using ex vivo-derived scaffolds compared to degradable synthetics. Natural polymers have intrinsic cellular adhesion moieties such as the arginine-glycine-aspartic acid (RGD) sequences on ECM peptides.\textsuperscript{25} This allows for relatively high cell seeding efficiency, and often rapid cellular repopulation of the scaffold, as cellular integrins recognize and bind to this peptide sequence found on fibronectin, laminin, and some collagens, etc.\textsuperscript{25} Furthermore, collagen is conserved among species, and as long as the tissue is appropriately decellularized and cellular debris is largely removed from the scaffold, the scaffold could theoretically be derived from xenogeneic sources, reducing potential ethical concerns and broadening the available sources.\textsuperscript{26}

**Rolled approach for TEVG.** Rolling planar materials to create a tubular scaffold is not a novel concept in the arenas of tissue engineering and regenerative medicine. Many groups have explored this approach, each with distinct methodologies and starting materials. Base materials can be derived from decellularized tissues that exist as thin sheets in the body (e.g. porcine small intestine submucosa (SIS) or porcine
urinary bladder matrix; UBM), produced by monolayers of cells in prolonged culture, or
generated synthetically using either natural or biocompatible synthetic polymers.

Xenografts created from animal tissues such as the SIS or UBM are similar to the
amnion in that they are thin tissues, but vary in compositional makeup and origin.\textsuperscript{27} SIS
is derived from porcine jejunum, its predominate collagen type is type I collagen and it,
unlike the amnion, is anisotropic.\textsuperscript{28} Porcine UBM has an intact basement membrane,
like the amnion, and it is primarily composed of collagen type IV and type VII.\textsuperscript{29} However, some concerns have arisen with regard to the safety of using these animal-
derived collagenous tissues. After observing cases of patients who suffered pain and
edema after having undergone surgical rotator cuff repair that used Restore SIS
implants, Zheng et al. found that extensive material characterization revealed that the
product was not completely devitalized but contained porcine DNA.\textsuperscript{30} Row et al. rolled
SIS into tubular scaffolds stabilized and seeded with cell-doped fibrinogen.\textsuperscript{31}

Termed “Sheet-Based Tissue Engineering”, or alternatively “tissue engineering
by self-assembly” (TESA), L’Heureux et al. have developed an approach that uses
autologously-derived fibroblasts cultured \textit{in vitro} to produce an ECM sheet eliminating
exogenous biomaterials.\textsuperscript{32} In this approach, sheets are wrapped around a support tube
and are fused together via cell-mediated processes over time. This graft has been
termed the Lifeline graft by Cytograft Tissue Engineering (Novato, CA, USA) and has
since been used in humans for arteriovenous shunts for hemodialysis access in a
clinical trial as early as September 2004.\textsuperscript{33} Despite the progress made towards
translating this approach to engineer vascular tissue, extended production times were
not ideal; patient-derived cells had to be cultured from six to nine months (mean
production time $7.5 \pm 0.7$ months). Moving forward, this research group has moved towards the evaluation of devitalized Lifeline grafts for allogeneic transplant such that the grafts may be stored for extended periods and available emergently.

**The Human Amnion**

**Anatomy and physiology of the amnion**

There are two placental membranes, the amnion and the chorion, that comprise the amniotic sac that support the developing fetus *in situ*. The fetal membranes are derived from the embryonic blastocyst of the zygote, and are thus genetically identical to the developing fetus. The chorionic membrane is derived from the trophoblast, while the amniotic membrane is derived from a portion of cells in the inner cell mass, classified as the amniotic ectoderm with the extraembryonic mesoderm contributing to the mesodermal layer of the amniotic membrane. The amniotic membrane is the innermost membrane, oriented towards the developing fetus. The amnion is an avascular tissue and although it is the thinner of the two placental membranes at only $50 \, \mu m$, it confers most of the strength and elasticity to the amniotic sac due to its biomolecular composition. This tissue is typically described as having five distinct strata based on compositional and microarchitectural distinctions. Starting from the innermost layer (oriented from the amniotic cavity towards the maternal decidua), the amniotic strata are: epithelium, basement membrane, compact layer, fibroblast/reticular layer and lastly, the spongy layer that makes contact with the chorionic membrane. Figure 1-2 shows a histological cross-section of the native placental membranes.

In more detail, the first layer is comprised of a simple cuboidal epithelium oriented inward toward the fetus. The amniotic epithelium is metabolically active with many important functions during parturition. They also are responsible for producing
and secreting amniotic fluid, growth factors and cytokines. These cells produce prostaglandins, and are one of the main sources of prostaglandin E₂, important for the initiation and maintenance of uterine contractions. These specialized cells also maintain the homeostasis of the amniotic fluid, keeping the pH at approximately 7.10. Amniotic epithelial cells lay on top of a basement membrane. Inferior to the basement membrane, there is a zone of dense connective tissue termed the lamina compacta; this compact stromal layer is the strongest layer of the amnion. Loose connective tissue is the final layer of the amnion in a zone called the zona spongiosa that consists of fibroblasts. Of note, a mesodermal derivative connecting the amnion to the subjacent chorionic membrane represents what some consider to be a “third” placental membrane.

Collagen is the main constituent of the amniotic membrane ECM. The reticular stroma is largely composed of type I collagen and type III collagen. Type IV collagen, as expected, is localized to the basement membrane, along with collagens type IV, VII, XV, XVI, and XVII. Fibronectin and laminin are abundant in the basement membrane, but are present throughout the tissue. Type VI collagen and type V collagen has also been identified on the stromal side of the amnion. Non-collagenous components of the hAM ECM include glycoproteins such as fibronectin, nidogen, laminins and proteoglycans such as perlecan. Fibrillin, agrin and elastin are other ECM constituents found in the basement membrane.

**Commercialization of amnion-based products for biomedical application**

As discussed, there are two membranous layers that comprise the amniotic sac that supports the developing fetus *in utero*: the amnion and the chorion. The placental membranes (interchangeably referred to as the fetal membranes) are considered
extraembryonic tissue and thus do not give rise to ethical concerns, and have been showing promise in the field of regenerative medicine. These tissues have been used as skin grafts for over 100 years to promote healing of superficial wounds.\textsuperscript{47} Since the 1970s, the broad clinical applicability of the fetal membranes has expanding, and has made what was once considered medical waste into an abundantly available source of material for biotechnological advancements.\textsuperscript{48} Figure 1-3 shows some examples of the targeted biomedical applications for which amnion-based therapies have been explored.

There is now there is an abundance of commercially available amnion- or fetal membrane-derived products on the market, Table 1-2. In addition to therapeutic applications, these placental membrane-based products are being investigated as platforms for stem cell and drug delivery.\textsuperscript{49-51} These are typically regulated by the FDA as human cell, tissue, and cellular- and tissue-based products (HCT/P).\textsuperscript{36} This regulatory pathway is much less rigorous, and thus much less expensive, than undergoing a pharmaceutical or medical device regulatory pathway. Furthermore, there have been remarkable successes in treating refractory wounds and joint pain, with mild-to-no documented adverse events.\textsuperscript{52} As a result, these amnion-based products appeal to companies within the biotechnology sector. While there is an extensive array of products on the market, most companies tend to be terse in their descriptions of processing methodology and often do not provide detailed information regarding outcome data for each proposed therapeutic application. Given the proprietary nature of processing methods, it can be difficult to determine relevant differences between distinct amnion-based allografts marketed by the same company, even after review of company websites and extensive literature search (including search of published patents).
Despite this, there are now nearly 200 clinical trials related to the study of amnion-based allografts registered on the NIH Clinical Trials website (clinicaltrials.gov), as yielded with the search criterion “amniotic membrane” as of September 2016 (excluding studies with unknown status or that were not relevant). A review published in 2015 performed a similar search of the registered trials in June 2015, and included “placental derived cells” in the search, finding 95 registered clinical trials.\textsuperscript{48} This is just one indicator of the growth of the field and clinical interest in the regenerative capacity of the amnion.

Evidence of immune tolerance of these extraembryonic tissues has long been provided based on studies that used fetal membranes as subdermal or peritoneal allografts (in 1954 and 1974, respectively) without any sign of an adverse immune response.\textsuperscript{53, 54} However, since then, there has been much research seeking to elucidate the mechanism responsible for the immune tolerance, regenerative potential, and other therapeutic effects of the amnion. These will be discussed in further detail below with a summary of the intrinsic bioactivity described in Table 1-3.

**Bioactivity of the Amnion**

The amnion possesses a high degree of bioactivity through both secreted and structural proteins.\textsuperscript{55} Much of its activity is a product of its anatomical position and physiological role in being a protective barrier between a mother and the developing fetus. It is thus not surprising that this tissue possesses features that allow for immunomodulation. Of note, the degree of bioactivity and the conferred properties are dependent on whether or not the cells are extracted from the tissue, in addition to how the tissue is further processed. Factors secreted from the epithelium, for example, would be diminished with decellularization, and the more aggressive the processing, the
more the attributes of the scaffold may deviate from the native amnion. This can often lead to seemingly conflicting evidence in the literature. However, processing does not necessarily alter the properties for the worse, especially since different tissue engineering and clinical applications require distinct material characteristics. The amnion can both promote and inhibit angiogenesis depending on the surface used.\textsuperscript{56}

Furthermore, the membrane can be made to be an anti-adhesive barrier\textsuperscript{57}, preventing post-operative adhesions or can be layered (requiring amnion-to-amnion adhesion) to develop more complex structures. These are just a few interesting examples of opposing material properties that arise from differences in material processing.

Decellularized amnion retains insoluble matrix proteins and despite its lack of cells, the ECM often contains an abundance of cytokines. One theory is that even secreted growth factors get retained within the proteoglycan networks of the matrix.\textsuperscript{58}

**Immunomodulatory properties**

*Antimicrobial activity.* It was first empirically discovered that amniotic membranes used in surgical and wound-healing applications decreased bacterial counts in infected sites. However, the mechanism was not initially known and was thought to occur due to the close association between the amnion, as a wound dressing, and the wound surface, thus providing a mechanical barrier.\textsuperscript{59} In the early 2000s, biochemical antimicrobial agents associated with the amniotic membrane have been identified and help explain the phenomenon viewed clinically for many years prior. Potent antimicrobial agents such as human β-defensins (HBD1-3), elafin (skin-derived antileukoproteinase) and secretory leukocyte protease inhibitor (SLPI) have been identified as produced by amniotic epithelial cells.\textsuperscript{60, 61} This biochemical activity is perhaps not surprising, given that protection against ascending infection is necessary to
maintain a healthy pregnancy. HBD1 is constitutively expressed, while HBD2 and elafin expression is upregulated by proinflammatory cytokine IL-1β.\textsuperscript{60, 62} Defensins are part of the innate immune system and are able to destroy both Gram-negative and Gram-positive bacteria, viruses and fungi.\textsuperscript{63, 64}

**Immunosuppressive activity.** In the early 1980s, The Lancet reported a study on the immunogenicity of subcutaneously implanted human amnion in seven healthy adult volunteers.\textsuperscript{65} Tissue biopsies were harvested after 16 to 54 days (timeframes were different for each individual), and it was found that none of the allografts elicited an acute reaction. Any tissue reaction ultimately noted was of ambiguous significance due to coincidental clinical events in several participants, and the concurrent foreign body reaction to the suture material. However, of specific interest for this study, volunteers did not produce anti-HLA antibodies, providing further evidence of allo-immune tolerance to the amniotic membrane, even with the cells intact. Immunologic tolerance, as detected \textit{in vitro} via an alloreactive splenocyte proliferation assay, was maintained against vacuum-dried forms of amniotic membrane, more so with an intact epithelium but still present when the tissue was completely devitalized.\textsuperscript{66}

The first hypothesis set forth to explain the immune privilege of the fetal membranes was reported by McIntyre et al. in 1979, proposing that an amniotic glycoprotein acted on lymphocytes to suppress the foreign body reaction.\textsuperscript{67} Since then, there has been much progress made in identifying the molecular players and their complex interactions with the cells of the immune system. Comprehensive reviews have detailed the progress made in elucidating these interactions.\textsuperscript{48, 68}
While the mechanisms for these immunomodulatory effects have not been fully elucidated, there have been many studies and hypotheses set forward to better understand what is occurring at the subcellular level. Briefly, amniotic cells have been shown to modulate lymphocytes, monocytes, neutrophils, and natural killer cells, immune cells of both innate and adaptive immunity. Amnion-derived cells were shown to suppress proliferation of activated T cells, and induce Th1 cytokines and T regulatory cells. Additionally, HLA-G has been reported to suppress myeloid, natural killer and immune cell surfaces through specific surface receptors. Immune-privilege may arise from constitutive expression of human leukocyte antigens (HLA), in particular the expression and secretion of HLA-G. HLA-G, also expressed in other immune-privileged tissues, interacts with inhibitory receptors to confer tolerance by T-regulatory cell modulation. Recently, a mechanism has been proposed for the role human amniotic mesenchymal cells have on modulating macrophage phenotype to confer immunomodulation.

**Anti-thrombotic activity.** Several glycosaminoglycans (GAGs) as well as growth factors and cytokines associated with the amniotic membrane are known to play a role in modulating surface thrombogenicity. Specifically, perlecan and hyaluronic acid are GAGs that have been found to inhibit coagulation. Pigment-epithelium derived factor (PEDF) IL-10 and MMP-9 are known to inhibit platelet aggregation. Blood compatibility of both the epithelial and mesenchymal surfaces of the amnion were studied in comparison to heparin-coated ePTFE and it was found that both sides have appropriate hemocompatibility based on static tests of platelet activation, clotting time, activated partial thromoplastin time, prothrombin time and percent hemolysis.
Anti-fibrotic properties

Fragmented, cryopreserved human amnion reduced postoperative adhesions following abdomino-pelvic surgery in a rat model\(^5\), and lyophilized sheets of amnion reduced postlaminectomy epidural adhesions in both rats\(^3\) and dogs\(^4\). Fresh and dried amnion grafts have been tested as an adjunctive therapy to prevent intrauterine adhesions in pilot human trials and showed promise compared to the control groups.\(^5\) In addition to prevention of surgical adhesions, dehydrated, acellular, layered amnion ECM has been investigated as a barrier membrane in bone tissue regeneration to prevent invasion of fibroblasts to promote bone growth in lieu of fibrotic tissue.\(^6\) Ricci et al. found that both fresh and cryopreserved hAM reduced liver fibrosis in rats following bile duct ligation.\(^7\)

Angiogenic modulatory properties (both support and inhibition of angiogenesis)

Similar to the conflicting reports on the amnions ability to serve as an anti-adhesive barrier, there are mixed reports in the literature regarding angiogenesis. However, careful study of the investigations into whether the amnion is anti-angiogenic or pro-angiogenic reveals that this property is a function of how the tissue scaffold is treated and where the microenvironmental conditions surrounding the implant, and can be modulated depending on the desired outcome.

The unprocessed, native amnion has been shown to produce anti-angiogenic factors. Some of the factors that have been identified as being produced by the amniotic membrane include pigment epithelium-derived factor (PEDF), tissue inhibitor of metalloproteinase (TIMP)-1, TIMP-2, and thrombospondin-1 (TSP-1).\(^8\)-\(^1\) Additionally, the ECM itself is composed of proteins such as laminin-1, laminin-5 and fibronectin, with known roles in suppressing vascularization.\(^2\) Complicating the current understanding of
the mechanism of what confers the anti-angiogenic property to the amnion is that both decellularized scaffolds and isolated amniotic membrane cells have both exhibited suppression of neovascularization.93,94

Although suppression of angiogenesis is desirable for applications such as corneal defect repairs, intuitively it would seem that this property would stunt wound healing and regenerative capacity. However, numerous amnion-based grafts have been shown to be pro-angiogenic, with specific pro-angiogenic factors identified such as platelet-derived growth factor (PDGF)-AA, PDGF-BB, placental growth factor (PLGF), granulocyte colony-stimulating factor (GCSF), and vascular endothelial growth factor (VEGF) in processed, dehydrated amnion.95,96 It is currently believed that the microenvironment contributes more to determining the angiogenic properties of amnion-based scaffolds than the processing methodology.

Physical properties

Intrinsic cell attachment sites. There are a multitude of factors responsible for cellular attachment and repithelialization of the amniotic membrane, in both its native and decellularized forms. Secreted factors, such as IGF-1, have been shown to promote the growth of endothelial cells, dermal fibroblasts and keratinocytes.97 Additionally, as exists in all ex vivo derived tissue scaffolds, short amino acid sequences, such as the RGD sequence, act as ligands for cell attachment. Additional molecules involved in cell adhesion include perlecan, a large heparan sulphate proteoglycan localized to the basement membrane of the hAM.44 This is significant, as one essential trait of an ideal scaffold is its ability to support cell attachment and proliferation.

Barrier function. The amnion, in both fragmented and membranous forms, has shown promise in preventing surgical adhesions and providing a temporary barrier to
fibrotic tissue formation. Rather than just acting as a physical barrier, this function is more likely secondary to its biochemical suppression of fibroblasts, and thus is described above in more detail in the section titled “Anti-fibrotic properties”.

