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AUTHOR CONTRIBUTION:

Dr. Reiffel: Data collection and analysis, manuscript preparation

Mr. Perez: Data collection and analysis

Dr. Fullerton: Data collection

Mr. Lekic: Data collection

Dr. Campbell: Data collection

Dr. Spector: Data analysis, manuscript preparation, overall project supervision

FINANCIAL DISCLOSURE:

None of the authors hold any commercial associations or conflicts of interest to disclose.

PRODUCTS:

1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (Advanced ChemTech Inc.; Louisville, KY)

4',6-diamidino-2-phenylindole dilactate (Invitrogen; Carlsbad, CA)

Acetic acid (EMD Chemicals; Gibbstown, NJ)

Alginate sodium (NovaMatrix; Ewing, NJ)

Alloderm (LifeCell; Branchburg, NJ)

Amphotericin B (MP Biomedicals; Solon, OH)

AxioCam MRm digital camera (Carl Zeiss Microscopy; Jena, Germany)

Basic Fibroblast Growth Factor (AA 1-155 Recombinant Human; Invitrogen)

Calcium chloride (Sigma-Aldrich; St. Louis, MO)

Calcium sulfate (Alfa Aesar; Ward Hill, MA)

CellLight Actin-GFP BacMam 2.0 (Invitrogen)

Chitosan (Spectrum Chemicals & Laboratory Products; Gardena, CA)

Collagen type I (MP Biomedicals)

DiI-Ac-LDL (Biomedical Technologies, Inc.; Stoughton, MA)

Dulbecco's Modified Eagle's Medium (Mediatech; Manassas, VA)

Endothelial mitogen (MP Biomedicals)

Fetal bovine serum (Mediatech)

Fibronectin active fragment (Peptides International; Louisville, KY)

Glycerol (VWR; Radnor, PA)

Hanks' Buffered Salt Solution (Lonza; Walkersville, MD)

Heparin sodium (Sigma-Aldrich)

Human aortic smooth muscle cells (Invitrogen)

Human umbilical vein endothelial cells (Invitrogen)

ImageJ software (National Institutes of Health; Bethesda, MD)

Integra® (Integra LifeSciences Corporation; Plainsboro, NJ)

LSM510 (Carl Zeiss Microscopy)

Matrigel (BD Biosciences; San Jose, CA)

Media 199 (Invitrogen)

N-hydroxysulfosuccinimide (Thermo Fisher Scientific; Waltham, MA)

Neonatal human dermal fibroblasts (Invitrogen)

Neutral buffered formalin (VWR)

Nunc Lab-Tek™ II 2-well Chamber Slide (Thermo Scientific; Rochester, NY)

Olympus Fluoview FV1000MPE multiphoton microscope (Olympus Corporation of the Americas; Center Valley, PA)

Penicillin/streptomycin (Mediatech)

Phosphate buffered saline (Mediatech)

Sodium hydroxide (Agilent Technologies, Inc.; New Castle, DE)

XenMatrix (Daval; Warwick, RI)

ZEN [Zeiss Enhanced Navigation] software (Carl Zeiss Microscopy)

Vascular Smooth Muscle Cell Optimization of Vasculogenesis within Naturally Derived, Biodegradable Hybrid Hydrogel Scaffolds

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Abstract

BACKGROUND—As vascularization represents the rate-limiting step in permanent incorporation of hydrogel-based tissue-regeneration templates, we sought to identify the material chemistry that would optimize endothelial cell adhesion and invasion into custom hydrogel constructs. We further investigated induction of endothelial tubule formation by growth factor supplementation and paracrine stimulation.

METHODS—Hydrogel scaffolds consisting of combinations of alginate, collagen type I, and chitosan were seeded with human umbilical vein endothelial cells (HUVEC) and maintained under standard conditions for 14d. Cell density and invasion were then evaluated. Tubule formation was evaluated following basic fibroblast growth factor (bFGF) addition or co-culture with human aortic smooth muscle cells (HASMC).

RESULTS—HUVECs demonstrated greatest cell-surface density and invasion volumes with alginate+collagen 10:1 w/w scaffolds ($p<0.05$). Supplementation with bFGF increased surface-density but neither invasion nor tubule formation. A significant increase in tubule content/organization was observed with increasing HASMC:HUVEC ratio co-culture.

