Published in final edited form as:

Ann Biomed Eng. 2016 December ; 44(12): 3435–3445. doi:10.1007/s10439-016-1662-y.

3D Printed Vascular Networks Enhance Viability in High-Volume Perfusion Bioreactor

Owen Ball¹, Bao-Ngoc B. Nguyen¹, Jesse K. Placone¹, and John P. Fisher¹

¹Fischell Department of Bioengineering, University of Maryland, College Park, MD

Abstract

There is a significant clinical need for engineered bone graft substitutes that can quickly, effectively, and safely repair large segmental bone defects. One emerging field of interest involves the growth of engineered bone tissue *in vitro* within bioreactors, the most promising of which are perfusion bioreactors. Using bioreactor systems, tissue engineered bone constructs can be fabricated *in vitro*. However, these engineered constructs lack inherent vasculature and once implanted, quickly develop a necrotic core, where no nutrient exchange occurs. Here, we utilized COMSOL modeling to predict oxygen diffusion gradients throughout aggregated alginate constructs, which allowed for the computer-aided design of printable vascular networks, compatible with any large tissue engineered construct cultured in a perfusion bioreactor. We investigated the effect of 3D printed macroscale vascular networks with various porosities on the viability of human mesenchymal stem cells *in vitro*, using both gas-permeable, and non-gas permeable bioreactor growth chamber walls. Through the use of 3D printed vascular structures in conjunction with a tubular perfusion system bioreactor, cell viability was found to increase by as much as 50% in the core of these constructs, with *in silico* modeling predicting construct viability at steady state.

Keywords

vascular; bioreactor; perfusion; hMSCs; 3D printed; modeling; computer-aided design

Introduction

Each year, there are nearly 15 million bone fractures¹ and 1 million bone grafting procedures² worldwide, and 185,000 limb amputations in the United States³. Autografts are the gold standard for harvesting bone tissue for implantation^{4,5}, but are severely limited by supply, and patients often experience donor site complications including post-surgical pain, infection, and scarring at the donation site.⁶ Allografts are the most widely available option for treating long bone defects.⁴ Fresh allografts are rarely used due to the potential for

*Corresponding Author: John P. Fisher, Ph.D., Fischell Family Distinguished Professor & Department Chair, Fischell Department of Bioengineering, University of Maryland, 2330D Jeong H. Kim Engineering Building, College Park, MD 20742, Phone: 301.314.2188, jpfisher@umd.edu, Web: <http://teblumd.net>.

Conflicts of Interest

J.P.F. and J.K.P. are both founders and co-owners of the company 3DBioWorks which focuses on the use of bioreactors for cell proliferation and differentiation.

serious infections such as HIV and Hepatitis C and the presence of immunogenic factors.⁴ Therefore, allogeneic bone requires processing prior to transplantation to reduce the risk of disease transmission, which also decreases the desirable biological activity of the tissue.⁴ Because of these complications, there is a significant clinical need for engineered bone graft substitutes. However, conventional tissue engineering techniques have so far been limited to tissue engineered constructs of less than 11 cubic centimeters⁷. Current research is limited by the need to implant large-scale tissue engineered bone (TEB) constructs with the necessary vasculature for long-term graft functionality.

Traditional static culture, which involves a TEB construct surrounded by static media, is largely limited by construct size. Under this condition, oxygen diffusion is limited to the outer 200 microns of the construct, resulting in the formation of a necrotic core. Many limitations of static culture are overcome by perfusion bioreactors, in which porous TEB constructs are cultured in adjustable reaction chambers. Media is then perfused through the chamber, following the path of least resistance, thus flowing around the scaffolds and thus applying shear stress to the encapsulated cells. However, this design requires a high degree of pore interconnectivity, and a tightly-fitted reaction chamber, both of which can prove to be difficult design constraints. Recent research has therefore focused on the development of TEB constructs complete with functioning vascular networks prior to *in vivo* implantation.