**Mechanical strength.** The biomolecular composition and organization of the ECM is responsible for the mechanical properties of the human fetal membranes. A high collagen content, organized within the amniotic stroma in an interlocking, anisotropic arrangement, confers structural integrity to the membrane in the face of multidirectional force. In humans and higher vertebrates in particular, there is a greater mechanical demand on the membranes due to the upright posture of the mother and this structure-function relationship results in the development of fetal membranes that are both strong and elastic.\(^{35}\)

**Elasticity.** The amnion is a highly elastic tissue due to the presence of elastic fibers within the tissue. Elastin is responsible for both providing both elasticity and resilience in tissues, and thus is responsible for compliance in vascular tissue. Studies have found that the elastin content in hAM is 2.1\(^{98}\) to 36\(^{23}\) of wet weight, where water contributes to as much as approximately 80% of the wet weight. This discrepancy is cited as being a result of difficulty in using biochemical approaches to quantify elastin content in tissue. Despite the difficulties in quantifying an accurate percent elastin that comprises the compositional makeup of the amnion, it is clear that the membrane is highly elastic.

**Part Two: Bioadhesives**

**Requirements of Tissue Adhesives in Vascular Tissue Scaffold Development**

Collagen types I, II, III, V, XI (fibrillar forms of collagen) and fibril-associated type IX collagen naturally form cross-links via lysyl oxidase(s) by acting on specific lysine
and hydroxylysine side chains. The degree of collagen cross-links within a tissue determines the physical strength and function of the tissue. In selecting which adhesives to investigate further for the best chance of success in our application, we outlined selection criteria in order to identify an appropriate adhesive for the application. Bioadhesives have to be both biocompatible and mechanically stable. The degree of biocompatibility and adhesive strength depends on the application. In binding extracellular matrices to develop vascular tissue scaffolds for regenerative medicine, it is difficult to balance the biocompatibility and mechanical requirements of the tissue adhesive. The cardiovascular system is a highly dynamic and mechanically stressful environment that requires robust adhesion capable of withstanding aqueous environments subjected to cyclic radial stress. Additionally, the binding agent must be biocompatible such that it, and its breakdown products, are not immediately cytotoxic nor adversely immunogenic. Furthermore, more stringent biocompatibility must be taken into consideration, as the graft is intended to make contact with blood, and thus must also be hemocompatible and not initiate thrombus formation. Another design consideration is that binding must not render the material inert. Cellular enzymatic degradation is essential for host cells to populate the graft and constructively remodel the scaffold into vascular tissue. In summary, a suitable adhesive would need to meet the criteria listed in Table 1-4.

**Physical Cross-Linkers**

Physical methods of crosslinking collagen scaffolds include dehydration (i.e. dehydrothermal treatment, lyophilization, or vacuum-desiccation), and photo-cross-linking (short wave UV irradiation or gamma irradiation). The primary advantage of using physical methods to cross-link collagen is that no potentially cytotoxic chemicals
are introduced into the system. Dehydration of collagen results in the formation of interchain peptide bonds. However, in some cases, there are changes to the physical structure of the protein and the overall scaffold that may alter the biological response. For example, dehydrothermal treatment results in denaturation of protein, which has the potential to influence host response to the biomaterial.

**Dehydrothermal treatment**

Dehydrothermal (DHT) treatment subjects collagenous scaffolds to elevated temperatures (>90 °C) while under vacuum in order to enhance the mechanical strength of the scaffold. Removal of water allows for intermolecular condensation reactions to take place between carboxyl and amino groups of adjacent amino acids (via esterification or amide formation). Subjecting collagen to these high temperatures breaks the hydrogen bonds that stabilize the triple helices, and result in denatured collagen.

**Lyophilization-induced cross-linking**

Lyophilization, another modality that desiccates the scaffold, relies on sublimation to dehydrate. It is used as a method to preserve the scaffold in order to prolong storage and facilitate transport. One study found that collagen type IV is preserved within the basement membrane of lyophilized hAM, but does result in a loss of total protein compared to native amnion and cryopreservation. Furthermore, similar to DHT treatment, there is protein denaturation that occurs both during the freezing phase as well as during the freeze-drying. Vacuum-desiccation, drying the tissue under vacuum without a pre-freeze phase, hopes to minimize tissue damage from crystallization and dry the tissue under vacuum.
**Photo-cross-linking**

Photo-cross-linking has been investigated as a method to cross-link collagen using various light sources and photosensitizers in hopes of minimizing collagen degradation. Ultraviolet irradiation (UV) at 254 nm for 15 min was found to cross-link collagen as effectively as DHT treatment, but found similar collagen fragmentation.\(^\text{106}\) UV irradiation produces crosslinks between collagen fibrils by generating free radicals on the phenylalanine and tyrosine residues.\(^\text{106, 107}\) In efforts to reduce this nonspecific chain scission, photosensitizers (such as Methylene blue) have been investigated to increase the thermal stability of collagen.\(^\text{108}\) Recently, research efforts have attempted to optimize UV irradiation such that integrin binding cites are preserved in order to preserve cellular compatibility and collagen bioactivity.\(^\text{109}\) Gamma irradiation of amnion has also been investigated and shown to have a significant impact on the physicochemical properties. A dose-dependent effect on the number of collagen cross-links was observed, but it was found that collagen fibril diameter was increased in a small percent (~5%) of collagen fibrils in the irradiated samples.\(^\text{110}\) Gamma irradiation, however, has an advantage of serving a dual purpose in that it also sterilizes the hAM scaffold.\(^\text{111}\)

**Synthetic Cross-Linkers**

**Aldehyde-based adhesives**

Glutaraldehyde and dialdehyde have been used to crosslink collagen in efforts to both decrease the thrombogenicity\(^\text{112}\) (by rendering the tissue inert), increase the mechanical strength of the scaffolds, and as a sterilizing agent. Chemical crosslinking does enhance the initial mechanical properties of decellularized vessels and lower the risk of infection. However, in developing scaffolds for vascular tissue engineering, at the
concentrations and chemicals that have been investigated and clinically translated, the approach does not allow for full integration of the scaffold with the vasculature. The vascular walls are prone to calcification, and have cytotoxic breakdown products. Also, these grafts tend to fail via aneurysmal development as cells are unable to digest and remodel the matrix of the cross-linked scaffold, resulting in imbalanced degradation and weakening of the wall in a non cell-mediated fashion.

**Cyanoacrylate adhesives**

Cyanoacrylates have the advantage of being effective adhesives in aqueous environments, and are notable for high binding strengths. Although these are effective glues, in biomedical applications the lack of biocompatibility is problematic. Cyanoacrylates are thought to be directly cytotoxic. In aqueous media, the cured adhesive degrades, producing formaldehyde as its toxic breakdown product. Despite these disadvantages, cyanoacrylate-based adhesives are commonly used in topical applications clinically, but are still investigated in preclinical trials for binding deep soft tissues in surgical applications due to their strong binding. Also, within this class of tissue adhesives, the longer-chain derivatives (i.e. butyl- and isobutyl-cyanoacrylate), have been found to be less toxic than the shorter-chain derivatives.

**Enzymatic and Biological Cross-Linkers**

**Enzymatic adhesives**

Fibrin-based sealants are the main biological adhesives currently used, but other proteins such as gelatin and albumin have also been used clinically for many years. Commercially available fibrin sealants come as two-component formulations with one part being thrombin in calcium chloride and the other part being a concentrated allogeneic fibrinogen with factor XIII. When mixed together, these components mimic
the last step of the coagulation cascade forming a covalently cross-linked fibrin network and provide hemostasis. Another group of enzymatic adhesives similar to fibrin are the transglutaminases, a family of enzymes that form covalent amide bonds between glutamine and lysine.\textsuperscript{117} Factor XIIIa (activated factor XIII, a cofactor in the polymerization of fibrin monomers) is actually a transglutaminase. One group found that treatment of amniotic membrane scaffolds with tissue transglutaminase (TG-2) improved the mechanical strength without compromise to the biological characteristics by forming stable intra-amnion $\varepsilon(\gamma$-glutamyl)lysine cross-linkages.\textsuperscript{118}

One of the drawbacks of biological adhesives is the risk of blood-borne pathogen transmission. As such, medical devices, such as the Vivostat\textsuperscript{®} system (Vivolution A/S Alleroed, Denmark), are able to formulate an autologous fibrin sealant derived from the patient’s blood.\textsuperscript{119} These autologously derived formulations take two days to prepare, and thus are not viable options for emergency procedures. Another potential drawback, depending on the clinical application, is that the strength of the adhesive bond may not be adequately strong for dynamic environments. Microbial transglutaminase has been approved for use in food.

Non-enzymatic adhesives

\textbf{Glucose-based adhesives.} Glycation of collagen renders collagen matrices stiffer via non-enzymatic based oxidative reactions that lead to the formation of advanced glycation end-products (AGEs).\textsuperscript{120} The accumulation of these AGEs have been implicated in age- and diabetes-associated pathologies of connective tissue. Therefore, the use of glucose and sugar-based crosslinking to alter the physiochemical properties of collagenous scaffolds have the potential to lead to impaired scaffold remodeling.
**Gelatin-based adhesives.** Gelatin is a form of collagen that has been irreversibly hydrolyzed and was one of the first polymers used as medical adhesive. Classified as a biologic or semisynthetic adhesive, it can be cured by photochemical, chemical or enzymatic crosslinking. Due to this diversity in formulation and application, it is worthy of its own category. Crosslinking is essential to modulate the mechanical integrity to stabilize gelatin hydrogels for biomedical applications as they swell in aqueous solutions and dissolve at approximately 35°C.

**Silica-Collagen Composite Scaffolds for Tissue Engineering**

It is believed that the binding between collagen and silica is mediated through electrostatic interactions and hydrogen bonds due to the negative charge of silicates.\textsuperscript{121, 122} One group found that there is a size dependence that helps determine the degree of interaction between the collagen and the nanoparticle; it was found that larger silica nanoparticles (SiNP) (80 nm) do not interact strongly with collagen, but that smaller SiNP (12 nm) form predictable formations around the protein fiber.\textsuperscript{123} The concentration dependence of silica on the functional properties of collagen-silica composite hydrogels has been studied.\textsuperscript{124} A silica-collagen composite hydrogen has been investigated \textit{in vitro} as a three-dimensional scaffold for bone tissue engineering\textsuperscript{125}, and also as biological dressings\textsuperscript{126}.

**Part Three: Summary and Present Objectives**

The clinical demand for enduring coronary artery bypass graft (CABG) materials inspired the overall aim of this work. We set out to develop and study an \textit{ex vivo}-derived tissue scaffold created from decellularized amnion rolled into a supportive, tubular structure to support the infiltration of host cells in order to allow the body to regenerate vascular tissue. Using this rolled approach, geometries of the scaffold could easily be
controlled and manipulated to suit each desired clinical application. For example, with just slight modification to the methodology, scaffolds developed for CABG procedures in adults could be configured to produce smaller scaffolds for correction of congenital heart defects in pediatric populations.

As work progressed on this rolled approach to develop a vascular graft, we found encouraging results in a preclinical model as fibrin-stabilized scaffolds were implanted into the carotid arteries of rabbits. Even though these were human placental membrane-derived scaffold, and the rabbits were not given immunosuppressive therapies, the scaffolds were not rejected and showed evidence of extensive remodeling after four weeks. However, from these investigations, it was determined that the handling properties of the scaffolds needed to be improved in order for this approach to be successful.

The weak handling properties stemmed from the nature of the approach; rolling the amnion results in a wall that formed of layered ECM. Without the addition of an adhesive or intervention, such as dehydration that does allow some inter-layer bonding, the scaffold will not retain its form and may delaminate. Although lyophilization of the graft does allow initial physical stability, the resultant scaffold is fragile and must be handled with care.

This dissertation details the progressive development of a rolled, tubular scaffold derived from the human amniotic membrane. In particular, mechanical characterization and cellular-scaffold interactions were studied to determine the feasibility of the approach. The objective of this dissertation is to investigate biocompatible adhesive that
would enhance the ECM-to-ECM binding such that the overall stability of the scaffold would be augmented, without compromise to the scaffold biocompatibility.

Figure 1-1. Photomicrograph of arterial cross-sections shows the layered anatomy of muscular arteries. (A) and (B) Show H&E stained arterial cross-sections from two distinct humans with varying degrees of neointimal hyperplasia. A) Demonstrates the three layers of a relatively healthy coronary artery. Note that the artery is collapsed and the lumen is not discernably patent as an artifact of processing. B) More progressive neointimal hyperplasia can be seen in this artery; the intima has become thickened, narrowing the lumen. C) Stained for elastin, the internal elastic lamina is easily observed. (B) and (C) are from the same artery. Magnification: 20x objective.
Table 1-1. Characteristics of an ideal vascular graft.

<table>
<thead>
<tr>
<th>Desired Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-immunogenic</td>
</tr>
<tr>
<td>Non-thrombotic lumenal surface</td>
</tr>
<tr>
<td>Mechanically stable</td>
</tr>
<tr>
<td>Compliance comparable to native vessel</td>
</tr>
<tr>
<td>Biodegradable</td>
</tr>
<tr>
<td>Non-toxic degradation products</td>
</tr>
<tr>
<td>Porous</td>
</tr>
<tr>
<td>Promotes cell adhesion, infiltration and proliferation</td>
</tr>
<tr>
<td>Promotes adaptation of physiological cellular phenotype</td>
</tr>
<tr>
<td>Good handling properties</td>
</tr>
<tr>
<td>Good suture retention</td>
</tr>
<tr>
<td>“Off-the-shelf” availability</td>
</tr>
<tr>
<td>Available in assorted lengths and diameters</td>
</tr>
<tr>
<td>Sterilizable</td>
</tr>
<tr>
<td>Complete integration with native vasculature with capacity of growth potential</td>
</tr>
</tbody>
</table>

Figure 1-2. Photomicrograph of native placental membranes. Hematoxylin and Eosin stained cross-section of placental membranes. Image taken on a camera coupled to an Olympus BX43 microscope, 20X magnification.
Figure 1-3. Potential clinical targets for amnion-derived therapeutics. The amnion is commercially available for surgical applications (reconstructive\textsuperscript{129}, hernia repair, adhesion prevention\textsuperscript{57, 85, 130} and spinal operations\textsuperscript{84}), acute and chronic wound healing\textsuperscript{96}, treatment of burns\textsuperscript{131-133} and ulcers (diabetic foot ulcers\textsuperscript{134, 135}, pressure and venous leg ulcers\textsuperscript{136}), and has numerous indications for ophthalmic use\textsuperscript{105, 118, 137-139}. Furthermore, it has shown promise in pre-clinical trials or small pilot studies for use in tendon or general musculoskeletal repair\textsuperscript{140, 141}, periodontal repair\textsuperscript{142}, vascular tissue regeneration\textsuperscript{128, 143, 144}, nerve grafting\textsuperscript{145, 146}, stroke treatment\textsuperscript{50}, vaginoplasty\textsuperscript{147}, repair of gastroschisis in newborns\textsuperscript{148} and liver disease management\textsuperscript{49, 76, 87}. Limited reports exist on the potential use of the amniotic membrane in pericardial closure.\textsuperscript{149}
Table 1-2. Partial list of commercially available amnion-based products.