CONCLUSIONS—Alginate+collagen 10:1 scaffolds allow for maximal cellularization compared with other combinations studied. Growth factor supplementation did not affect HUVEC invasion or morphology. Paracrine signaling via co-culture with HASMC stimulated endothelial tubule formation and vascular proto-network organization. These findings serve to guide our future endeavors towards fabrication of pre-vascularized tissue constructs.

INTRODUCTION

Biocompatible hydrogels have recently emerged as an attractive building block from which to fabricate artificial tissue replacements, both in the form of random-porosity tissue regeneration templates or potentially pre-vascularized three-dimensional tissue constructs. In contrast to commercially available tissue regeneration scaffolds comprised of decellularized dermis of allogenic or xenogeneic origin (i.e., Alloderm® or XenMatrix®, respectively), biocompatible hydrogels can be fabricated from a broad range of materials into virtually any shape or size, are readily chemically modified to suit specific purposes (i.e., via covalent growth factor addition),¹ and are sufficiently porous to allow for more rapid vascularization.²

Despite their frequent use, commercially available hydrogel-based tissue regeneration templates are limited not only by their low tensile strength (and resultant poor surgical handling characteristics), but also by the rate at which they become vascularized. As vascularization represents the rate-limiting step in permanent incorporation of implanted

constructs,³ any means of accelerating this process could potentially result in meaningful clinical advances in patient care. Accordingly, the specific material chemistry that promotes maximal endothelial cell adhesion and invasion into hydrogel-based tissue regeneration scaffolds (which translates to more rapid vascularization, scaffold incorporation, and ultimately wound healing) has not yet been definitively identified.

We therefore evaluated *in vitro* multiple candidate biocompatible biodegradable hydrogel combinations in an effort to identify the hydrogel material chemistry that would optimally support endothelial cell adhesion as well as invasion into the construct bulk. Based upon these data, we next investigated the potential for induction of endothelial tubule formation within our “optimal” hydrogel by utilizing *in vitro* techniques designed to simulate the complex signaling environment of the “vasculogenic” milieu, namely exogenous growth factor supplementation with basic fibroblast growth factor (bFGF) and paracrine instruction via co-culture techniques with vascular smooth muscle cells.

MATERIALS AND METHODS

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were cultured in Media 199 supplemented with 20% fetal bovine serum (FBS), 90µg/mL heparin sodium, 50µg/mL endothelial mitogen, 1% penicillin/streptomycin (P/S), and 2.5mg/L amphotericin B. Human aortic smooth muscle cells (HASMC) were cultured in Media 199 supplemented with 20% FBS, 90µg/mL heparin sodium, 25µg/mL endothelial mitogen, 1% penicillin/streptomycin, and 2.5mg/L amphotericin B. Cells were maintained in an incubator at 37°C in a humidified environment containing 5% CO₂.

Hydrogel and Scaffold Preparation

Alginate solution was prepared from alginate sodium 4% w/v in phosphate buffered saline (PBS) and sterilized via filtration (0.2µm). Alginate hydrogels were fabricated by cross-linking alginate solution with autoclaved calcium sulfate 2% w/v (CaSO₄) in deionized water (dH₂O) combined in a 2:1 ratio. Chitosan 4% w/v solution was prepared by dissolving powdered chitosan in acetic acid 0.01% v/v, titrating to pH 7.0 with sodium hydroxide (NaOH), and filter sterilization. Type I collagen 1% w/v solutions were prepared by dissolving collagen type I in filtered acetic acid 0.01% over ice and titrating to pH 7.0 with NaOH. Hybrid hydrogels containing alginate and chitosan, alginate and collagen, or alginate, chitosan and collagen were prepared by combining alginate with chitosan and/or collagen at the desired ratio prior to calcium crosslinking.

Immediately after the addition of CaSO₄, scaffolds were cast by injecting 1mL of the final solution into each well of a 2-well chamber slide, resulting in scaffolds approximately 20mm × 20mm × 2mm in dimension. Scaffolds were then immersed in calcium chloride (CaCl₂) 1% w/v in PBS overnight and allowed to cure.