Vascular networks are a key component of any biological system. In fact, cells within the human body are restricted to a distance of 100–200 μm from the nearest capillary.^{8,9} Vascular systems provide cells with oxygen and glucose transport necessary for respiration, as well as an efficient means of waste removal.^{8,10,11} The human body has demonstrated the ability for vascular tissues to spontaneously invade implanted tissue.^{8,12} However, host vasculature invades from the outside of an implanted scaffold inward, and thus the time required to achieve sufficient vascularization depends on the thickness of the implant. Spontaneous vascular ingrowth has been measured on the order of a few hundred nanometers per day,¹² thus requiring several weeks to vascularize any constructs. During this time, implantable constructs quickly develop a necrotic core.^{8,10,11,13} Those cells that do not undergo apoptosis experience extreme nutrient gradients, with cells on the periphery of the construct consuming much greater levels of nutrients than those cells embedded deeper within the core. The unequal metabolic rates cause cells to release different signals, thus resulting in non-uniform differentiation of stem cells in these constructs.¹²

Here, a biomimetic macro-vascular network was created using stereolithography 3D printing to integrate with a tissue engineered hydrogel scaffold. Currently, constructs designed of any size greater than the oxygen diffusion limit developed necrotic cores when cultured to clinically relevant sizes. In this study, we aim to incorporate a rigid macroscale vascular structure connected to a tubular perfusion system (TPS) bioreactor's media supply. The TPS model is a perfusion bioreactor that utilizes a bottom-up approach, which helps ensure that all cells have adequate nutrient supply and waste removal. Human mesenchymal stem cells (hMSCs) are encapsulated within alginate beads and media is then able to freely perfuse the void space between beads, applying shear stress and enhancing proliferation, differentiation, and mineralization.^{14–16} These type of perfusion systems have yielded constructs on the order of 200 cm^3 ¹⁷. The integration of the 3D printed vascular network will allow for the

culture of high-volume aggregated tissue engineered construct within a perfusion bioreactor. Ultimately, this macroscale vasculature allows for sustained viability of cells throughout the aggregate while allowing the host vasculature to infiltrate from both ends, resulting in a fully vascularized construct. The objective is to model this system *in silico* to determine the optimal porosity and branching to increase construct viability, and to evaluate these results *in vitro*. The combination of a perfusion bioreactor with pre-established vasculature will provide advantages over both traditional static culture and various different means of dynamic culture that do not comprise any vascular networks.

Materials and Methods

Cell Culture

Human mesenchymal stem cells (hMSCs) (passage=3) were purchased from RoosterBio (Frederick, MD) and cultured in hBM-MSC Basal Medium with hBM-MSC Media Booster GTX (RoosterBio) following the manufacture's protocol. Cells were stored at 37°C and 5% CO₂ and passaged every 4–5 days. After the initial growth phase, cells were cultured in high glucose DMEM with L-Glutamine (Gibco, Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS, Invitrogen), 1% v/v antibiotic-antimycotic (Gibco), and 0.1 mM nonessential amino acids (Invitrogen) with a media change every day. Cells were stored at 37°C and 5% of CO₂.

Alginate Bead Formation

hMSCs were homogenously mixed into 2% w/v alginate (Sigma) solution as described previously¹⁴ and used to make alginate scaffolds by adding the mixture dropwise into a suspension of 0.1M calcium chloride (Sigma) (130,000 cells/individual scaffold, 80 million cells per experimental group). Figure S1 a–b.

TPS Bioreactor Assembly

Alginate scaffolds were divided into experimental (dynamic culture) and control (static culture) groups. The bioreactor was set-up as described previously¹⁸ utilizing a ¾" reaction chamber (Figure 1). The ¾" reaction chamber wall was then slid over top of the inlet connector/printed network assembly. Approximately 600–750 alginate beads were loaded from the top to fill each reaction chamber (Figure S1c). Media flow was driven by an L/S Multichannel Pump System (Cole-Parmer) at a flow rate of 27 mL/min, as determined by scaling up a previously determined optimum value of 3 mL/min in a ¼" tubing system^{14,19}. Static scaffolds were cultured in six-well plates (10 scaffolds per well). The medium was changed daily.