<table>
<thead>
<tr>
<th>Company</th>
<th>Product Name</th>
<th>Characteristics/ Application Type</th>
<th>Intended Application</th>
<th>Acknowledged Uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alliqua® Biomedical</td>
<td>Biovance®</td>
<td>Dehydrated amniotic membrane</td>
<td>Wound repair</td>
<td>Acute and chronic wounds, DFUs, pressure ulcers, arterial ulcers, burns, surgical use</td>
</tr>
<tr>
<td>Alphatec Spine®</td>
<td>AmnioShield®</td>
<td>Membrane; Purion® processed</td>
<td>Wound covering, soft tissue surgery</td>
<td>Post-laminectomy epidural scarring, barrier membrane</td>
</tr>
<tr>
<td>AmnioGenix™</td>
<td>AmnioDryFlex</td>
<td>Dehydrated, sterilized amnion; DryFlex® processed</td>
<td>Dental defects; adhesion barrier</td>
<td>Laminectomy, nerve, dura protection, craniotomy, discectomy</td>
</tr>
<tr>
<td>Amniox® Medical</td>
<td>NEOX® 100</td>
<td>Cryopreserved AM; CryoTek™ Membrane or particulate (Neox Flo)</td>
<td>Wound covering</td>
<td>Minor and superficial dermal wounds</td>
</tr>
<tr>
<td></td>
<td>CLARIX® 100</td>
<td>Cryopreserved AM; CryoTek™ Membrane, wrap or particulate (Flo)</td>
<td>Surgical covering, wrap, or barrier</td>
<td>Soft tissue surgery (orthopedic, spinal, etc)</td>
</tr>
<tr>
<td>Applied Biologics, LLC</td>
<td>FlöGraft®</td>
<td>Cryopreserved, injectable AF</td>
<td>Soft tissue defect filler, wound repair</td>
<td>Bone fractures, joint pain, tendinitis, chronic wounds, inflammation, strains</td>
</tr>
<tr>
<td>BioD, LLC</td>
<td>BioDFactor™</td>
<td>Injectable; micronized, dehydrated AM/CM; CryoPrime™ processed</td>
<td>Articular injections</td>
<td>Tissue voids and defects, periodontal defects</td>
</tr>
<tr>
<td>BioDFence™</td>
<td></td>
<td>Hydrated, membrane</td>
<td>Soft tissue surgery, adhesion barrier</td>
<td>Nerve wrap, laminection, tendon and soft tissue repair</td>
</tr>
<tr>
<td>BioD Restore™</td>
<td>BioGraft®</td>
<td>Injectable; micronized amnion allograft; CryoPrime™ processed</td>
<td>Articular injections, soft tissue injuries</td>
<td>Tendinitis, OA, planatar fasciitis, muscle tears, inflamed nerves</td>
</tr>
<tr>
<td>Bio-Tissue®</td>
<td>AmnioGraft®</td>
<td>Membrane; CryoTek® cryopreserved, on carrier paper</td>
<td>Ocular wound repair</td>
<td>Corneal defect; partial stem cell deficiency; chemical burns; pterygium; dry eye</td>
</tr>
<tr>
<td></td>
<td>AmnioGuard®</td>
<td>Membrane; CryoTek® cryopreserved</td>
<td>Ophthalmic surgery</td>
<td>Glaucoma shunt graft</td>
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<tr>
<td>Celgene</td>
<td>Acelagraft™</td>
<td>Decellularized, lyophilized, gamma-irradiated, amniotic membrane</td>
<td>Ophthalmic surgery</td>
<td>Superficial corneal diseases, dry eye</td>
</tr>
<tr>
<td>Integra®</td>
<td>BioFix®/ BioFix® Plus</td>
<td>Dehydrated, decellularized, amniotic membrane grafts; HydraTek® processed</td>
<td>Wound repair, surgical use, soft tissue defect filler</td>
<td>Acute and chronic ulcers</td>
</tr>
<tr>
<td>IOP Ophthalmic Keera Srl</td>
<td>AmbioDisk™, Ambio2™</td>
<td>Cleaned, dehydrated and sterilized; sutureless AM overlay graft</td>
<td>Ophthalmic defects</td>
<td>Corneal erosion, neurotropic ulceration, ocular burns, keratitis</td>
</tr>
<tr>
<td></td>
<td>AMX: Amniotic Membrane eXtract</td>
<td>Lyophilized membrane</td>
<td>Ophthalmic surgery</td>
<td></td>
</tr>
<tr>
<td>Company/Tag</td>
<td>Product Name</td>
<td>Characteristics/ Application Type</td>
<td>Intended Application</td>
<td>Acknowledged Uses</td>
</tr>
<tr>
<td>---------------------------</td>
<td>--------------</td>
<td>--------------------------------------------------------------------------------------------------</td>
<td>----------------------------------------------------</td>
<td>-----------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Liventa Bioscience, Inc</td>
<td>AmnioClear™</td>
<td>Large AM allograft (15 cm x 15 cm); aseptically processed and sterilized</td>
<td>Surgical use, chronic wound care</td>
<td>Soft tissue repair, diabetic wounds; fascia repair</td>
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<tr>
<td>MiMedx®</td>
<td>AmnioFix®</td>
<td>Dehydrated, AM/CM; Purion® processed; denuded epithelium - Micronized, membrane, or wrap</td>
<td>Tendon, nerve and soft tissue repair, adhesion barrier</td>
<td>Plantar fasciitis; prostatectomy – prevent ED and incontinence; laminectomy – reduce adhesions</td>
</tr>
<tr>
<td></td>
<td>EpiFix®</td>
<td>Dehydrated, layered amnion/chorion; Purion® processed - Membrane or particulate</td>
<td>Acute and chronic wound care, soft tissue repair</td>
<td>DFUs, venous leg ulcers, plantar fasciitis</td>
</tr>
<tr>
<td>MTF Wound Care®</td>
<td>AmnioBand™</td>
<td>Dehydrated, layered amnion/chorion membrane</td>
<td>Acute and chronic wound care; surgical applications</td>
<td>DFUs, venous ulcers, pressure ulcers, vascular ulcers, burns</td>
</tr>
<tr>
<td>Noveome biotherapeutics</td>
<td>Amnion-derived Cellular Cytokine Solution (ACCS)</td>
<td>Injectable</td>
<td>Dental defects, ocular applications</td>
<td>Gingivitis, dry eye, burns</td>
</tr>
<tr>
<td>NuTech® Medical</td>
<td>NuShield™</td>
<td>Dehydrated; amnion/chorion; BioLoc™ processed - Membrane, wrap or on-lay graft Native AM; Allo-Fresh™ processed</td>
<td>Adhesion barrier, wound covering, soft tissue healing</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Affinity®</td>
<td>Injectable; amnion and AF</td>
<td>Orthopedic defects</td>
<td>Chondral defect</td>
</tr>
<tr>
<td></td>
<td>NuCel®</td>
<td>Cryopreserved placental allograft</td>
<td>Wound dressing</td>
<td>Lumbar fusion as DBM, OA</td>
</tr>
<tr>
<td>Osiris Therapeutics, Inc.</td>
<td>Grafix ®</td>
<td></td>
<td></td>
<td>Chronic wounds&lt;sup&gt;151&lt;/sup&gt;, DFUs&lt;sup&gt;152&lt;/sup&gt;, venous leg ulcers, pressure ulcers, burns</td>
</tr>
<tr>
<td>Snoasis Medical</td>
<td>BioXclude™</td>
<td>Dehydrated, sterilized amnion/chorion overlay graft, Purion® processed</td>
<td>Dental defects and oral surgery</td>
<td>Alveolar ridge preservation, tooth loss</td>
</tr>
<tr>
<td></td>
<td>BioCover™</td>
<td>Dehydrated amnion/chorion composite graft; Purion® processed</td>
<td>Oral surgery</td>
<td></td>
</tr>
<tr>
<td>SurgiLogix</td>
<td>AmnioFLEX</td>
<td>Injectable; cryopreserved amnion and AF</td>
<td>Soft tissue barrier, soft tissue defect filler</td>
<td>OA, tendinitis, tendon, ligament, muscle, fascia, nerve, cartilage repair</td>
</tr>
<tr>
<td>Tides Medical</td>
<td>AmnioHeal® Plus</td>
<td>Dual-layered membrane; dehydrated</td>
<td>Wound covering, surgical applications</td>
<td>DFUs, pressure ulcers, venous stasis ulcers, burns</td>
</tr>
<tr>
<td></td>
<td>Artacent™ Flex</td>
<td>Dual-layered membrane; dehydrated; epithelial layer oriented outward on both sides</td>
<td>Surgical barrier</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Artacent™ Wound</td>
<td>Dual-layered membrane; dehydrated</td>
<td>Wound covering</td>
<td>DFUs, pressure ulcers, venous stasis ulcers, burns</td>
</tr>
</tbody>
</table>

AF - amniotic fluid, AM - amniotic membrane, CM - chorionic membrane, DFU – diabetic foot ulcer, DBM - demineralized bone matrix, ED - erectile dysfunction, OA - osteoarthritis
### Table 1-3. Intrinsic properties of amniotic membrane-derived scaffolds

<table>
<thead>
<tr>
<th>Property</th>
</tr>
</thead>
<tbody>
<tr>
<td>Provide physical support for cellular repopulation</td>
</tr>
<tr>
<td>Contain intrinsic cell attachment sites</td>
</tr>
<tr>
<td>Low immunogenicity</td>
</tr>
<tr>
<td>Mitigate fibrosis</td>
</tr>
<tr>
<td>Reduce inflammation</td>
</tr>
<tr>
<td>Antimicrobial</td>
</tr>
<tr>
<td>Promote wound healing</td>
</tr>
<tr>
<td>Degradable</td>
</tr>
<tr>
<td>Support or inhibit angiogenesis</td>
</tr>
<tr>
<td>Non-tumorigenic</td>
</tr>
<tr>
<td>Promote epithelialization</td>
</tr>
</tbody>
</table>

### Table 1-4. Summary of key requirements of tissue adhesives for vascular tissue scaffold development

<table>
<thead>
<tr>
<th>Adhesive Requirements</th>
<th>Description of Criterion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biocompatibility Criteria</td>
<td>Cytocompatible adhesive and breakdown products</td>
</tr>
<tr>
<td></td>
<td>Not adversely immunogenic</td>
</tr>
<tr>
<td></td>
<td>Hemocompatible</td>
</tr>
<tr>
<td></td>
<td>Adequate systemic compatibility</td>
</tr>
<tr>
<td></td>
<td>Renal excretion and clearance</td>
</tr>
<tr>
<td></td>
<td>No biomass accumulation</td>
</tr>
<tr>
<td>Mechanical Criteria</td>
<td>Effective at binding soft tissue</td>
</tr>
<tr>
<td></td>
<td>Enduring adhesion in aqueous environments</td>
</tr>
</tbody>
</table>
CHAPTER 2
GENERAL MATERIAL AND METHODS

In this chapter, commonly used experimental and analytical methods are described. Methods specific to distinct chapters will be detailed within the relevant chapter.

Experimental Methods

Placental Tissue Procurement and Processing

De-identified, human, placental tissues were obtained from the Labor & Delivery department at UF Health Shands Hospital at the University of Florida (Gainesville, FL, IRB approval #64-2010). Mid-membrane amnion (Figure 2-1) were isolated from the chorionic membrane by blunt dissection and washed in DI H₂O to initiate the decellularization process, modified from Wilshaw et al. 23, 128, 153 Peri-placental and rupture site-associated membrane were discarded. Amnion were devitalized by two freeze/thaw cycles (frozen at -82ºC, thawed at 37ºC), followed by incubation in 0.1% (w/v) sodium dodecyl sulfate (SDS) in PBS under agitation (100 RPM) for 24 hours. SDS was removed from the amnion in three subsequent PBS washes, each 1 hour on an orbital shaker plate at 100 rpm. Amnion ECM were treated with 50 U/mL deoxyribonuclease I (DNase I; from bovine pancreas, Sigma) in DI H₂O at 37ºC supplemented with 10mM MgCl₂ for two hours. Next, decellularized amnion were washed three times in PBS to remove residual enzyme and cellular debris from the acellular matrix, Figure 2-2. Sterilization was achieved using peracetic acid (PAA; 0.01%, Sigma-Aldrich) supplemented with 4% (v/v) EtOH for one hour, followed by thorough washing in sterile buffered saline under agitation until pH was balanced at pH 7.4.
Preparation of Silica Nanoparticle Dispersions

Commercially available colloidal silica nanoparticles were purchased from Sigma and used in a range of dilutions, without surface modification (Ludox TM-50, Sigma). Ludox® is a registered trademark of W.R. Grace & Co.-Conn. According to manufacturer product information, the aqueous colloidal SiO$_2$ stock solution contains 50% silica by weight and is stabilized with sodium hydroxide (SiO$_2$/Na$_2$O ratio of 200-250; Sigma-Aldrich specification sheet). It is a monodisperse preparation of negatively charged, amorphous silica 22 nm in diameter. Particle size and zeta potential were analyzed in double distilled water (DDH$_2$O) on Nicomp Nano ZLS Z3000 particle analyzer (Particle Sizing Systems Inc., Santa Barbara, CA, USA; Figure 2-3) Serial half dilutions of the nanoparticle dispersion were prepared in DDH$_2$O immediately prior to use. For use in preparation of sterile scaffolds, diluted samples were passed through a 0.22 μm mixed cellulose esters (MCE) membrane syringe filter (Fisherbrand. Fisher Scientific). The resulting concentration of each preparation was confirmed by testing small representative samples by both 1) determining the lyophilized weight of from a sample of known volume and 2) testing the absorbance at 295 nm and comparing it to a standard curve of known concentrations, Figure 2-4.

Preparation of Amniotic Membrane Scaffolds

The decellularized amnion was cut into the appropriate rectangular dimensions to achieve the selected numbers of layers for the desired wall thickness for any given internal diameter (which is determined by the external diameter of the mandrel). The flat rectangular hAM was treated with either the SiNP dispersions or with pH-matched PBS based on the unit area of scaffold (5 μm/cm$^2$), then rolled tightly around the mandrel. Rolled scaffolds were frozen at -86°C for 12 hours and transferred to a Millrock Bench-
Top Freeze-Drier (Millrock Technology, Kingston, NJ) for freeze-drying between 4 and 8 mT for 24 hours.

**Cell Isolation and Culture**

**Primary smooth muscle cell isolation and culture**

Primary human vascular smooth muscle cells (SMC) were explanted from human umbilical arteries\(^{154}\) obtained from the umbilical cords of at least three distinct human placentas to reduce phenotypic variability (IRB approval #64-2010).\(^{155}\) Histology of a human umbilical cord is shown in Figure 2-5 to show the two umbilical arteries and one umbilical vein supported by Wharton’s Jelly. Briefly, human umbilical arteries were dissected from each cord and opened along the longitudinal axis. Arterial lumens were scraped to remove the endothelium. Next, the tissue was cut into small pieces (approximately 1 mm\(^2\)) and placed into 25 cm\(^2\) tissue culture flasks with the intimal surface of the sections oriented down. Cells typically migrated from the tissue within two weeks. Cells were cultured in low glucose Dulbecco’s modified Eagle Medium (DMEM; Hyclone, Thermo Scientific) supplemented with 1% Penicillin-Streptomycin (Hyclone, Thermo Scientific), and 10% fetal bovine serum (FBS; Gemini BioProducts). Cells were maintained at 37°C in 5% CO\(_2\) and media was exchanged every two to three days. Cells were passaged when they reached approximately 75% confluence using Accutase (Innovative cell technologies, Inc.), and were used between passage 3 to 6.

**Wharton’s jelly stem cell isolation and culture**

Wharton’s Jelly Stem cells (WJSC) were explanted from human umbilical cords in a similar procedure to the explantation of smooth muscle cells as described above. Human umbilical cords were opened longitudinally and Wharton’s Jelly, the glistening stromal tissue in between the umbilical vessels was carefully cut from the cord into
pieces approximately 1-2 mm². Tissue was placed in tissue culture flasks with no specific orientation. Cells were cultured in DMEM supplemented as described above and maintained similar to the primary smooth muscle cells, but were used at passage 3.

Analytical Methods

Imaging: Cellular and Scaffold Visualization

Histological analysis

Samples were embedded in optimum cutting temperature media (OCT; Tissue-Tek, Sakura Finetek USA). Cassettes were held over the vapors of an isopropanol bath contained in liquid nitrogen. Samples were equilibrated to -20°C, and sectioned on a cryostat with the blade perpendicular to cross-sections of tissue. Tissue sections were 5-8 μm thick. Sections were stained with hematoxolin and eosin (H&E) according to standard protocol, or Masson’s Trichrome (Richard-Allen Scientific; Kalamazoo, MI). Images were obtained on a Zeiss AxioImager M2 upright microscope outfitted with a Zeiss AxioCam HRc digital camera (Zeiss, Thornwood, NY).

Fluorescent staining

Qualitative assessment of cell viability was done via staining with the LIVE/DEAD® assay, calcien AM (Invitrogen) and ethidium homodimer-1, as per manufacturer’s instructions and visualized by fluorescent microscopy, using the GFP and DsRed filters, respectively. Living cells were counted from three random regions per sample with NIH ImageJ to provide a more quantitative assessment of cell viability. Cell nuclei were co-stained using 4’,6-diamidino-2-phenylindole (DAPI; Invitrogen) according to manufacturer’s instructions. Images were obtained on a Zeiss AxioImager M2 upright microscope using a Zeiss Axiocam HRm Rev. 3 monochromatic digital camera module (Zeiss, Thornwood, NY).
Scanning electron microscopy (SEM)

Samples were dehydrated by lyophilization for 48 h as described above and cut using a sharp blade and mounted on adhesive stubs. Samples were sputter coated with palladium-gold (DeskV, Denton Vacuum, Moorestown, NJ), and imaged via scanning electron microscopy at 3.0 kV (Hitachi SU-5000 FE-SEM).

Biochemical Analysis

Cell proliferation and metabolic activity

Quanti-iT PicoGreen assay was used to determine the cell density as per manufacturer’s instructions (Invitrogen, USA). A calibration curve was created by preparing DNA from known concentrations of SMC using a five-point standard curve. Samples were first weighed and then digested in papain (papaya proteinase I) solution (125 µg/mL papain, 5 mM L-cystein, 5 mM EDTA in PBS) for 24 h at 60°C to break down the peptide bonds of the extracellular matrix. Samples were centrifuged, and the supernatant was incubated with the PicoGreen reagent. Sample excitation and emission fluorescence wavelengths for dsDNA bound to PicoGreen are 502 nm and 523 nm, respectively (Synergy 2 Plate reader, BioTek, USA). The fluorescence value of the reagent with control samples (no seeded cells) is subtracted from that of each sample to yield fluorescence versus DNA concentration.