Additional collagen 1% and alginate 4% scaffolds were prepared for the purpose of surface characterization. Alginate scaffolds were crosslinked as above, while collagen scaffolds were crosslinked at 37C for 30 minutes. Following crosslinking, scaffolds were immersed in Media 199 prior to imaging.

Surface Characterization

The surfaces of Alginate 4% and collagen 1% scaffolds were imaged with an Olympus Fluoview FV1000MPE multiphoton microscope. A femtosecond-pulsed titanium/sapphire laser at 780 nm was used to excite the specimen. Optical sections were obtained using a

water immersion 10× magnification objective. Color coding and post-processing of images was completed using ImageJ software.

RGD Modification

Scaffolds were RGD (Arg-Gly-Asp) modified according to the methods of Rowley et al.⁴. Briefly, 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (sulfo-NHS) were combined in a 2:1 ratio in 40mL dH₂O. The reaction was allowed to proceed for 5 minutes prior to the addition of 1mg fibronectin active fragment (RGDS). Scaffolds were then submerged in this solution for 20 hours.

Following modification, scaffolds were rinsed several times with Hanks' Buffered Salt Solution (HBSS) and allowed to equilibrate in the appropriate cell culture medium overnight.

Adhesion Assay

Hydrogel scaffolds were fabricated to contain alginate alone (A), a 1:1 and 2:1 w/w combination of alginate and chitosan (A/Ch), a 10:1 and 5:1 w/w combination of alginate and collagen (A/C), and a 10:2:5 w/w combination of alginate, collagen and chitosan (A/C/Ch). All scaffolds were seeded with 3.0×10^5 HUVECs per well and maintained under standard cell culture conditions with daily media changes for 2 weeks.

On day 14, HUVECs were labeled with DiI-Ac-LDL according to the manufacturer's instructions. Scaffolds were fixed in 10% neutral buffered formalin supplemented with CaCl₂ 1.0% w/v for 30 minutes, rinsed with dH₂O, and mounted with glycerol 90% (VWR) in dH₂O and beeswax.

Six randomly-selected representative images (each of identical dimensions) of 4 samples of each material combination were obtained using confocal microscopy (LSM510 running ZEN software) under rhodamine filter and 543nm laser. Cell surface density in each image was quantified (as cells/high-powered field [HPF]) and compared between groups.

Invasion Assay

Three-dimensional z-stacks were created through the thickness of the hydrogel from each of the 24 representative fields corresponding to the six hydrogel combinations (Figure 1). Component images were converted to binary images within ImageJ and the area occupied by fluorescence in each slice was measured using a particle counting algorithm and plotted as a function of the distance from the scaffold surface. Volumes of invasion were extrapolated from these data by applying the trapezoidal rule with uniform grid to estimate the area under each invasion curve and compared between groups.

Addition of bFGF

A/C (10:1) scaffolds were fabricated and seeded as above. Beginning on the third day of culture, control HUVEC media was exchanged daily for media containing 1, 5, 10 or 20ng/mL bFGF (AA 1–155 Recombinant Human). Scaffolds were labeled and fixed on day 14. Adhesion and invasion were quantified as above.

Vascular Smooth Muscle Co-Culture

HASMCs were transduced with baculovirus to produce an actin-green fluorescent protein (GFP) fusion construct (CellLight Actin-GFP BacMam 2.0) according to the manufacturer's instructions. A/C (10:1) scaffolds were fabricated as above, seeded with 3.0×10^4 , 6.0×10^4 , 1.5×10^5 , or 3.0×10^5 HASMCs and cultured in HASMC media. On the third day of culture,

scaffolds were seeded with 3.0×10^5 HUVECs and media was exchanged for HUVEC media. Scaffolds were labeled with DiI-Ac-LDL and fixed on culture day 17. Prior to mounting with glycerol, specimens were labeled with 4',6-diamidino-2-phenylindole dilactate (DAPI) according to the manufacturer's instructions. The three-dimensional organization of the resulting cellular structures were evaluated using confocal microscopy (543nm laser) in conjunction with an AxioCam MRm digital camera to incorporate widefield DAPI-fluorescence images into the confocal stack.