Construct Aggregation

Alginate scaffolds were exposed by removing the outlet connector. Then acellular liquid alginate at 37 °C was injected in increments of 10 mL into the void space between alginate beads. Following each alginate injection, 10 mL of 0.1 M CaCl₂ was injected in a similar manner, aspirating excess CaCl₂ after crosslinking. This technique was repeated 3–5 times, allowing a 5 minute period for alginate gel formation in between each injection. After the final injection of alginate and CaCl₂, the system was left to aggregate for 15 minutes, the

outlet connector was reattached, and the entire system transported back to the incubator where it was perfused again. (Figure S1d).

The statically cultured beads were aggregated by placing a 3 inch section of platinum-cured silicone rubber tubing on a sterile surface. Statically cultured beads were then placed into this tubing and aggregated in the same manner as described above. Once aggregated, the construct was removed from the tubing and placed into a 50 mL Falcon tube, which was filled with media and kept at 37 °C.

SolidWorks Geometry Generation

SolidWorks was utilized to create all structures to be printed. Final network design consisted of three vertical branches, each of which split into three more horizontal branches, for a total of nine branches (Figure 2). Major dimensions were a length of 67.72 mm, and a cylindrical diameter of 19.05 mm. Pore diameters were 750 µm and pore spacing was determined through COMSOL simulations. Scaffold geometries for the multilevel vascular network were chosen based upon a minimum spacing on the order of a single bead diameter. Further the overall size was constrained based upon the TPS bioreactor system which utilizes modular tubing as the bioreactor chambers. Connectors were 3D printed to connect the vasculature to the TPS bioreactor media source (Figure S2).

COMSOL Mass Transport Analysis

To determine the effect of network porosity on oxygen concentrations within aggregated constructs, a 2D steady-state model of the construct was developed in COMSOL Multiphysics 4.2. The effects of convection and diffusion on pre-aggregation “free” beads have been previously investigated in depth and shown to provide necessary levels of oxygen to encapsulated cells.^{14,20} For this reason, these COMSOL studies focused on convection and diffusion throughout the aggregated construct. Furthermore, alginate, while highly porous, has very low permeability ($k=1\times10^{-17} \text{ m}^2$ ^{21–23}). The relationship between flow rate and permeability is given by Darcy’s Law (Equation 1).

$$Q = -\frac{kA}{\mu} \frac{\Delta P}{L} \quad (1)$$

Where Q is the flow rate, μ the dynamic viscosity of the media (0.78 centipoise)¹⁴, L is the length, and ΔP is the pressure drop. Previously, flow rate through a single bead was calculated to be $3 \times 10^{-7} \text{ mL/min}$.²⁴ Based on this minimal flow rate, and taking into consideration both the free flow path that the printed network allows the media and the complete size of the alginate construct, convection through the aggregated construct was neglected. COMSOL convection and diffusion equations (Equation 2), based on Fick’s Law, are therefore simplified, as the fluid velocity, u , is assumed to be zero.

$$N_i = -D_i \Delta C_i + C_i u \quad (2)$$

Where N_i is the flux (mol/m²-s) through material “i”, D_i is the diffusivity of material “i”, and C_i is the concentration of the diffusing species within material “i”.

Due to the presence of metabolically active cells within each alginate bead, a nutrient sink exists within the model. This sink is modeled through Equations 3 and 4, where Equation 3 is the Michaelis-Menten reaction kinetics equation, and Equation 4 is the concentration sink equation utilized by COMSOL.