Cellular metabolic activity was assessed using the AlamarBlue assay (AB; Invitrogen, USA) as per the manufacturer’s instruction. The percent reduction of resazurin to resorufin was used as an indicator of cellular metabolic activity (as determined by measuring the fluorescence at 590 nm). Controls were run at each time point and included 0% reduced and 100% resazurin conditions, created by incubating the 10% AB solution without cells and autoclaving a solution of 10% AB, respectively.
When combined with cell density data obtained from PicoGreen analysis, metabolic activity per cell could be ascertained.

**Glycosaminoglycan (GAG) content analysis**

Samples were digested in papain as described above. Glycosaminoglycan content was determined using dimethylmethylene blue assay on digested samples (Sigma Aldrich, St Louis MO, USA) and calibrated using chondroitin sulfate as a standard.\(^{159, 160}\)

**Scaffold Biomechanics**

Tensile properties were assessed using an Instron uniaxial testing rig (Model 5542 Norwood, MA, Instron) with a 50 N load cell, Figure 2-6. For analysis of all mechanical data, the assumption of material incompressibility was made.

**Uniaxial tensile test**

Strips of tissue were hydrated for 30 min in PBS, and then securely clamped using the top and bottom grips of the Instron with a gauge distance of seven centimeters. Thickness of each sample was measured using digital calipers. Sample tissue strips were tensioned until failure at a constant rate of 2 mm/min.

**Hoop stress test**

Tissue ringlets were hydrated in PBS for 24 hours and cut into an oblong shape maintaining a 5:1 (L:W) ratio.\(^{128}\) Ringlets were mounted onto stainless steel hooks, and were progressively tensioned to reach a preloaded force of 5 mN. Ringlets were then tensioned until failure at 5 mm/min. Recorded load and displacement values were normalized to attain stress and strain. Stress, \(\sigma = F/A\) where \(F\) is the recorded load and \(A\) is the cross-sectional area of the sample.
Suture retention strength

The tissue was held securely in place by the bottom grip of the Instron. A 3-0 vicryl suture (Ethicon, Somerville, NJ) was threaded through the sample, secured to the force transducer and elongated at 1.33 mm/s. The force at failure was determined.\textsuperscript{32}

Statistical Analysis

At minimum, three independent experiments were conducted at each time point for biochemical assays, and five independent samples for mechanical studies. All quantitative results are expressed as mean ± standard deviation. Student’s t-test and one-way analysis of variance (ANOVA) followed by Tukey post hoc test was used where appropriate for statistical analysis; \( p < 0.05 \) was considered statistically significant (confidence level ≥ 95%).

Figure 2-1. Placental tissue procurement and dissection of amnion from the mid-zone region. A) Demonstrates the amnion still attached to the chorion and placenta in the orientation it would have been found \textit{in situ}. B) The amnion being peeled from the chorion shows the peri-placental and part of the mid-zone membrane. Photo courtesy of author.
Figure 2-2. Photograph of one segment of acellular amnion. Four 9-layered 3.2mm diameter grafts could easily be made from this membrane. Photo courtesy of author.
Figure 2-3. Characterization of Ludox TM-50 Nanoparticles. A) Particle size analysis by dynamic light scattering in water with intensity-weighted Gaussian distribution analysis yields a mean diameter of 38.5 ± 17.2 nm. B) With number-weighted analysis, the mean diameter is 11.7 ± 5.24 nm. C) Transmission electron microscopic image shows particle diameters of approximately 25 nm. Magnification: 200,000x. Scale bar represents 100 nm.

Zeta potential (in water) = -56.5 ± 2.26 mV
Figure 2-4. Absorbance spectra of Ludox® TM-50 nanoparticle dispersions prepared in H₂O.

Figure 2-5. Primary cell explantation from the human umbilical cord. (A) The structure of the human umbilical cord is demonstrated with an H&E stained cross-section. The umbilical cord is composed of two umbilical arteries and one vein supported by the GAG-rich Wharton’s Jelly that confers resistance to compression. (B) Small sections of tissue are dissected from the Wharton’s Jelly and cultured in vitro to create primary explants of Wharton’s Jelly stem cells.
Figure 2-6. Uniaxial mechanical testing: (A) single sheet and (B) hoop stress testing. Uniaxial testing of single sheets is shown from an angle (Ai) and from the front (Aii) of the Intron testing rig. Photo courtesy of author.
CHAPTER 3
PILOT ASSESSMENT OF A HUMAN EXTRACELLULAR MATRIX BASED VASCULAR GRAFT IN A RABBIT MODEL

Herein we describe a small-diameter vascular graft constructed from rolled human amniotic membrane (hAM), with in vitro evaluation and subsequent in vivo assessment of its mechanical and initial biologic viability in the early postimplantation period. This approach for graft construction allows customization of graft dimensions, with wide-ranging potential clinical applicability as a nonautologous, allogeneic, cell-free graft material.

Acellular hAMs were rolled into layered conduits (3.2-mm diameter) that were bound with fibrin and lyophilized. Constructs were seeded with human smooth muscle cells and cultured under controlled arterial hemodynamic conditions in vitro. Additionally, the acellular hAM conduits were surgically implanted as arterial interposition grafts into the carotid arteries of immunocompetent rabbits.

On in vitro analysis, smooth muscle cells were shown to adhere to, proliferate within, and remodel the scaffold during a 4-week culture period. At the end of the culture period, there was histologic and biomechanical evidence of graft wall layer coalescence. In vivo analysis demonstrated graft patency after 4 weeks (n = 3), with no hyperacute rejection or thrombotic occlusion. Explants displayed histologic evidence of active cellular remodeling, with endogenous cell repopulation of the graft wall concurrent with degradation of initial graft material. Cells were shown to align circumferentially to resemble a vascular medial layer. The vascular grafts were shown to provide a

supportive scaffold allowing cellular infiltration and remodeling by host cell populations in vivo. By use of this approach, "off-the-shelf" vascular grafts can be created with specified diameters and wall thicknesses to satisfy specific anatomic requirements in diverse populations of patients.

Introduction

Atherosclerotic cardiovascular disease remains the leading cause of mortality worldwide, with arterial bypass grafting representing an important therapeutic intervention. While autologous arteries have the highest success rates as bypass grafts, inessential and non-diseased arteries are in short supply, such that the need for enduring vascular replacements remains an urgent clinical priority. Tissue-engineering by self-assembly (TESA) represents a strategy for vascular graft assembly that utilizes autologously-derived cells to secrete ECM sheets that can subsequently be rolled into vascular constructs. Motivated by the clinical successes of this approach in non-urgent procedures, herein we investigate a similar, yet expeditious, approach to develop a rolled vascular graft that could be available on an emergent basis for allogeneic use.

Similar to the pioneering TESA cell-sheet technology described by Dr. L’Heureux et al., thin ECM sheets of human amniotic membrane (hAM) can be rolled around a supportive mandrel into multilayered wall conduits. Owing to its perinatal origin, the human amnion does not elicit an adverse immune response even in its native, cellular form, making it an ideal matrix for regenerative therapies. The hAM has the additional advantage of abundant availability, and has been clinically used in other applications with success for over a century. While a cell-mediated fusion of the layers has proven successful in preliminary studies, this study investigated the use
of freeze-drying in conjunction with a fibrin sealant to bind adjacent layers.\textsuperscript{128, 166}

Without the need for pre-population with autologous cells, the conduits would be readily available, easily transportable, and storable extended time periods.

With a rolling approach, vascular graft dimensions can be prepared to custom specification. An acellular graft should be able to withstand surgical implantation, be tolerated by the host, and support host cell remodeling of the scaffold into vascular tissue. As such, our research question was three-fold: 1) can a vascular graft derived from decellularized human amnion mechanically endure initial surgical implantation into arterial systemic circulation, 2) will such a vascular graft survive into the early postoperative period without eliciting hyperacute rejection and/or thrombosis, and 3) will such a vascular construct demonstrate histologic evidence of early cellular remodeling reminiscent of native vascular architecture.

**Methods**

**Experimental Design**

Human amniotic membranes were decellularized and rolled into tubular conduits for \textit{in vitro} and \textit{in vivo} investigation. For preliminary study of early graft remodeling \textit{in vitro}, grafts were seeded with human smooth muscle cells and cultured for 28 days to assess the influence of pulsatile fluid forces on cell-mediated changes to scaffold biochemical and biomechanical composition. For the \textit{in vivo} study, the \textit{hAM} grafts were implanted in rabbit carotid arteries over a 4-week time period to assess initial host tolerance of the graft as well as early cellular remodeling. Study design and methods conform to the \textit{Guide for the Care and Use of Laboratory Animals} (NIH Publication No. 85-23). This study was performed after obtaining approval from the University of
Graft Preparation

Human amniotic membranes were separated from the chorion by blunt dissection and decellularized as previously described using sodium dodecyl sulfate and DNAse I.\(^\text{23, 170}\) hAM ECM was cut into 60-mm x 60-mm sheets, rehydrated with 50 mg/mL fibrinogen and rolled around 3.2-mm outer diameter tubing. The resultant six-layered hAM scaffolds were then treated with thrombin to catalyze the formation of fibrin and freeze-dried. Samples were then washed in successive PBS rinses (5, 15, 40 min, 1 hour, 6 hours) prior to a final freeze-drying step. Samples were rehydrated sterile PBS 30 min prior to implantation.

In Vitro Studies

Cell expansion and culture

Primary human smooth muscle cells were explanted from umbilical arteries and cultured at subconfluent densities as previously described.\(^\text{128}\) Based on previous work that optimized cell seeding density (unpublished), the ablumenal surface of the graft was seeded at a density of 600 cells/mm\(^2\).\(^\text{128}\) Dynamic culture was performed in custom bioreactors connected within a dual circuit perfusion system. Two peristaltic pumps were used to independently control the lumenal and ablumenal flow circuits. Lumenal flow rate and pressure were ramped over the first ten days to reach a final maximum pressure of 120 mmHg and pulse rate of 1 Hz, while the ablumenal flow was maintained at 5 mL/min throughout the duration of the four week culture period.
Biochemical and histological analysis

Samples were digested using 125 µg/mL Papain (Spectrum, Gardena, CA) for 24 h at 60 °C. SMC proliferation was assessed, via quantification of total construct DNA, using the Quanti-iT PicoGreen DNA assay (Invitrogen, Oregon, USA). GAG content was assayed using dimethylmethylen blue (Sigma Aldrich, St Louis MO, USA).\(^{23, 160, 171}\) Acellular scaffolds were imaged under SEM as previously reported.\(^{128}\) Samples were embedded in Neg-50 media, frozen, sectioned and stained with hematoxylin and eosin using standard protocols.

Biomechanical analysis

Tensile properties were assessed using an Instron uniaxial testing rig (Model 5542 Norwood, MA with Version 2.14 software) as previously reported.\(^{128}\) Burst pressure was determined using a modified syringe pump to increase lumenal pressure within the 6-cm long scaffolds until failure. The distal end of each construct was sealed with a pressure transducer immediately downstream of the scaffold to determine the ultimate pressure at failure. Each test was digitally recorded to measure vessel distention at a given pressure using NIH ImageJ software.

In Vivo Studies

Graft implantation

Acellular grafts were prepared from human amnion as described above to match the inner diameter and wall thickness of New Zealand White rabbit carotid arteries (approximately 3.2-mm and 300-µm, respectively). Scaffolds were implanted in the common carotid arteries of Male New Zealand White rabbits using an anastomotic cuff technique as previously described, with polyethylene cuffs (3.0–3.5 kg; n = 3; see Supplemental Figure A-1).\(^{172}\) The four-week length of implantation was selected
following preliminary evaluation of the extent of graft remodeling after one week of implantation (n=1).

All operations lasted approximately 15 minutes on average, used aseptic technique and were performed under an operating microscope. Prior to graft implantation, 1,000 units of IV heparin were administered. Anesthesia was initiated by 30.0-mg/kg IM ketamine hydrochloride and maintained by inhaled isoflurane (2.5–3.0%) through endotracheal intubation. Perfusion through the carotid arteries was recorded using an ultrasonic transducer (model T106, probe 2SB; Transonic Systems Inc, Ithaca, NY). Neurological assessment of each rabbit appraised potential cerebrovascular compromise. No post-surgical medical therapy was necessary.

**Histological analysis**

Explanted tissue was fixed in 10% formalin, paraffin embedded and sectioned. Samples were stained with Masson’s Trichrome using standard histological techniques. Mid-graft sections were used to evaluate the thickness of the wall. Wall thickness was measured using NIH ImageJ software.

**Statistical Analysis**

All data are presented as mean values +/- standard deviation (SD) from at least three independent experiments (n=3) for biochemical assays and at least five independent experiments (n=5) for biomechanical tests. Statistical analysis was performed using a student t-test with significant differences corresponding to a P<0.05 (confidence level ≥95%).

**Results**

Figure 3-1A shows the change in gross appearance between a single dry ECM sheet and laminated sheets of increasing thicknesses. Initial durability testing verified
acellular grafts withstood 24 hours of 1 Hz pulsatile perfusion culture at approximately 80 to 120 mmHg without delamination and had burst pressures of 1430 +/- 250 mmHg. Cross-sectional views of the rolled scaffolds under SEM display the smooth surface of the amniotic basement membrane that forms the ablumenal surface of the tubular scaffold (Figure 3-1B). Association of adjacent layers that form the six-layered wall can be observed at a higher magnification (Figure 3-1Bi). As an example of variation in rolled geometry, scaffolds with internal diameters (ID) of 3.2 mm, 4.2 mm and 6 mm are shown in Figure 3-1C. Figure 3-2A details the overall approach of graft preparation, with panel 3-2Aiv showing a scaffold in a bioreactor for in vitro analysis.

**In Vitro Studies: Cell Repopulation and Early Graft Remodeling**

The schematic diagram of the dual-perfusion circuit is shown in Figure 3-2B. A downstream pressure regulator was used to maintain a maximum lumenal pressure of 120 mmHg from day 10, as shown in Figure 3-2C. Upon initial rehydration the translucent acellular tubular scaffolds collapse, (Figure 3-3A) however, after four weeks of dynamic culture the construct is visibly opaque and able to maintain a tubular conformation without additional mechanical support (Figure 3-3B). A histological assessment of the acellular scaffold (Figure 3-3C) and the dynamically stimulated construct (Figure 3-3D) shows that the initially laminated wall coalesced after 28 days in perfusion culture. Cell densities and glycosaminoglycan (GAG) content each displayed statistically significant increases between days 14 and 28. Cell density increased seven-fold from 13.7 to 97 million cells per gram of the cultured construct and GAG content increased from 5.4 to 37.6 mg/g of wet tissue in the final two weeks of culture (Figures 3-3E and 3-3F, respectively).
Remodeling of the constructs’ laminated walls over the 4-week perfusion culture period results in changes in the representative load-extension profile as compared to acellular grafts, Figure 3-4A. Constructs displayed increased modulus values (stiffer) with a single fracture point when tensioned to failure after four weeks of perfusion culture. Figure 3-4B is a schematic of a stress-strain curve to demonstrate the regions of the curve from which the mechanical properties were derived. After 28 days of perfusion culture, constructs maintained the initial scaffolds tensile properties with trends towards a decreasing physiological modulus (from 120±54.6 kPa to 60±26.0 kPa, Figure 3-4C) and an increasing Young’s modulus (from 694±227 kPa to 1290±593 kPa, Figure 3-4D). No significant changes were noted in the scaffolds’ ultimate tensile strength from 854±272 kPa as an acellular scaffold to 878±447 kPa by the conclusion of the study at 28 days (Figure 3-4E).

**In Vivo Studies: Ultrasound and Histomorphologic Evaluation**

Acellular human-derived grafts were interposed into the carotid arteries of four New Zealand white rabbits. Grafts were described by the surgeons to have acceptable handling properties. Complete reperfusion was visualized grossly and confirmed by ultrasound, Figure 3-5A and 3-5C. No erratic behavior was observed in the neurological assessments and there was no statistically significant change in animal weight. No acute rejection occurred and no thrombotic response was detected by ultrasound. At day 28 explantation, one graft exhibited non-occluding thrombus formation at the distal anastomosis while the other two appeared grossly patent.

Gross appearance of the graft in the longitudinal view is also shown immediately prior to explant, Figure 3-5B. At explantation, grafts were enveloped by a thin, highly vascularized tissue, and displayed pulsation patterns synchronous with the adjacent
native carotid artery. Doppler ultrasound at explant showed graft patency at four weeks (Figure 3-5D). No statistically significant changes were noted in average local blood flow rate through the graft over the course of the implantation (28±21 mL/min at implantation and 23±19 mL/min at four weeks; Figure 3-5E). The mean internal luminal diameter decreased, without statistical significance, from 3.2 to 2.5 mm and the wall thickness of the graft at the midpoint increased significantly from approximately 300 to 500 μm (Figure 3-5F and 3-5G respectively).