Statistical Analysis

Adhesion as functions of matrix material and bFGF concentration were evaluated using one-way analysis of variance (ANOVA) followed by a *post-hoc* Tukey-test with a family error rate of $\alpha=0.05$. Invasion as a function of matrix material was evaluated using a Kruskal-Wallis test adjusted for ties followed by a *post-hoc* Dunn's test with family error rate of $\alpha=0.05$. Data are expressed as mean $\pm$ SEM. A p-value less than 0.05 was considered statistically significant.

RESULTS

Surface Characterization

Multi-photon imaging of alginate 4% (data not shown) and collagen 1% scaffolds (Figure 2) revealed their surfaces to be relatively smooth with occasional small pores, but devoid of large cracks or crevices.

Adhesion Assay

HUVEC adhesion was found to be significantly different between matrix materials ($p<0.001$). After 14 days of cell culture, alginate hydrogels (5.0 ± 0.9 cells/HPF) demonstrated the lowest HUVEC surface density of all groups, followed by A/Ch (1:1) (8.4 ± 1.2 cells/HPF). When alginate was combined with chitosan in a 2:1 ratio, cells were nearly confluent and took on a spindle-shaped morphology. Cell surface density for this scaffold set (126.0 ± 18.5 cells/HPF) was greater than A and A/Ch (1:1) but less than A/C (10:1) and (5:1) scaffolds (250.7 ± 18.2 cells/HPF and 225.6 ± 23.9 cells/HPF, respectively), and not different from A/C/Ch scaffolds (155.4 ± 27.0 cells/HPF). Values obtained for A/C (10:1) and (5:1) were not statistically different from each other (Figures 3–4).

Volume of Invasion

Hydrogels consisting of alginate alone or A/Ch 1:1 did not demonstrate evidence of invasion and were excluded from further analysis. The Kruskal-Wallis test demonstrated the invasion volumes of the remaining 4 matrix materials to be significantly different from each other ($p=0.001$). Hybrid hydrogels consisting of A/C (10:1) demonstrated the greatest invasion volumes ($9.21 \times 10^5 \pm 2.97 \times 10^5 \mu\text{m}^3$), followed by A/C (5:1) ($5.45 \times 10^5 \pm 3.72 \times 10^5 \mu\text{m}^3$), A/Ch (2:1) ($2.68 \times 10^5 \pm 1.53 \times 10^5 \mu\text{m}^3$), and A/C/Ch ($4.83 \times 10^4 \pm 3.31 \times 10^4 \mu\text{m}^3$). The multiple comparisons test demonstrated that the invasion volume of A/C (10:1) scaffolds was significantly greater than those of A/Ch (2:1), A/C (5:1), and A/C/Ch scaffolds (Figure 5). Of note, spontaneous HUVEC tubule formation was not seen with any hydrogel combination.

Addition of bFGF

The addition of varying concentrations of bFGF to HUVEC culture media on day 3 of culture on A/C (10:1) scaffolds resulted in statistically significant differences in cell surface density ($p<0.001$). Specifically, the multiple comparisons test demonstrated that cell surface density following supplementation with bFGF 20 ng/mL (372.5 ± 37.4 cells/HPF) was

significantly greater than density following supplementation with bFGF 0ng/mL (250.7 ± 18.2 cells/HPF), 1 ng/mL (179.0 ± 17.4 cells/HPF), 5 ng/mL (198.5 ± 17.2 cells/HPF), and 10 ng/mL (273.3 ± 22.9 cells/HPF). Cell counts corresponding to 0, 1, 5 or 10 ng/mL were not statistically different from each other (Figure 6). However, there was no difference in invasion volume between groups, nor was HUVEC tubule formation seen (data not shown).

Vascular Smooth Muscle Co-Culture

HUVEC co-culture with HASMCs on A/C (10:1) scaffolds resulted in distinctive changes in HUVEC phenotype with increasing HASMC proportions (Figure 7). When cultured at a 10:1 ratio with HASMCs, HUVECs remained as single isolated cells and did not coalesce to form tubules. At a 5:1 ratio, HUVECs demonstrated mixed phenotypes including isolated single cells as well as the occasional formation of small tubule-like structures with lumens. Further increasing the HASMC ratio to 2:1 and 1:1 resulted in a significant increase in aggregation to larger and more organized networks of lumen-containing tubules. Of note, DAPI-positive, DiI-Ac-LDL-negative HASMCs comprised a significant structural component of these networks (Figure 8) and were demonstrated to be in direct physical contact with HUVECs when GFP-labeled (Figure 9).