Significant graft remodeling was noted on histologic examination between one and four weeks post implantation (Figure 3-6A and 3-6B, respectively). This was evidenced by changes in observable mural cell density, connective tissue deposition, and overall increased consolidation of graft layers over time. The external (adventitial) layers of the construct were histologically notable for a homogenous pattern of organized connective tissue deposition, associated with an interspersed, uniform-appearing population of cells with mild to moderate density throughout the stroma. These cells and connective tissue pattern corresponded to construct layers demonstrating consolidation and coalescence. In contrast, the internal (lumenal) layers of the construct demonstrated a pattern of loose, disorganized connective tissue deposition associated with a high-density cell population. Cell distribution within the wall of the explant is easily visualized in Supplemental Figure A-2A. Circumferentially aligned cellular populations, elongated in the direction of the dotted line in Figure 3-6Bi, appeared to morphologically approximate a vascular medial layer and displayed strong positive reactivity for α-actin (Supplemental Figure A-2B). Figure 3-6C highlights the apparent remnants of the original six-layered hAM graft, reduced to only two discrete
collagen-dense layers. Small capillaries were seen forming in the periphery of the vascular wall, as noted grossly in situ at the time of explant and on subsequent histological analysis (Figure 3-6Ci, arrows). Immunohistochemical study with CD31 demonstrated equivocal reactivity for endothelial elements along the construct lumen, but verified the establishment of peripheral microvasculature (Supplemental Figure A-2B).

Inflammatory cell infiltrate was histologically identified. A patchy distribution of predominately mononuclear and plasmacytoid inflammatory cells were identified within the wall of the construct. Rather than a trans-mural distribution, the inflammatory cells were seen to preferentially infiltrate along the identifiable ECM layers near the lumen, as well as radially along the external adventitial-like zone. Isolated neutrophils and eosinophils were focal and rare.

Discussion

The goal of these investigations was to conduct a preliminary feasibility assessment of utilizing a cell-less, human-derived scaffold in service of small-diameter vascular regeneration. This pilot study assessed, on both an in vitro and in vivo basis, three essential scaffold requirements for guided tissue regeneration: 1) maintenance of structural integrity, 2) lack of thrombosis or hyperacute rejection, and 3) promotion of cellular remodeling to suggest an attempt to approximate arterial vasculature.

The in vitro analyses focused primarily on the feasibility of cellular repopulation in a layered construct and the consequences of the repopulation on the grafts' mechanical stability and biochemical changes under controlled conditions. Unlike the acellular scaffold that collapses post-rehydration without external physical support, cultured constructs displayed a qualitatively robust tubular conformation, with layers coalescing
into a homogenous wall upon gross and histologic inspection. Statistically significant increases in GAG concentration and cell density suggest that the scaffold provides a hospitable environment for cellular integration and proliferation, potentially facilitating extensive ECM remodeling.

The coalescence of graft layers after four weeks in biomimetic culture correlated with increasing material stiffness and ultimate tensile strength. Biomechanical parameters were on the same order of magnitude as those described of healthy human coronary arteries, which served as the target vessel for graft replacement during in vitro investigation. Karimi et al. found that healthy human coronary arteries have elastic moduli of 1.55±0.26 MPa and ultimate tensile strengths of 1.44±0.87 MPa (as compared to the 1.29±0.59 MPa and 0.88±0.45 MPa, respectively, found in the present study). Physiological modulus of the cultured constructs, 0.06±0.03 MPa, was lower than that of published data for native human vessels, 1.48±0.24 MPa, and the acellular scaffolds, 0.120±0.0546 MPa, suggesting the construct is more compliant during early stages of remodeling when compared to the native vessel. This is consistent with increasing GAG concentration within the collagenous construct. However, vascular compliance is dynamic and known to be adaptive to the hemodynamic environment.

The in vivo investigations served as a pilot proof-of-principle for targeting specific anatomical locations, evaluating early scaffold remodeling, and testing dynamic fatigue. The acellular grafts endured the mechanical forces associated with surgical implantation and with the immediate exposure to the hemodynamic stress of systemic circulation. In this study, rabbits were immunocompetent and no post-surgical pharmacological treatment was administered. No hyperacute rejection of the biomaterial
was noted clinically (as confirmed by the lack of vascular compromise on Doppler ultrasound following implantation), and all grafts remained patent over 28 days. No cerebrovascular compromise was noted. The histologic paucity of neutrophils and eosinophils suggests the absence of a nonspecific acute inflammatory response and allergic reaction, respectively. The absence of multinucleated foreign body giant cells formation was noted, suggesting the lack of a classic foreign-body response to the graft.

Histologic analysis showed organized remodeling of the hAM layers, with complete cellular integration of circumferentially-aligned, elongated, alpha-actin positive population of cells, interspersed amid newly-formed collagen. The pattern of connective tissue deposition from ablumen to lumen appears consistent with organized and still-active stromal remodeling, with appreciable capillary network formation in the graft periphery. It is interesting to note that the pattern of “early” connective tissue deposition near the lumen is associated with yet-discrete construct layers, suggesting a temporal heterogeneity to mural remodeling, with lumenal consolidation occurring as a late-stage event.

The described rolling methodology allows for a significant degree of patient-centered graft customization, effectively expanding the clinical applicability of this amnion-derived biomaterial. Fibrin adhesives can also be manufactured from a human origin, improving the feasibility of clinical translation. Diameter, length, wall thickness and shape can be easily adjusted to generate custom vascular grafts for routine or complex vascular reconstruction in a variety of anatomical sites and patient populations. This could include arteriovenous grafts to allow hemodialysis access for patients suffering from end stage renal disease, coronary artery bypass grafts to
prevent or palliate ischemic heart disease, and personalized grafts for modified Fontan’s procedures to treat complex congenital heart disease in pediatric populations.

Important areas for future work include detailed evaluation of graft functional remodeling capacity (e.g., via phenotypic assessment of both stromal and inflammatory cellular elements), as well as study of graft longevity in vivo. These preliminary investigations focused on early remodeling to characterize the initial thrombogenicity and cellular integration whereas longer in vivo analysis would be necessary to determine the resolution of these early remodeling events. Longer implantation times would additionally help to elucidate the biological significance of the trending increase in wall thickness, potentially differentiating between progressive graft stenosis versus transient phenomena of early remodeling.

**Conclusions**

This approach has multiple advantages: the material is derived from abundantly available human ECM and it can be readily prepared into an acellular graft of a wide range of geometries. With endogenous biological components, the ECM is readily degradable and, in theory, should allow for in situ growth. This growth potential makes the scaffold particularly appealing for use in various applications, from treatment of adult coronary artery disease to vascular reconstruction of complex congenital heart disease in pediatric patients. As a preliminary investigation, these works have demonstrated that grafts possess adequate mechanical integrity, postoperative immunologic tolerance lasting at least four weeks, and evidence of cellular remodeling approximating the native vascular architecture. Further investigation that details cellular behavior, inflammatory infiltration and graft longevity will aid in our understanding of in situ graft remodeling. As human tissue in a rabbit model, the human amnion-derived ECM scaffold showed signs
of constructive remodeling and, with further investigation, holds promise as a platform for allogeneic vascular tissue reconstruction.

Figure 3-1. ECM scaffold characteristics. Laminated amnion-derived ECM scaffolds allow tailored geometries for guided tissue regeneration. A) Flat sheets of lyophilized sheets of amnion with increasing number of layers are visualized by increasing opacity. B) SEM cross-section of a scaffold wall comprised of six lyophilized layers shows the degree of layer-to-layer interaction, better visualized with 10x magnification (Bi). C) Three tubular scaffolds of different geometries, from left to right, with internal diameters (ID) of 3.2 mm, 4.2 mm and 6 mm. Figure adapted with permission from the Journal of Vascular Surgery.
Figure 3-2. Graft preparation and *in vitro* conditioning. A) Decellularized amniotic membranes (Ai) were freeze-dried, followed by rehydration with fibrinogen (Aii). Scaffolds were then rolled and thrombin was applied to catalyze the cross-linking. Lastly, the scaffolds were freeze-dried (Aiii). Scaffolds were mounted in bioreactors (Aiv) prior to seeding. B) Constructs mounted in bioreactors were connected in the dual-perfusion circuit as shown schematically. C) An incremental lumenal flow regime was applied to allow time for initial cellular adhesion to the scaffold surface. As the flow rate was increased, the check valve was used to regulate the pressure of the flow circuit such that a maximum lumenal pressure of 120 mmHg was obtained 10 days following cell seeding. The ablumenal flow was held constant at 5 mL/min throughout the study. Figure adapted with permission from the Journal of Vascular Surgery.
Figure 3-3. *In vitro* morphological and biochemical evaluation. A) Hydrated, acellular scaffolds were translucent in appearance and collapsed upon rehydration, but after four weeks in dynamic perfusion culture constructs were grossly opaque (B). C) Histological sections of the initial laminated acellular scaffold wall (with high powered view inset), was shown to coalesce following 28 days in perfusion culture (D; hematoxylin-eosin). E) A significant 7-fold increase in cell density (from 13.7 to 97 million cells per gram of wet tissue from the cultured graft) occurred between 14 and 28 days of culture. F) Similarly, glycosaminoglycan (GAG) content increased significantly from 5.4 to 37.6 mg/g of wet tissue between days 14 and 28. Figure adapted with permission from the Journal of Vascular Surgery.
Figure 3-4. Biomechanical properties of *in vitro* constructs. A) Representative failure profile of graft ringlets tensioned until failure show typical failure profiles of acellular grafts displayed a lower failure point than constructs cultured for four weeks in pulsatile perfusion bioreactors. Constructs cultured for 28 days displayed a distinct failure point. B) Biomechanical parameters were calculated from the regions highlighted in a stress-strain curve schematic. Biomechanical analysis of grafts *in vitro* showing the C) physiological modulus, D) Young’s modulus, and E) the ultimate tensile strength of the acellular scaffolds (before culture) and after 28 days of biomimetic culture with SMC. Figure adapted with permission from the Journal of Vascular Surgery.
Figure 3-5. *In vivo* pilot study. Human amnion-derived grafts were interposed in rabbit carotid arteries. Gross morphology: longitudinal view of a representative graft A) at implantation and B) at harvest 28 days later. At explantation, grafts were surrounded by a loose thin cover of highly vascularized tissue. The blue tubing in B represents a 12.6 mm length. Analysis by Doppler ultrasound: representative longitudinal views of grafts at implantation and harvest (C and D, respectively) reveal the patency of the graft after 4 weeks. The white arrowheads indicate the location of the polyethylene cuffs. E) Mean flow rate at implantation (‘Implant’) and after 28 days (‘Harvest’) does not change significantly. F) The internal diameter (ID) of the lumen decreased, but not significantly but the G) average wall thickness was significantly increased. Figure adapted with permission from the Journal of Vascular Surgery.
Figure 3-6. Histological evaluation of explanted grafts. A-C) Histology of graft explants at one (A) and four weeks (B and C) stained with Masson's Trichrome. Cell population through the total thickness of the wall is seen in B, with circumferential alignment (as indicated by the dashed line in Bi) and newly deposited ECM demonstrating the extent of remodeling that occurs between weeks one and four. C) Progressive inward remodeling of the layered graft was seen and the remaining discernable layers of the original graft wall are indicated by the numbered arrows near the lumen. At higher power (Ci), the arrowhead highlights red blood cells and arrows point to longitudinal cross-sections through newly formed capillaries. Figure adapted with permission from the Journal of Vascular Surgery.
CHAPTER 4
BINDING EXTRACELLULAR MATRICES IN AQUEOUS ENVIRONMENTS USING SILICA NANOPARTICLES

Robust binding of soft tissues in aqueous environments remains a challenge in both the clinical setting of minimally abrasive wound closure, and in the fabrication of scaffolds within regenerative medicine. Some approaches in regenerative tissue engineering utilize 3-dimmensional scaffolds that mimic the intended tissues structure, whilst supporting cellular infiltration and guiding tissue regeneration. Using a planar extracellular matrix (ECM) derived from human amniotic membrane, these investigations create multilayered scaffolds. Although this scaffold is relevant to versatile tissue-engineering applications, as a proof of concept, these investigations focus on the development of a tubular scaffold in service of vascular tissue regeneration. In order to maintain structural integrity within the aggressive hemodynamic environment the laminated structure requires stable binding without the use of toxic treatments or cross-linkers that limit innate healing.

By adsorbing silica nanoparticles (SiNP) to the scaffold at the macromolecular level, we explore the ability of SiNP to enhance the binding of multilayered tubular ECM scaffolds. Rectangular sheets of human amniotic membrane were decellularized using 0.1% w/v SDS, treated with SiNP, rolled and subsequently lyophilized to generate scaffolds with tubular geometries. Mechanical properties were assessed using hoop stress testing in order to define binding strength and mechanism of failure. Results show that SiNP-treated tubular ECM grafts maintain their shape upon rehydration, increase tensile strength, and change the mechanism of failure from interfacial slipping to single-point fracture. The ability to construct adherent layers of ECM capable of
withstanding aqueous environments would provide means to develop scaffolds of complex geometries with tunable mechanics.

**Introduction**

*Ex vivo*-derived tissue scaffolds have shown promising preclinical and clinical results, but their therapeutic application has been limited by their native anatomic geometry. For example, the human umbilical vessels have been investigated for use as scaffolds in the context of nerve\textsuperscript{178,179}, musculoskeletal\textsuperscript{158} and vascular\textsuperscript{180} tissue engineering. Human umbilical vessels possess many favorable properties in service of tissue regeneration, including minimal immunogenicity, endogenous cell adhesion sites, and with appropriate preparation, retention of their native vascular ultrastructure and extracellular matrix (ECM) components.\textsuperscript{28} However, these vessels have limitations as scaffolds, in that their diameter is set and subject to biological variability. As the inner diameter of the human umbilical vein (HUV) is approximately 4-6-mm, it is not suitable for utilization as a scaffold for smaller-diameter applications, such as coronary or carotid artery bypass – both areas of great clinical need. Thus, the ability to produce *ex vivo*-derived scaffolds with customizable dimensions, without the use of harsh chemical treatments that may compromise their ultrastructure and biocompatibility, would harness the benefits of *ex vivo*-derived tissue, while extending the range of clinical applicability.

Our approach is to roll devitalized amniotic membrane to develop a tubular scaffold for vascular tissue regeneration.\textsuperscript{128} With this strategy, tubular scaffolds with tunable diameters and wall thicknesses can be achieved to match the existing vasculature.\textsuperscript{166} This perinatal tissue possesses many of the same beneficial attributes as the HUV, and has also been shown to exhibit anti-inflammatory properties.\textsuperscript{181} Preclinical investigation of this rolled, tubular scaffold in a rabbit model showed that
stabilization with a fibrin sealant and lyophilization resulted in a scaffold that 1) was not acutely rejected and 2) exhibited extensive remodeling over the four-week implantation.\(^{153}\) Despite these encouraging preliminary results, the surgeons noted that the graft required delicate handling during surgical implantation in order to prevent delamination. A critical determinant of success is the robust binding of the mural layers to withstand surgical implantation, aqueous environments, and cyclic radial stress encountered in the circulatory system.

While treatment with chemical cross-linkers such as glutaraldehyde has been noted to bind and stiffen ECM matrices, it has also renders tissues inert and thus incapable of constructive cellular repopulation and remodeling, with aneurysmal formation a common adverse outcome with its application.\(^{112, 182}\) Medical adhesive technology is an active area of research, where the clinical demand for biocompatible glues capable of binding soft tissue in aqueous environments is currently driving the identification and study of novel compounds. As such, Leibler et al. reported on the use of commercially available silica nanoparticles as a biocompatible adhesive for tissues and hydrogels.\(^{183}\) The principle is based on the theory of nanoparticle adsorption to the polymeric ECM components between tissues, so as to form an adhesive layer that dissipates energy when the polymeric strands are strained, increasing adhesive strength.\(^{184}\) A successful scaffold for tissue regeneration relies on physicochemical properties to achieve biocompatibility and to guide cellular remodeling.

The focus of this work was to evaluate the physical material properties of a silica nanoparticle-augmented ECM scaffold; while past studies have noted preliminary biocompatibility, future investigations may further evaluate its biological implications.\(^{185}\)
Our primary objective was to study the concentration-dependence of silica on ECM-to-ECM adhesion, and its resultant influence on the physical properties of the bulk SiNP-ECM composite scaffold. To do this, we assessed the dose-dependence of silica on ECM-to-ECM binding by evaluating hoop strength and radial mechanical properties of scaffolds. These mechanical tests were repeated at six months to evaluate the stability of the adhesion in an aqueous environment. To isolate the influence of SiNP within the scaffold (as opposed to at the interface of each mural layer), tensile mechanics of single sheets were tested.