DISCUSSION

Optimization of cellular and vascular invasion of tissue regeneration templates remains one of the formidable challenges of tissue engineering.^{2,3} The clinical significance of this limitation is immense: the long duration currently required for permanent scaffold integration translates to an extended need for dressing changes and wound care, a resultant high financial burden, and an increased length of time during which the newly-placed scaffold remains susceptible to infection. Furthermore, the limitations of such scaffolds are further amplified when applied to a suboptimal wound bed. Much work has therefore been invested in addressing the challenge of vascularization of biocompatible hydrogel-based tissue regeneration scaffolds, particularly as it pertains to vessel formation within. Several techniques have emerged as potential solutions, with varying degrees of success.

The simplest approach involves specifically tailoring the scaffold material chemistry^{3,5-8} or its internal three-dimensional architecture.⁹ A slightly more complex and likely prohibitively expensive strategy entails growth factor doping of the scaffold, either through covalent bonding to the hydrogel matrix,^{10,11} or addition to otherwise standard cell culture medium.

Understanding that cellular paracrine signaling is a crucial component of vasculogenesis and angiogenesis, co-culture techniques have similarly been utilized to encourage vessel formation within hydrogel constructs. Multiple cell types have been assessed in culture with endothelial cells and observed for their ability to induce assembly of proto-microvascular networks, including vascular smooth muscle cells,¹² adipose-derived mesenchymal stromal cells (ASC),^{13,14} fibroblasts of dermal¹⁵⁻¹⁸ or pulmonary¹⁹ origin, or a combination of dermal fibroblasts and skeletal myoblasts.²⁰

For example, Verseijden et al.¹³ demonstrated that human ASC/HUVEC spheroids formed non-lumen containing “prevascular structures” *in vitro* and an increased number of host-derived (mouse) and total (human- and mouse-derived) vessels compared with ASC-only spheroids *in vivo*. Sukmana et al.¹⁸ utilized parallel polymer monofilaments embedded within a fibrin gel as a guide for HUVEC orientation and found that co-culture with fibroblasts resulted in a greater number of cell-cell connections and cells per fiber length than either control samples or samples with growth-factor (i.e., VEGF or bFGF) addition.

Lastly, Lesman et al.²⁰ examined vascularization of fibrin constructs with or without poly(L-lactic acid)(PLLA)/polylactic-glycolic acid (PLGA) sponge reinforcement utilizing both co-culture (HUVEC+skin fibroblast [SF]) and tri-culture (HUVEC+SF+C2 skeletal myoblast) techniques. Interestingly, tri-culture systems demonstrated less vessel maturity compared with co-culture systems.

Thus, despite a great deal of work invested in this endeavor, few have demonstrated successful spontaneous (i.e., non-guided) tubule formation,^{14,15,19,20} and more importantly none have yet incorporated these techniques in the fabrication of larger, clinically relevant three-dimensional constructs such as artificial tissue to be used in place of vascularized autologous tissue for transfer. While cells within thin (< 1mm) onlay wound-healing modalities such as hydrogel scaffolds or even autogenous split-thickness skin grafts are able to survive prior to full vascularization by means of simple imbibition of oxygen and nutrients, the process of diffusion is insufficient to adequately sustain cells within the bulk of larger or thicker constructs.^{21,22} Therefore, the process of vascularization is the primary rate-limiting step in the creation of artificial vascularized tissues for uses such as solid organ transplant or microvascular free tissue transfer.

Towards this end, in previous work we have reported a simple yet elegant technique for the creation of architecture critical for the fabrication of pre-vascularized tissue constructs (containing macrovascular inflow/outflow channels confluent with an internal microfluidic network recapitulating a “capillary bed”)²¹ that utilizes sacrificial sucrose structures embedded within a polydimethylsiloxane (PDMS) bulk construct. However, translation of this technique to the clinical realm requires substitution of non-biodegradable PDMS with biocompatible biodegradable materials. In addition, construct implantation and anastomosis to host vasculature requires the presence of an endothelialized lining to support long-term thrombosis-free blood flow. Lastly, the optimal bulk material would not only support endothelial cell survival but would encourage further spontaneous construct vascularization via invasion and spontaneous vasculogenesis.