**Methods**

**Preparation of Amniotic Membrane Scaffolds**

Placental tissues were obtained from the Labor & Delivery department at UF Health Shands Hospital at the University of Florida (Gainesville, FL, IRB approval #64-2010). Amnion were isolated from the chorionic membrane by blunt dissection and washed in DI H₂O to begin the decellularization process, modified from Wilshaw et al.\(^{23, 153, 166}\). Briefly, amnion were devitalized by incubation in 0.1% sodium dodecyl sulfate (SDS) in PBS under agitation for 24 h. SDS was removed from the amnion in three subsequent PBS washes, and treated with 50 U/mL deoxyribonuclease I in DI H₂O supplemented with 10 mM MgCl₂ for 2 h. The devitalized amnion were washed in PBS, strained and cut into 6 cm x 10 cm rectangular sections for rolling. Manufactured Ludox TM50 colloidal silica nanoparticles (SiNP), were diluted in DI H₂O to the desired concentration immediately prior to use (Sigma-Aldrich). These are monodisperse, 25-nm diameter particles with a strong negative surface charge.\(^{187}\) Planar, rectangular hAM-ECM were treated with uniform application of SiNP dispersions (5 μL/cm²) then rolled for ten revolutions around the 3.2 mm diameter mandrel. Concentrations are
expressed as dry micrograms of nanoparticles applied per square millimeter of amnion.

Rolled scaffolds were frozen at -86°C for 12 h and lyophilized for 24 h (Millrock Technology, Kingston, NJ). Initial mechanical testing assessed scaffolds with a range of SiNP concentrations (0 μg/mm², 4.38 μg/mm², 8.75 μg/mm², 17.5 μg/mm² and 35 μg/mm²), while the remaining experiments compared the mechanically optimized nanoparticle concentration (8.75 μg/mm²) to control scaffolds. 35 μg/mm² corresponds to the stock concentration of Ludox silica nanoparticles (0.7 g/mL).

**Mechanical Characterization**

Tensile properties were assessed using an Instron uniaxial testing rig (Model 5542 Norwood, MA, Instron) with a 50 N load cell. 5-mm segments of 10-layered rolled grafts or 10 cm x 1.5 cm single-layered strips were hydrated in PBS for 24 h prior to testing. Samples were progressively tensioned to reach a preloaded force of 5 mN, then tensioned until failure at a constant rate of 5 mm/min (tubes) and 2 mm/min (strips). Sections of the same rolled grafts were stored at room temperature in PBS under sterile conditions for six months, and similar hoop stress testing evaluated stability of the binding to extended exposure to aqueous environments. Load and displacement values were normalized to attain stress-strain curves. Stress (σ) was calculated based on the following formula: σ = F/A where F is the recorded load and A is the cross-sectional area of the sample normal to the applied force. Yield strain was defined to be the strain at the first occurrence of a stress-strain slope equal to zero. Strain at final hoop rupture was defined to be the strain at which the force dropped below 5 mN.

Suture retention strength tests were performed as indicated by the ANSI 7198 standard. Tubular scaffolds prepared with 8.75 μg/mm² and without nanoparticles were hydrated and cut into 2-cm segments (n=5). Each specimen was clamped onto the
base of the Instron mechanical testing rig. A single 3-0 vicryl taper SH suture was looped through one wall, 2 mm from the end of the vessel segment, secured to the upper grip and pulled at a constant rate of 80 mm/min. Suture retention strength was determined as the maximum force recorded prior to suture pullout.

**Scanning Electron Microscopy (SEM) Imaging**

Samples of 10-layered tubular scaffolds were prepared both with and without 8.75 μg/mm² SiNP in H₂O, dehydrated by lyophilization as described above and sectioned using a sharp blade to obtain a transverse section from the center of the graft. Samples were sputter coated with palladium-gold (DeskV, Denton Vacuum, Moorestown, NJ), and imaged via scanning electron microscopy at 3.0 kV (Hitachi SU-5000 FE-SEM).

**Silica Saturation and Retention**

Stability of nanoparticle-ECM fiber interaction in solution was studied both with and without pre-wash lyophilization. Weighed sections of freeze-dried, acellular amnion were incubated for 60 s in 0.7 mg/mL Ludox TM-50 under gentle agitation. Half of the samples were subsequently incubated in H₂O on an orbital shaker plate for 30 min. The other samples were lyophilized for 48 h prior to the 30 min incubation in H₂O. Following incubation, samples were lyophilized, weighed and silica retention was determined based on the change in weight following the wash in water using the following equation:

\[
\text{Silica retained (μg/mm}^2\) = (W – W₀)/ \text{scaffold dimensions}
\]

Where \(W\) represents the dry weight of the scaffold following the wash and lyophilization of the SiNP-doped sample and \(W₀\) is the initial dry weight of sample.
Statistical Analysis

Experiments were run in no less than triplicate, with five samples at minimum per condition tested for mechanical evaluation. Results are reported as mean ± standard deviation. A student t-test was used to test the means for statistical significance when only one condition was evaluated against a control. One-way ANOVA was used in all other cases unless otherwise specified to determine differences between scaffolds treated with a ranging concentration of nanoparticles. When significance was detected by ANOVA (P < 0.05), Tukey post-hoc analysis was used to determine significant differences between each tested condition. Statistical significance was set at α = 0.05.

Results

Ludox TM-50 silica nanoparticles were assessed for their ability to bind extracellular matrices in aqueous environments, in an effort to evaluate their potential as an adhesive for fabrication of ex vivo-derived tissue scaffolds directed toward applications in tissue engineering and regenerative medicine. Rectangular sections of devitalized human amniotic ECM, treated with silica nanoparticles (0-35 μg/mm²), were rolled into tubular scaffolds and subsequently lyophilized (Figure 4-1). Scaffolds’ mechanical properties were assessed in order to evaluate the concentration-dependent effect of the silica nanoparticles.

Biomechanical Analysis of SiNP-Augmented Scaffolds: Hoop Stress Testing

Functional assessment of the scaffolds’ mechanical robustness was determined by tensioning segments of the 10-layered tubular samples until failure (Figure 4-2). Young’s modulus and peak stress displayed similar SiNP concentration-dependent trends. Control samples (without nanoparticles) failed without appreciable rupture at an average of approximately 0.5 ± 0.5 MPa, and continued to unravel until ultimate failure.
Increased Young’s modulus and peak stresses were seen with intermediate nanoparticle concentrations of 4.38 through 17.5 μg/mm² SiNP, with no statistically significant differences identified between conditions in either mechanical parameter (Figure 4-2A and B). However, at the highest tested concentration, 35 μg/mm² SiNP, a significant decrease in peak stress and modulus were observed relative to the intermediate conditions (2.5 ± 1.6 MPa and 3.2 ± 2.4 MPa, respectively), albeit still significantly greater than control values (0.51 ± 0.50 MPa and 0.98 ± 1.0 MPa, respectively). Strain at both initial yield and final hoop rupture were evaluated (Figure 4-2C). Marked differences between initial yield and final hoop rupture were noted to occur in the control samples, where the yield point occurred at lower strain values than final failure, after unraveling. The same was observed, to a lesser degree, in samples saturated with high nanoparticle concentration. Representative stress-strain curves (Figure 4-2D), and photographs of control (0 μg/mm² SiNP) and 8.75 μg/mm² SiNP scaffolds (Figure 4-2E and F, respectively) provide comparative visualization of stress-strain profiles and gross appearance immediately following rupture.

**Durability of SiNP-Mediated Binding Following Extended Hydration**

Tensile hoop tests were repeated on samples stored in PBS (at room temperature) for six months to evaluate binding strength after extended durations in an aqueous environment, (Figure 4-3). Control samples (without nanoparticles) had delaminated at the six-month time period and were unable to be tested. Insufficient lengths of 35 μg/mm² SiNP grafts were stored and thus were not included in these investigations. To facilitate comparison of the results after six months, results from the initial tests (24 h post hydration) are repeated on the graphs (Figure 4-2A and B). All conditions had decreased Young’s moduli and peak stress after six months. Scaffolds
prepared with 4.38 μg/mm² and 8.75 μg/mm² SiNP had decreased Young’s moduli and peak stress after six months. Scaffolds prepared with 17.5 μg/mm² SiNP did not have statistically significant changes in these mechanical properties. After six months, the Young’s modulus of the 4.38 μg/mm² SiNP scaffolds decreased by 37%, whereas the 17.5 μg/mm² SiNP scaffolds only decreased by 10% (Figure 4-3C). Comparison between the peak stress after six months hydration and 24 h are presented in Figure 4-3D.

**Biomechanical Analysis: Bulk Material Properties**

Based on the results of the hoop stress analysis and qualitative handling properties all remaining experiments used the optimized SiNP concentration of 8.75 μg/mm². The tensile strength of single amnion sheets was evaluated to assess the effect of nanoparticles on the bulk material properties independent of layer-to-layer binding (Figure 4-4). Qualitatively, SiNP samples did not stretch or contract laterally as control tissue, but maintained the J-shaped stress-strain curve of viscoelastic soft tissues (Figure 4-4A, B and C). Similar to the hoop strength tests, samples with SiNP at 8.75 μg/mm² displayed a significant increase in tensile strength and stiffness compared to control scaffolds (Figure 4-4D and F).

**Comparative Assessment: Gross Observations, Suture Retention Strength and SEM Analysis**

Upon hydration, tubular scaffolds without nanoparticles collapsed and required careful handling to prevent delamination. By contrast, hydrated scaffolds treated with SiNP maintained a robust tubular structure on a bench top, even when taken out of solution (Figure 4-5A and 4-5B). SiNP treated samples exhibited a significant increase in suture pullout force compared with controls (164 ± 41 to 114 ± 14 grams-force,
respectively; Figure 4-5C). SEM imaging revealed the association of the mural layers (Figure 4-6), demonstrating that SiNP treatment results in layers that, in most locations, are more closely associated with adjacent layers, with each layer appearing moderately thicker and denser when compared to the untreated control.

**Silica Retention to Amnion ECM under Agitation**

To better understand the non-linear relationship between increasing SiNP concentration and scaffold mechanical strength, maximal SiNP retention was investigated. Single layers of freeze dried hAM samples were incubated in a concentrated SiNP dispersion (0.7 g/mL), then either rinsed immediately or refreeze-dried then rinsed. Scaffolds rinsed immediately after the initial SiNP incubation period retained $-0.12 \pm 0.24 \mu g/mm^2$ SiNP, whereas scaffolds freeze-dried prior to washing retained $26.7 \pm 5.55 \mu g/mm^2$ SiNP (Figure 4-7).

**Discussion**

Tubular scaffolds were generated by rolling sections of devitalized amniotic membrane and stabilized using silica nanoparticles. It was seen that the nanoparticles reinforce the ECM protein matrix both at the interface of two distinct ECM surfaces (as discovered by the hoop testing) and within the ECM itself, as evidenced by the testing of the mechanical properties of single sheets. The change in mechanism of failure from interfacial slipping to distinct fracture is equal in importance to the enhanced hoop strength in those composite SiNP-hAM scaffolds as it demonstrates a change from adhesion failure—the layers of the wall are behaving similar to that of a bulk material. Furthermore, this failure occurs well beyond the forces that it would encounter naturally in the cardiovascular system. SEM evaluation shows that there is no appreciable
difference in thickness between the scaffold walls, however, there is less apparent void space between each layer in the SiNP-ECM composite scaffold.

The stiffness of the control sample, 0.98 ± 1.1 MPa, was most similar to published values of Young’s modulus found in healthy human coronary arteries, 1.55 ± 0.26 MPa.\textsuperscript{173} SiNP-ECM scaffolds were significantly stiffer, but were similar to the 8.0 ± 3.0 MPa Young’s modulus reported by a different research group for human internal thoracic arteries.\textsuperscript{190} These published values for human arteries may differ due to differences in testing methodology or perhaps due to differences in the tissue source. However, it is well known that following implantation, the graft is remodeled by host cells rendering the mechanical parameters of the graft a dynamic process. Furthermore, this relatively increased graft stiffness may serve a purpose in future development of this scaffold. For example, the membrane could be perforated prior to rolling to enhance mass transport of nutrients and cellular repopulation, and still maintain acceptable hoop strength with deceased scaffold stiffness.

The enhancement in mechanical strength was not linearly proportional to concentration of the applied nanoparticle solution, but was optimized in the range of 4.5 μg/mm\textsuperscript{2} to 17.5 μg/mm\textsuperscript{2}. At 35 μg/mm\textsuperscript{2}, a statistically significant drop in peak stress and Young’s modulus is evident. This is hypothesized to occur due to oversaturation of the nanoparticles in between the layered collagenous wall preventing the nanoparticles to bridge polymeric chains on adjacent ECM layers.

This corresponds to the results quantifying the maximum amount of silica retained in the amnion matrix. It was found that when the scaffold is saturated in a high concentration silica nanoparticle solution, 26.7 μg/mm\textsuperscript{2} of silica are retained. Another
interesting finding was that for the scaffold to retain the nanoparticles within the scaffold in an aqueous environment, the treated scaffold must first be lyophilized. Samples similarly incubated in high concentration nanoparticle solution that were not first lyophilized retained no detectable silica within the matrix. This suggests that the lyophilization step results in chemical changes to the nanoparticles resulting in enhanced SiNP-scaffold interactions able to withstand agitated washing. This provides insight into how the nanoparticles bind ECM in aqueous environments and the necessity of the lyophilization step; it suggests SiNP are not physically entrapped within the amniotic membrane ECM fibers, nor strongly adsorbed to the ECM until the composite scaffold is freeze-dried, resulting in stronger physiochemical ECM-SiNP interactivity. However, it is worth mention that investigations into silica retention were done using planar sections of amnion. In rolled scaffolds treated with 35 μg/mm², 100% efficiency in nanoparticle coating of the scaffold is not likely, but physical entrapment of lyophilized nanoparticles might be possible within the layered scaffold. Decreased peak strength and stiffness at the highest tested concentration, again supports that dense application of nanoparticles prevents cohesion of the mural layers.

By testing the mechanical strength of the scaffolds after six months in an aqueous environment, bond durability was assessed. These investigations demonstrated resistance of the bond to extended periods of hydration. Decreased mechanical integrity over that timeframe was anticipated for an ECM-based scaffold as the matrix proteins degrade over time due to enzymatic or hydrolytic degradation. Implanted, cells originating from the host would likely infiltrate the scaffold and contribute to matrix remodeling, as is known to occur in biodegradable scaffolds.
Hydrated SiNP-ECM scaffolds retain a tubular shape and do not collapse when taken out of solution. This qualitative enhancement to scaffold handling properties was reinforced by the suture retention strength: 164 ± 41 gmf, of the SiNP-ECM composite graft, is comparable to the suture retention strength of the internal thoracic artery reported in the literature, 138 ± 50 gmf.192

Future work needs to be done to evaluate how the change in scaffold stiffness influences cell-scaffold interactions and cellular remodeling of the tissue construct.

Figure 4-1. Scaffold development. (A) Process flow diagram for scaffold development. Steps iv, v and vi are detailed below in photographs: (B) nanoparticle solution is applied uniformly to the amnion except on the section that will become the lumenal surface. (C) The rectangular section of amnion is rolled around the mandrel to create a tubular scaffold. (D) Lyophilized scaffolds created with two diameters (3.2 mm, Left; 6.35 mm, Right) demonstrate ease in customizing scaffold size using this approach. (E) The change in gross appearance of between 3.2 mm-diameter scaffolds without nanoparticle treatment (far Left) and with scaffolds created with increasing concentrations of applied nanoparticle solutions are best appreciate following hydration. Scale bar: 1 cm. Photos courtesy of author.
Figure 4-2. Biomechanical characterization of 10-layered scaffold ringlets. Tubular, 5-mm graft segments of scaffolds prepared with increasing concentrations of SiNP were tensioned at 5 mm/min until failure. (A) Young’s Modulus, (B) peak stress, and (C) strain at both yield and failure show a non-linear response between SiNP dose and tensile mechanical properties. (D) Representative stress-strain curves show SiNP-treated scaffolds (solid line) have enhanced mechanical strength compared to control scaffolds (without SiNP, dashed line). (E) Photograph of representative scaffold without nanoparticles shows failure by interfacial slipping, while (F), nanoparticle-treated scaffolds (4.38 – 17.5 μg/mm²) failed by fracture. Photos courtesy of author.
Figure 4-3. Stability of SiNP-ECM scaffolds in aqueous environments for extended periods. Biomechanical characterization of 10-layered scaffold following six-month incubation in H$_2$O. (A) Young’s Modulus and (B) peak stress of scaffolds hydrated for six months are compared with scaffolds hydrated for only 24 h. Percent similarity of the mechanical parameter between the differing periods of hydration is reported at the top of each concentration of silica. Control samples, without nanoparticle treatment, had delaminated by the six-month time point.
Figure 4-4. Uniaxial tensile biomechanics of decellularized amnion sheets. Photographs of representative ECM sheets mid-test (A) without nanoparticles and (B) with nanoparticles (8.75 μg/mm²). (C) Representative stress-strain curves. Statistically significant differences exist between grafts with and without nanoparticle treatment in the following parameters: (D) Young’s Modulus, (E) change in pre- and post-test width, and (F) Peak stress. Photo courtesy of author.
Figure 4-5. Improvement in handling properties of SiNP-treated scaffolds. Photographs of 10-layered scaffolds (hydrated, 3.2 mm diameter): (A) without SiNP, and (B) with SiNP (8.75 μg/mm²). (C) Suture retention strength of 10-layered scaffolds is improved with nanoparticle treatment. Photo courtesy of author.
Figure 4-6. Scanning electron micrographs of cross-sectional view of the 10-layered scaffold wall (A) without SiNP and (B) with SiNP (8.75 μg/mm²). Micrographs at increased magnification are below each condition: (C) without SiNP and (D) with SiNP.
Figure 4-7. SiNP retention on the decellularized amnion following 30 min agitated wash. SiNP-doped samples were either washed immediately (labeled: “Without FD”) or washed after lyophilization (labeled: “FD”).
CHAPTER 5
BIOCOMPATIBILITY OF SILICA-ECM COMPOSITE SCAFFOLDS

Introduction

Through early studies and optimization we have found a concentration at which nanoparticles not only augment the mechanical strength, but also change the mode of failure from interfacial slipping to fracture in a tensile hoop stress test. As promising as this new technology is for the mechanical integrity of the scaffold, the biocompatibility, specifically the cyto- and hemocompatibility, have yet to be extensively tested. As we are altering the chemical composition of the amnion ECM with both the decellularization and nanoparticle treatment, it is important to study how this will influence the way cells and blood will interact with the scaffold, with particular attention paid to the impact this approach will have on the material’s activation of circulating platelets and other elements of coagulation.