It is with this larger goal in mind that we evaluated the hydrogel combinations best suited to this task. Although abundant, fibrin- or collagen-based matrices are extremely low in tensile strength and have unattractive handling characteristics, making them unsuitable materials from which to fabricate surgically-relevant replacement tissues. For this reason, the more firm and resilient alginate was selected as the building block from which to fabricate our scaffolds.

However, despite RGD-modification (which serves to increase the substrate’s positive charge), the nanoporous alginate hydrogel²³ did not satisfactorily support cell growth. Increasing positive charge and likely the pore structure with the addition of cationic chitosan²⁴ not only increased endothelial cell adhesion, but invasion into the scaffold as well. Lastly, the incorporation of even small amounts type I collagen resulted in a statistically significant increase in endothelial cell adhesion and invasion that are not due to faults in the scaffolds’ surface topography. Such a hybrid hydrogel composition utilizes both the low-cost and structural integrity of alginate-based hydrogel scaffolds and the cellular appeal of microporous native type I collagen.²⁵ This finding is in agreement with Girandon et al.,²⁶ who report increased adipose-derived stem cell viability when cultured on collagen sponges compared with alginate hydrogels. For these reasons, the remainder of our study (i.e., bFGF and co-culture assays) was performed using the A/C 10:1 combination.

In the current study, standard culture medium supplementation with bFGF did not result in an increase in endothelial cell invasion or tubule formation. However, a significant increase in HUVEC cell surface density was seen at higher (20ng/mL) concentrations. This latter

finding is similar to the results seen Sukmana et al.¹⁸ However, in contrast to the present study, Sukmana et al. reported an increase in HUVEC migration with the addition of FGF. This discrepancy is likely due to differences in the models used. Incongruencies between studies such as these are not altogether unexpected as the processes of vasculogenesis and angiogenesis are complex and likely require an equally complex signaling pattern for their instruction.

While others have reported that endothelial cell co-culture with dermal fibroblasts stimulates tubule formation within hydrogels,^{19,20} we did not observe this following HUVEC and neonatal human dermal fibroblast co-culture (*data not shown*). Instead, desired results were only seen when co-culture with smooth muscle cells of vascular origin was performed. However, it is not surprising that vascular smooth muscle cells, cells closely related to and possibly even derived from pericytes,^{27,28} and not fibroblasts of dermal origin, would be more likely to guide the complex process of vasculogenesis. However, these variations could potentially be due to differences in experimental models.

For example, in the studies by Velazquez et al.¹⁵ and Nakatsu et al.¹⁶ in which HUVECS formed capillaries when co-cultured with fibroblasts, the two cell types were spatially separated by a hydrogel “barrier” and did not participate in direct cell-cell interactions. In contrast, Chen et al.¹⁹ reported the formation of “capillary networks” when HUVECS and fibroblasts were co-cultured in proximity. However, this group utilized fibroblasts of pulmonary and not dermal origin. Lastly, many studies utilized the surgically undesirable fibrin^{16,18–20} as the ECM component. The effect of matrix composition and architecture cannot be underestimated, as ECM offers not only chemical but structural cues to cells located within,³ any of which could potentially alter cellular signaling and response.

In conclusion, HUVEC adhesion and invasion was greatest within alginate/collagen type I hybrid hydrogels (10:1 w/w combination) compared with the other hydrogel chemistries studied. High-dose growth factor supplementation resulted in an increase in HUVEC surface density, but no difference in invasion or phenotype. Lastly, paracrine signaling via endothelial cell co-culture with HASMC (and not fibroblasts of dermal origin) stimulated tubule formation and network organization. These findings will serve to guide our future endeavors towards the formation of biocompatible, hydrogel-based pre-vascularized tissue constructs to be used in place of current avascular tissue regeneration templates so as to achieve more rapid host incorporation. Furthermore, these tissue-engineering strategies may be applied to the future fabrication of artificial vascularized autologous tissue for transfer.

Our study is not without limitation. Despite the low cost and biocompatibility of alginate based hydrogel constructs, the extended time course of biodegradation remains a relative drawback for certain scenarios. In future work, we will evaluate our pre-vascularized hybrid hydrogel constructs in an *in vivo* wound-healing model.