Modulating Material Thrombogenicity

Blood compatibility is arguably the more important determinant of success for a vascular prosthesis, especially in the application of small-diameter vessels (ID < 6 mm). Blood compatibility is influenced by material surface roughness, and chemical composition of the surface, among other variables. The endothelium in healthy vessels has a negative charge due to GAGs and polysaccharides such as chondroitin sulfate and heparin sulfate.\textsuperscript{193} There have been many different approaches attempted in the effort to obtain nonthrombogenic surfaces. Approaches include the development of a quiescent endothelium, surface modification by covalent attachment of known anti-coagulants such as heparin or direct inhibitors of thrombin,\textsuperscript{194, 195} and incorporation of drug-eluting elements into the scaffold itself. Alternatively, approaches to promote the

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development of a quiescent endothelium are also being explored. For example, methods incorporating vascular endothelial growth factor (VEGF) on the graft surface aim to promote endothelialization of the surface.

*In vitro* testing of biomaterial thrombogenicity is also an evolving field. There is no standardization in testing methodology with respect to directives on which anti-coagulant to use (if any) when drawing blood, which blood components to use (whole blood or isolated platelets), or testing conditions (i.e. static versus dynamic). Most studies use static methods of evaluation, such as the Lee-White method for measuring clotting time. However, shear stress is known to influence the coagulative response, prompting research into dynamic methods of testing platelet-material interactions. Our lab has previously reported a method to evaluate cell-material interactions in real-time under shear conditions using a modified parallel plate flow chamber; this method is used in this study to evaluate the dynamics of platelet-material interaction.

**Previous Studies of Silica Cytotoxicity**

Toxicity has been found to be dependent on physiochemical properties and experimental methodology including route of administration, particle size, porosity, particle surface area, and surface charge. With so many variables influencing cytotoxicity, the toxic effects of silica nanoparticles remains an area of debate. Some studies emphasize the biocompatibility of silica, sometimes even using silica as a negative control for cytotoxicity. Other studies have identified silica nanoparticles as being toxic to cells. However, in the application of an interfacial adhesive, nanoparticles will effectively be bound within a collagenous matrix, thus limiting cellular exposure.
**Methods**

Human amnion was decellularized and rolled into ten-layered scaffolds as described in chapter two, with several notable exceptions. First, scaffolds were rolled with the amniotic sub-epithelial layer oriented upward such that it would comprise the lumenal surface. As such, the smooth, sub-epithelial basement membrane layer was examined in the subsequent experiments for cyto- and hemocompatibility. Scaffolds were prepared using similar silica nanoparticle concentrations as in Chapter four (0 μg/mm², 4.38 μg/mm², 8.75 μg/mm², 17.5 μg/mm² and 35 μg/mm²). Figure 5-1A-D shows photographs of the rolling process.

**EC Isolation and Culture**

Human umbilical vein endothelial cells (HUVEC) were isolated from cords using collagenase perfusion as described by Jaffe et al.\textsuperscript{203} Prior to reaching confluence, primary EC were passaged using Accutase with 0.5 mM EDTA (Innovative Cell Technologies). Experiments used mixed EC populations collected from at least three donor cords. Media was replenished every two to three days with VascuLife basal medium supplemented with VEGF LifeFactors kit (LifeLine Cell Technologies) and 100 U/mL penicillin/streptomycin. HUVECs were used experimentally between passages P2 and P4.

**HL-60 Cell Culture**

HL-60 cells (a human promyelocytic leukemia cell line) transfected with a lentiviral vector expressing green fluorescent protein (GFP) were generously donated by Dr. Christopher Cogle at the University of Florida. Cells were cultured in suspension in DMEM supplemented with 20% FBS and 100 U/mL penicillin/streptomycin.
Evaluation of Cytocompatibility

HUVECs on tissue culture plastic (TCP)

Approximately 50,000 HUVECs (P4) were seeded into each well of 24-well plates. After cells reached a confluent monolayer, the culture medium was replaced with freshly prepared 0.5 mL SiO$_2$ nanoparticle dispersions diluted in the culture medium (0.1 μg/mL, 1 μg/mL, 10 μg/mL, 100 μg/mL, and 1 mg/mL). Cells without exposure to nanoparticles (0 μg/mL) served as a control. The cells were incubated for 24 hours at 37°C in a 5% CO$_2$ incubator. At 24 hours, the nanoparticle-doped media was aspirated from the wells and the cells were gently washed with PBS and incubated with 10% Alamar Blue in media for four hours at 37°C to quantify relative metabolic activity. Supernatants were collected and fluorescence was read at excitation 530 nm, emission 590 nm by a Synergy 2 multi-detection microplate reader (BioTek Instruments, Inc., Winooski, Vermont, USA). Percent reduction data was normalized to the control and presented as percent cell viability.

Following quantification of metabolic activity, cells were washed with PBS and stained using the Live/Dead cell viability assay according to manufacturers protocol (Invitrogen). Multidimensional image acquisition was used to take images using channels to record calcien AM (green; living cells), ethidium homodimer-1 (red; dead cells), and DAPI (blue; cell nuclei). All conditions were performed in triplicate.

HUVECs on scaffolds

Ten-layered scaffolds (3.2 mm diameter) were aseptically prepared both without and with SiNP (8.75 μg/mm$^2$). Scaffolds were opened longitudinally and then cut into 1 cm x 1 cm sections. Scaffolds were placed in 24-well plates, pre-wetted with DPBS, and seeded with HUVECs (approximately 50,000 cells/well). After allowing cells to adhere
overnight, seeded constructs were transferred into fresh wells (such that the cells adhered to the well plate would not interfere with the results of subsequent study). Twenty-four hours after seeding the scaffolds, metabolic activity was assayed using Alamar Blue as described above. Metabolic activity was similarly assayed on day three, five, seven and fourteen. Results are shown percent increase in metabolic activity relative to the metabolic activity on day one. Endothelial cell media was changed every two to three days.

**Transmission Electron Microscopy**

Lyophilized, tubular scaffolds prepared with Ludox TM50 silica nanoparticles (8.75 μg/mm²) and without nanoparticles were fixed in glutaraldehyde. Fixed samples were embedded in a plastic mold, sectioned on an ultramicrotome and mounted on copper grids. The samples were stained with uranyl acetate and lead citrate and imaged on a JEM-1400Plus Transmission Electron Microscope (JEOL USA, Inc.).

**Contact Angle Measurements**

Lyophilized, tubular scaffolds were opened along the longitudinal axis using a scalpel (prepared with 0 μg/mm², 8.75 μg/mm², 17.5 μg/mm², 35 μg/mm², and 70 μg/mm² SiNP). Sections were opened and fixed to a base with the lumenal surface facing up. Drops of distilled H₂O (25 μL) were pipetted onto the lumenal surface of the scaffold and immediate photographed using a Nikon D200 (Melville, NY). The angle at the interface of the droplet and scaffold surface was quantified using NIH ImageJ software (Bethesda, MD).

**Leukocyte Detachment Assay**

HL-60 cells were induced to differentiate towards neutrophil-like phenotypes by adding 1.3% dimethyl sulfoxide (DMSO) to the culture medium for three days. Samples
of grafts were prepared and cut longitudinally and then mounted into custom modified parallel plate flow chambers that accommodate tissue surfaces and real-time visualization in a fluorescent microscope through a transparent viewing window to study scaffold-cell interactions (Figure 5-1E and F). In the flow chamber, 8 cm x 1 cm acellular hAM scaffolds were oriented with the denuded basement membrane oriented upward. Differentiated HL-60 cells (20,000 cells/cm²) were incubated on the scaffolds for five hours at 37°C, and imaged at 15 locations along the scaffold. PBS was then perfused through the chamber for five minutes at a constant rate of 1 mL/min to achieve a shear stress of 10 dyn/cm². Images were taken at a single point every 15 seconds to quantify the temporal neutrophil detachment from the scaffold. After the five minutes, the scaffold was again imaged at 15 locations. Neutrophil detachment was quantified from the images using ImageJ software (NIH).

**Blood Collection and Processing**

Human venous blood was collected from healthy adult volunteers whom had given their IRB-approved informed consent. Fifty (50) mL blood was collected in tubes containing 10 U of heparin per mL using a 21-gauge needle and carefully handled prior to use to avoid activation of platelets and lysis of RBC. All participants acknowledged they had not taken medication that alters platelet function in the ten days prior to blood collection.

**Dynamic Evaluation of Platelet-Material Interaction**

Whole blood was used for dynamic evaluation of platelet-material interactions. Whole blood was fluorescently labeled using acridine orange (20 μg/mL) incubated for 10 min (Invitrogen, Cat. No. A3568; 10 mg/mL solution). A syringe pump perfused a steady, laminar flow of blood over the graft in custom designed tissue-based parallel
plate flow chambers, and into a waste container for five minutes. The flow rate was 1 mL/min to achieve a shear stress of 10 dynes/cm². Samples were visualized on Zeiss AxioImager M2 upright fluorescence microscope with a Zeiss AxioCam Hrm Rev 3 digital camera operated by AxioVision software version 4.8; images were taken at 15 second intervals. After five minutes of whole blood perfusion, the scaffold was gently washed with perfused PBS for one minute (0.5 mL/min). Images were then taken at 15 locations along the length of the scaffold. NIH ImageJ software was used to quantify number of platelet aggregations (defined as a grouping of three or more platelets, and assumed to be caused by activated platelets), and percent coverage (by utilizing thresholding in NIH ImageJ software).

**Assay for Hemolysis**

Whole blood was centrifuged to separate the packed red blood cells (RBC) from the buffy coat and plasma (200 g for 5 min). Packed RBCs were diluted in the ratio of 0.2:4 RBCs:PBS to create a hematocrit of approximately 5%. 200 μL diluted RBCs were incubated with 800 μL of freshly prepared SiNP dispersions diluted in PBS (0.1 μg/mL, 1 μg/mL, 10 μg/mL, 100 μg/mL, and 1 mg/mL). Samples prepared with 800 μL of water and PBS served as positive and negative controls, respectively. Samples were incubated at room temperature under gentle agitation for two hours, and subsequently centrifuged at 200 g for 5 minutes. Ruptured RBCs stained the plasma red by the release of hemoglobin. The absorbance of the supernatant at 541 nm was used to quantify the percent of lysed RBCs using the following formula: %hemolysis = 

\[
\frac{[(\text{absorbance of the sample} - \text{negative control sample}) / (\text{positive control absorbance} - \text{negative control absorbance})]}{100}.^{204}
\]
Results

Evaluations of Cytotoxicity

After direct exposure to SiNP at a large range of doses, HUVEC cell viability detected by assay of metabolic activity revealed dose-dependent cytotoxicity (Figure 5-2). At SiNP concentrations of 10 μg/mL and under, HUVEC metabolic activity was not affected. At 100 μg/mL, approximately 50% of the cells were viable. At 1 mg/mL, there were no viable cells detected.

TEM imaging allowed for high magnification of scaffold ultrastructure. Figure 5-3 shows control scaffolds and scaffolds treated with nanoparticle dispersions (at the concentration determined in Chapter 4 to be the optimal concentration to enhance scaffold mechanics, 8.75 μg/mm²). Distinct layers of ECM and nanoparticles can be visualized. Nanoparticles are not dispersed evenly in the radial direction of the scaffold, and are not identified within the denser collagenous regions of the ECM.

Scaffolds were prepared and cut into 1 cm² sections as shown in Figure 5-4A. HUVECs cultured on SiNP-doped scaffolds did not exhibit the same decrease in cell viability as seen when HUVECs on TCP were directly exposed to SiNP. HUVECs on SiNP-doped scaffolds exhibited higher metabolic activity than HUVECs on control samples on days five, seven and fourteen (Figure 5-4B). HUVECs on silica-treated scaffolds displayed increases in metabolic activity through day seven and then remained unchanged between day seven and day fourteen.

Scaffold Wettability

Surface wettability is known to influence hemocompatibility of materials. Contact angle measurement of water on scaffolds treated with silica nanoparticles (8.75 μg/mm², 17.5 μg/mm², 35 μg/mm², and 70 μg/mm²) served as an assay of surface
wettability. It was found that there was a trending increase in surface hydrophobicity with increasing nanoparticle concentration. Control scaffolds were hydrophilic (contact angle < 90°) with a mean contact angle of 74° ± 10° (Figure 5-5). All grafts treated with nanoparticles were hydrophobic.

**Differentiated HL-60 Detachment**

Differentiated HL-60 cells were incubated on scaffolds for five hours and then imaged along the scaffold. Then, scaffolds were perfused with saline at 10 dyn/cm² for five minutes and again imaged. Leukocyte detachment was quantified and the results are shown in Figure 5-6. Initial leukocyte attachment to the SiNP-treated scaffolds and control scaffolds were not statistically different. After 5 minutes of perfusion, more cells were washed from the silica-treated scaffolds relative to the controls.

**Platelet Adhesion**

Scaffolds were mounted in the modified parallel-plate flow chambers and perfused with whole blood at 10 dyn/cm² for five minutes. Images were collected every 15 seconds to quantify dynamic platelet adhesion and aggregation over the five minute time period. Figure 5-7 shows the dynamic percent surface coverage by platelets and an index of aggregation size (% surface coverage divided by number of aggregates). It was observed that in the first two minutes, nanoparticle-treated scaffolds had lower surface coverage and little to no aggregation, but by the end of the five minutes, there were no significant differences between the conditions. The index of aggregation size demonstrated trends of increasing size of platelet aggregates in all scaffolds.

**SiNP-Induced Hemolysis**

Packed red blood cells were incubated with nanoparticle dispersions prepared in DPBS (0.1 μg/mL, 1 μg/mL, 10 μg/mL, 100 μg/mL, and 1 mg/mL). Packed red blood
cells incubated with DPBS and H₂O served as the negative and positive controls, respectively. Disruption to the RBC membrane results in hemoglobin release into the supernatant, and allowed for quantification of nanoparticle-induced damage to RBCs (Figure 5-8). No detectable RBC lysis was seen in samples with concentrations of 10 μg/mL and under. When incubated with silica nanoparticles at higher concentrations (100 μg/mL), hemolysis was observed. However, hemolysis does not occur to a significant degree until SiNP concentrations of 1 mg/mL are incubated with red blood cells.

**Discussion**

When utilized as an adhesive for building ECM scaffolds in the context of regenerative medicine, nanoparticles are bound to proteins within the matrix. As the scaffold is degraded following implantation, nanoparticles will be released into the bloodstream, making contact with native cells. The physiochemical properties of the nanoparticle surface will most likely altered and thus cytotoxicity, and more generally, biocompatibility, would best be evaluated through *in vivo* study. However, these investigations aimed to achieve a preliminary understanding of the potential toxicity that silica nanoparticles and silica nanoparticle-stabilized ECM grafts may cause, and to elucidate the concentration at which these nanoparticles are toxic.

Toxicity of silica nanoparticle on endothelial cells was evaluated in two approaches: direct exposure of HUVECs to an array of concentrations of nanoparticle dispersions, and by culture over time on scaffolds prepared with nanoparticles. Direct exposure to cells on tissue culture plastic demonstrated toxicity at much lower nanoparticle concentrations (100 μg/mL, or with 0.5 mL per well, and each well having a surface area of 2 cm², 0.25 μg/mm²) as compared to those cultured on scaffolds
prepared with nanoparticles 8.75 μg/mm² (5 μL of silica dispersions of 175 mg/mL), which demonstrated no apparent toxicity to the cells. Interestingly, before the HUVEC-seeded constructs were moved to new wells, it was found that cells on the TCP surrounding the SiNP-enhanced constructs demonstrated altered morphology around the tissue (Supplemental Figure A-3).

Both methods of evaluating cytotoxicity yield valuable information, but their limitations must be addressed. Culturing cells directly on the scaffolds is more representative of how the cells and scaffolds (and thus nanoparticles) would interact in the application of a tissue scaffold; however, it is also important to identify any potential toxicity that may result from cell interaction with unmodified nanoparticles. Although cells cultured on nanoparticle-doped scaffolds help to evaluate potential toxicity and influence to cellular behavior, it is important to keep in mind that this model is two-dimensional and static, whereas the actual graft in vivo would represent a three-dimensional construct in a dynamic environment. As such, the two-dimensional model may not be perfectly representative of the toxicity afforded by nanoparticles in a dynamic, in vivo context.

The ability of the cells to survive and grow for two weeks on the scaffold may partially be explained by the TEM images, which show the nanoparticles remain between the layers of the scaffold and might not come into direct contact with the cells. However, with the surface wettability and differences in growth patterns observed between 8.75 μg/mm² scaffolds and the control, it is evident that the addition of nanoparticles is changing the physiochemical makeup of the surface, even if they are not diffusing through layers.
The addition of nanoparticles changes the ex vivo-derived scaffold from hydrophilic to hydrophobic in a concentration-dependent manner. There is evidence that surface wettability plays an important role in hemocompatibility of materials as it influences protein adsorption and thus thrombogenicity.\textsuperscript{205} Albumin and fibrinogen have small negative charges at physiological pH, thus resulting in minimal adsorption to silica surfaces.\textsuperscript{206} Plasma proteins, especially fibrinogen, are believed to mediate platelet adsorption to surfaces and thus, in turn are responsible for thrombus formation.\textsuperscript{207} This may represent the mechanism responsible for the delay in platelet activation on nanoparticle-treated scaffolds.

\textit{In vitro} immunogenicity testing tends to have a focus on monocyte and lymphocyte recruitment and phenotype. However, neutrophils have been shown to play an important role in xenograft rejection.\textsuperscript{208} In an effort to predict how SiNP may influence or contribute to graft rejection, neutrophil behavior was studied. While there was no detected difference in initial adherence of neutrophil-like cells to nanoparticle-treated scaffolds and controls, the cells were less strongly adherent to the nanoparticle scaffolds as fewer cells remained attached following perfusion.

Niknejad et al. studied the hemocompatibility of the amniotic membrane in relation to PTFE and found that both surfaces of the amnion demonstrated minimal platelet activation and no hemolysis.\textsuperscript{82} To study how the addition of nanoparticles to the scaffold may influence graft thrombogenicity, whole blood was perfused over the scaffold and platelet adhesion and aggregation (as a marker of platelet activation) was dynamically quantified. It was found that SiNP\textsuperscript{+} grafts resisted platelet aggregation for approximately 90 seconds, and overall had fewer aggregates, but that each aggregation
was relatively larger compared to those on the control scaffolds. Control scaffolds demonstrated a pattern of consistent aggregation from multifocal origins, whereas SiNP+ scaffolds typically had one, or several larger foci that began to form after approximately 90 seconds and then increased in size similar to those of the control scaffolds.

Vascular graft materials must not be hemolytic. As the scaffold is degraded, one possible fate of the nanoparticles is that they may be released into the blood stream. Ludox TM50 silica nanoparticles at concentrations of 10 μg/mL and lower do not result in any direct toxicity to cellular membranes of RBCs. There is likely toxicity to RBCs at concentrations higher than 100 μg/mL, as hemoglobin is detectable in the 100 μg/mL condition, but not significant until concentrated an additional order of magnitude. Study of nanoparticle-induced hemolysis has similar limitations as the *in vitro* testing of cytotoxicity discussed earlier for nanoparticles used in this application; after interaction with ECM-protein of the graft, interstitial fluid, and proteins within the blood, the nanoparticles are likely to be modified, with a high probability that the particle surface charge, size and other physicochemical properties will be altered.

With the results from these investigations, we have identified that there is a dose-dependent cytotoxicity and hemotoxicity of unaltered Ludox TM50 nanoparticles. However, when entrapped within the ECM of the scaffold, the tolerance of cells to higher concentration of nanoparticles is increased. Furthermore, there is some evidence that the nanoparticles confer decreased thrombogenicity relative to the control scaffolds. However, testing the hemocompatibility and cytotoxicity would need to be tested in preclinical trials to make definitive conclusions.
Figure 5-1. Preparing nanoparticle-enhanced ECM scaffold methodology. A) Rectangular section of decellularized amniotic membrane ECM is cut and placed with the sub-epithelial layer oriented upward. B) One revolution around a 3.2 mm OD tube is rolled and nanoparticle dispersion is applied to the membrane (175 mg/mL; 5 μL/cm²). C) After the nanoparticle dispersion is evenly spread over the membrane, the membrane is rolled to 10 layers. D) Rolled amnion grafts are then frozen at -86 °C and lyophilized. E) For material surface testing, lyophilized scaffolds are opened longitudinally and placed in the custom bioreactor shown in (F). Photo courtesy of author.
Figure 5-2. Cytotoxicity of Ludox TM50 silica nanoparticles on HUVEC in two-dimensional culture. A) Percent viability of HUVECs exposed to nanoparticles for 24 hours at an array of silica nanoparticle concentrations. B) Cells after removal of nanoparticles, stained with Live/Dead stain at corresponding silica concentrations shows living cells (green), dead cells (red) and cell nuclei (blue). Images were taken at 20x magnification.
Figure 5-3. Transmission electron microscopy (TEM) images of nanoparticle-enhanced scaffolds. Control samples without nanoparticles show the layered amniotic ECM on the left (A,C,E), with micrographs of nanoparticle-ECM grafts at each corresponding magnification on the right (B,D,F), where discrete layers of ECM and nanoparticles can be visualized. Each row increases in magnification. Images (A) and (B) are taken at 5,000x magnification, scale bar represents 2 μm. (C) and (D) are higher power images at 12,000x, scale bar represents 1 μm. The interface of the nanoparticles with the amniotic ECM is better visualized at 30,000x magnification of (F), with (E) showing the control at the same magnification, scale bar: 500 nm. G) shows the nanoparticles at the interface at 100,000x magnification, scale bar: 100 nm.
Figure 5.3. Continued
Figure 5-4. Cytotoxicity of HUVECs cultured on silica nanoparticle-enhanced grafts over two weeks. A) Grafts prepared with silica nanoparticles and control grafts were prepared, cut into 1-cm² sections, and cultured with HUVECs for two weeks. B) Metabolic activity over time, relative to the metabolic activity at 24 hours shows the % increase in metabolic activity of the cells on the construct over time. Symbols: * represents significant differences between the two conditions at the specified time point; ♯ indicates significant increase relative to previous time point. Photo courtesy of author.
Figure 5-5. Scaffold wettability as a function of increasing nanoparticle concentration. A) Surface contact angle with water was measured on scaffolds prepared with increasing nanoparticle concentrations. A1) Photograph demonstrating methodology of measuring the contact angle. B) Representative photographs of water droplet on surface of each condition. Photographs courtesy of author.
Figure 5-6. Leukocyte detachment following saline perfusion. A) Quantification of differentiated HL-60 cells attached to the scaffold surface after 5 hours of static culture (time = 0) and after 5 minutes of exposure to constant flow (time = 5). B) Representative photomicrographs of GFP⁺ leukocytes (green) attached to control (top row) and SiNP-treated grafts (bottom row), before and after exposure to five minutes of flow at 10 dyn/cm² (left and right, respectively).
Figure 5-7. Characterization of platelet attachment and aggregation. A) Dynamic quantification of platelet surface coverage over SiNP-treated grafts compared to control grafts over five minutes. B) The percent surface coverage normalized by number of aggregations serves as an index of aggregate size. C) Representative images show acridine orange-stained blood cells adhered to the scaffold during the five minutes of exposure to flow of whole blood at 10 dyn/cm².
Figure 5-8. Quantification of hemolysis induced by silica nanoparticle dispersions. A) Percent hemolysis at increasing nanoparticle concentrations as determined by the absorbance of the supernatant at 541 nm. B) Corresponding representative photographs of each condition shows the hemoglobin released into the supernatant when RBC membrane integrity was compromised. Negative and positive controls are shown on the left (RBC incubated in PBS and water, respectively). Photo courtesy of author.
CHAPTER 6
CONCLUDING REMARKS AND FUTURE DIRECTIONS

Summary

Tissue scaffolds derived from ex vivo tissues, such as the human umbilical arteries, have notable benefits that make them well suited for vascular grafting. Being of extraembyronic origin, these tissues afford a unique degree of immune privilege; additionally, they exhibit intrinsic cell binding sites and already possess the native microarchitecture and structural organization of the target tissue. However, the diameter of such scaffolds is predetermined by their anatomy, and is subject to inter-individual biologic variability. As a base scaffold-material to create tubular vascular scaffolds, the human amniotic membrane demonstrates many of the same benefits of the human umbilical vein, and additionally overcomes the limits of the vein by virtue of the rolled approach that produces it. Through the rolled approach, the scaffold's diameter, wall thickness, and even geometry can be customized to suit the targeted native vasculature. Thus, the applicability of the amniotic membrane, rolled into a tubular scaffold for vascular tissue regeneration, was studied.

One of the most important factors for the success of this rolled approach is effective binding of the mural layers. This not only allows the graft to withstand the aggressive hemodynamic environment, but also improves its handling properties, in turn allowing for enhanced stability during surgical implantation. This problem has been addressed in previous research investigations by our lab. It was clear that an effective interfacial tissue adhesive to bind the mural layers into a cohesive unit would be paramount for the success of the approach. Chapter 3 describes one of the tissue adhesives we explored, fibrin glue, an enzymatic cross-linker. In this chapter, a fibrin-
stabilized rolled amniotic membrane graft was studied \textit{in vitro} and implanted as an arterial interposition graft in rabbit carotid arteries with the goal of evaluating the ability of the scaffold to guide vascular tissue regeneration. With these investigations we found that the amnion-based graft was supportive of cellular adhesion and proliferation. Additionally, human-derived grafts were tolerated in rabbits without immunosuppressive therapy without adverse inflammatory reaction or thrombotic occlusion. After one month, mural layers had coalesced and were populated with endogenous cellular populations. The results of this preclinical pilot study were promising for use of the amnion-based graft. However, it was discerned from the study that the handling properties, although deemed acceptable by an experienced vascular surgeon, were suboptimal and could be improved. Thus, fibrin sealant, although demonstrated excellent biocompatibility, did not meet the mechanical criteria for an interfacial adhesive in this application.

The search for a more mechanically robust interfacial adhesive, effective in hydrated conditions and on porous soft tissues, identified reports of colloidal silica nanoparticles utilized to adhere hydrogels. In Chapter 4, the mechanical properties of silica-ECM composite scaffolds were investigated for a range of silica concentrations in an effort to both optimize the physical properties of the vascular scaffold, and also to explore the mechanism by which the silica nanoparticles were binding to and enhancing the composite scaffold. It was found that there was a non-linear dose dependence on silica concentration and enhancement of ultimate tensile strength. However, perhaps more important than the enhanced mechanics was the change in mechanism of failure, from interfacial slipping of the mural layers to discrete fracture of the tested ringlets.
Additionally, qualitatively, the scaffolds were much easier to handle and retained their tubular shape even when removed from hydration.

After identifying an adhesive that met the mechanical demands of the scaffold by effectively binding layers of ECM and improving its handling properties, it was necessary to evaluate the biocompatibility of the silica nanoparticle adhesive. This was explored in Chapter 5. Cytotoxicity was evaluated by directly exposing endothelial cells grown in two-dimensional culture on tissue culture plastic to silica nanoparticles. Cell viability was unchanged until exposed to concentrations of 100 μg/mL or greater. Cells cultured on SiNP-ECM scaffolds were viable and grew for two weeks. Hemocompatibility was also evaluated and similarly, a concentration-dependent toxicity ultimately observed.

Future directions to advance this work are proposed below.

**Future Directions**

**Modifications to Modulate Cellular Remodeling of the Scaffold**

**Engineering micro-porosity to enhance cellular migration**

Rapid cellular infiltration throughout the thickness of the wall of the vascular graft is controlled by driving factors such as the availability of nutrients, and is important for constructive vascular remodeling. As the addition of silica nanoparticles in service of binding the layers of the wall markedly increases the mechanical strength and modulus of the graft, the introduction of patterned perforations in the amnion prior to rolling should be investigated as a way to both enhance cellular migration and modulate the compliance of the graft. It is believed that mismatched compliance between grafting materials and the native vasculature is partly responsible for the development of intimal hyperplasia.\(^{209}\) Perforations could be added via an automated process such as by using
a CO₂ laser (an example of laser-patterned microporosity is shown in Figure A-4), or by manually puncturing the membrane using needles fixed to mechanically “stamp” a pattern of perforations without the use of heat.

**Simplifying scaffold processing – generating pre-populated tissue engineered constructs**

Recently, distinct populations of amniotic membrane cells have been investigated for their use as stem cells in regenerative medicine as knowledge about their stemness is evaluated.²¹⁰ Perhaps, as the amnion is pre-populated with mesenchymal stem cells, the decellularization and sterilization steps may be unnecessary, provided sufficient cell populations can tolerate treatment with silica nanoparticles (or a different tissue adhesive) and lyophilization. Procured amnion would need to be washed in saline and antibiotics and would likely require patterned microporosity to encourage cellular migration. While this approach would require much optimization and preliminary investigation, it may confer a further advantage to this rolled approach in generating vascular allografts as the ECM would be altered less than in the current approach and it would take advantage of the stem cells resident to the amniotic membrane.

**Exploration of bioactive nanoparticles**

The identification of silica nanoparticle suspensions as an effective adhesive opens new avenues for advancing the functionality of the scaffold. Nanotechnology is an evolving field with great potential for exciting applications. The nanoparticle adhesive could be developed to serve a dual purpose. For example, a suspension of mesoporous silica nanoparticles could be loaded with growth factors, pharmaceuticals (such as anti-coagulants, etc) or functionalized to enhance biocompatibility. Another potential avenue of investigation is to utilize nanoparticles as a tool to allow non-invasive imaging.²¹¹
Pre-Clinical Evaluation

While constructive remodeling was noted in a pre-clinical trial of fibrin-stabilized amniotic membrane grafts (described in Chapter 3), it would be necessary to confirm that this rapid remodeling is conserved in the silica nanoparticle-stabilized grafts (described in Chapters 4 and 5). Furthermore, long-term implantations would better help predict host response and feasibility of the approach.
Figure A-1. Scaffold implantation technique. Grafts were interposed in rabbit carotid arteries using an anastomotic cuff technique previously described by Jiang et al.\textsuperscript{172} 1.0 cm of the common carotid artery was resected (i). A cuff was placed around each end (ii), the native artery was everted over the cuff, and fixed using suture ties (iii). Lastly, wrapped sutures were used to secure the graft around the everted arteries (iv).
Figure A-2. Immunohistochemical study of explanted grafts. A) DAPI (blue) staining of cell nuclei shows the distribution of cell density throughout the wall. B) Merged photomicrograph of α-actin (red) and the endothelial marker CD31 (PECAM-1; green) in the vascular media-like zone of the graft displayed positive reactivity for alpha-actin with localized CD31 positivity corresponding to microvasculature. Magnification A = 5x, B = 20x.
Figure A-3. HUVEC elongation and alignment perpendicular to nanoparticle-doped scaffold. Photomicrographs of HUVECs seeded on scaffolds were imaged on a light microscope 12 hours post-seeding. A) and (C) show a straight edge and corner of control scaffolds (without nanoparticles). B) and (D) show the straight edge and corner of nanoparticle-enhanced scaffolds. Scale bar represents 500 μm.
Figure A-4. Engineered microporosity. Photo of laser micropatterned pores of two different sizes (left). The pore size can be visualized by microscopy (right). Photo courtesy of author.


BIOGRAPHICAL SKETCH

Leslie Alexis Goldberg was born in Arlington, Texas in 1987. She attended Rice University (Houston, Texas) and graduated in 2009 with a Bachelor of Science in bioengineering with a focus in bioinstrumentation, imaging and optics. During her undergraduate training, she served as a research assistant in the lab of Dr. Michael Raghunath at the National University of Singapore during the summer of 2007, and in the lab of Dr. Cherie Stabler at the University of Miami in 2008. Upon completing her Bachelor of Science, she had the opportunity to conduct research in the Brain Computer Interface Institute at Tsinghua University in Beijing, China during the 2009-2010 academic year as a Chinese Government Scholar.

She began her studies in the MD-PhD dual degree program at the University of Florida College of Medicine (Gainesville, Florida) in August 2010. After completing the first two years of her medical education, she began her graduate studies with her advisor, Dr. Peter S. McFetridge, in the Tissue Engineering and Regenerative Medicine Laboratory. She received her Doctor of Philosophy from the University of Florida Department of Biomedical Engineering in December 2016.