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Figure 1.

Illustration of method for quantification of invasion. 3-dimensional z-stacks were created through the thickness of each hydrogel (*top, left*) and separated into component slices (*bottom*). The area occupied by fluorescence in each slice (typically $3.14\mu\text{m}$ in thickness) was measured using a particle counting algorithm and plotted as a function of the distance from the scaffold surface (*top, right*). Scale bar= $200\mu\text{m}$ (*top, left*), $100\mu\text{m}$ (*below*).

Figure 2. Multi-photon imaging of collagen 1% scaffolds revealed their surfaces to be relatively smooth with occasional small pores, but devoid of large cracks or crevices. *Scale bar=200 μ m.*

Figure 3.

Representative images from adhesion assay for alginate (*top, left*), alginate and chitosan combined in 1:1 (*top, right*) and 2:1 (*middle, left*) ratios, alginate and type I collagen combined in 10:1 (*middle, right*) and 5:1 (*bottom, left*) ratios, and the combination of alginate, collagen and chitosan combined in a 10:2:5 ratio (*bottom, right*). Di-Ac-LDL-labeled HUVECs (*red*) are most confluent on alginate/collagen 10:1 and 5:1 hydrogels. Scale bar=100 μ m.

Figure 4.

Quantification of the adhesion assay demonstrated HUVEC adhesion to be significantly different between matrix materials ($p < 0.001$). Of the hydrogel chemistries evaluated, the combination of alginate and type I collagen in 10:1 and 5:1 w/w ratios resulted in the greatest cell surface densities. A=alginate; C=collagen; Ch=chitosan; HPF=high-powered field. Statistical significance ($p < 0.05$) compared with alginate denoted by *, with alginate/chitosan (1:1) denoted by †, with A/Ch (2:1) denoted by §, and with A/C/Ch denoted 10:1 by ‡.

Figure 5.

Volumes of invasion were significantly different between matrix materials ($p=0.001$). The multiple comparisons test demonstrated invasion of A/C (10:1) to be significantly greater than those of A/Ch (2:1), A/C (5:1) and A/C/Ch. A and A/Ch (1:1) hydrogels did not demonstrate evidence of invasion (*data not shown*). A=alginate; C=collagen; Ch=chitosan. * denotes statistical significance ($p<0.05$).

Figure 6.

The addition of varying concentrations of bFGF to HUVEC culture media on day 3 of culture on A/C (10:1) scaffolds resulted in statistically significant differences in cell surface density ($p < 0.001$). Cell surface density following supplementation with bFGF 20 ng/mL was significantly greater than those corresponding to 0 ng/mL, 1 ng/mL, 5 ng/mL, and 10 ng/mL, the latter of which were not statistically different from each other. *bFGF*=basic fibroblast growth factor; *HPF*=high-powered field. * denotes statistical significance ($p < 0.05$).

Figure 7.

HUVEC co-culture with HASMCs on A/C (10:1) scaffolds resulted in distinctive changes in HUVEC phenotype with increasing HASMC proportions. When cultured at a 10:1 ratio with HASMCs, HUVECs remained as single isolated cells and did not coalesce to form tubules (*top, left*). At a 5:1 ratio, HUVECs demonstrated mixed phenotypes including isolated single cells as well as the occasional formation of small tubule-like structures (*top, right*). Further increasing the HASMC ratio to 2:1 (*bottom, left*) and 1:1 (*bottom, right*) resulted in a significant increase in aggregation to larger and more organized networks of lumen-containing tubules. Only HUVECs are labeled with DiI-Ac-LDL (*red*), while nuclei of both HUVECs and HASMCs are stained with DAPI (*blue*). Scale bar=100 μ m.

Figure 8. DAPI-positive (*blue*) DiI-Ac-LDL-negative (*red*) HASMCs comprised a significant structural component of endothelial tubules. *Scale bar=100 μ m*

Figure 9.

Co-culture of HASMCs (*green*) and HUVECs (*red*) at a 1:1 ratio resulted in the formation of tubules comprised of both cell types in close proximity. Tubules demonstrated lumens when imaged along the axial plane (*inset at 2× magnification of larger image*). Arrow marks site of cross-sectional imaging. DAPI=blue; scale bar=100μm.