$$R_i = N_0 \frac{C}{C + K_{MM}} \quad (3)$$

$$\Delta(-D_i \Delta C_i) + u \Delta C_i = R_i \quad (4)$$

Here, R is the rate of consumption of oxygen (mol/m³-s), N_0 is the oxygen consumption rate (0.012 μmol/10⁶ cells/h)¹⁴, and K_{MM} is the Michaelis-Menten saturation constant (0.011 mol/m³)¹⁴. It is important to note that the user must define R as a negative value, since it represents a consumption rate. Additionally, while the entire space confined between the bioreactor wall and the network should be modeled as alginate, only the beads themselves should be modeled as metabolically active. Converting the oxygen consumption to account for our cell seeding density and bead volume yielded a consumption rate of 1.45×10^{-5} mol/m³-s. For this analysis, networks that yielded oxygen concentrations below .04 mM (4% O₂) were considered to be non-functional, based on the concentration of oxygen in human capillary blood (~5%),²⁵ in human tissues/organs (~2%–9%),^{25,26} and in bone marrow (~4%–7%)²⁷. While research has demonstrated conflicting information on the effect of low oxygen tensions on MSC differentiation, and it is generally accepted that low oxygen tensions favor chondrogenesis to osteogenesis,²⁸ the effect of these low oxygen concentrations on differentiation was not taken into account. This can be justified by the results of several experiments which demonstrate increased osteogenesis and proliferation^{14,15,18} pre-aggregation. Therefore, post aggregation oxygen concentrations need only support cell viability, as constructs will only be aggregated after significant differentiation and mineralization have occurred.

The diffusivity of oxygen was modeled as 2.56×10^{-9} m²/s in media, and 2.08×10^{-9} m²/s in alginate.¹⁴ Media, which is contained in a flask constantly exposed to air and travels through 1/8" gas-permeable tubing, was assumed to be saturated water at 37 °C and 0.21 mM, as calculated from Henry's Law (Equation 5) and the Van't Hoff equation (Equation 6).

$$C_{aq} = \frac{P}{K_H} \quad (5)$$

$$K_H(T) = K_H(T^*) \exp \left[-\frac{\Delta H_{sol}}{R} \left(\frac{1}{T} - \frac{1}{T^*} \right) \right] \quad (6)$$

Where C_{aq} is the concentration of oxygen dissolved in media, P is the partial pressure of oxygen in air, K_H is the Henry's Law constant, H_{sol} is the enthalpy of dissolution, R is the universal gas constant, T^* is 298 K, and T is 310 K (37 °C). Henry's Law constants and enthalpy of dissolution values were obtained from the National Institute of Standards and Technology.

The air-saturation assumption further served to allow for the pores to be modeled by simply imposing constant surface oxygen concentrations of 0.21 mM at each pore. Similarly, a constant surface concentration was imposed on the outer boundary of the reaction chamber wall. The EShell network, was modeled as non-permeable ($D = 1 \times 10^{-100} \text{ m}^2/\text{s}$) to provide modeling for even the least permeable materials.

To mimic an *in vivo* environment, low-permeability fluorinated ethylene propylene (FEP) tubing (McMaster-Carr, Robbinsville, NJ), in addition to the highly permeable platinum-cured silicone (PCS) tubing (Cole-Parmer, Vernon Hills, IL), typically used with TPS bioreactors. The diffusivity values utilized in calculations were $4.174 \times 10^{-11} \text{ m}^2/\text{s}$ for FEP and $2.357 \times 10^{-8} \text{ m}^2/\text{s}$ for silicone.

Experimental Group Determination

Taking into account the results of the COMSOL aggregated diffusion studies 5 experimental groups were determined. First, a static control was utilized. This group featured cell-encapsulated alginate beads cultured in 6-well plates until Day 1, upon which they were aggregated and incubated in a 50 mL Falcon tube. Other groups were cultured in a TPS bioreactor. Parameters for all groups are listed in Table 1. Three groups were cultured in FEP tubing to mimic the *in vivo* environment, where gas exchange on the periphery of these constructs would be limited. The final group was cultured in highly gas permeable PCS tubing, to demonstrate the *in vitro* efficacy of these constructs.

3D Printing

All 3D printing was completed using a Digital Light Processing (DLP) stereolithography printer (EnvisionTec, Dearborn MI). A clear, bioinert polymer, EShell 300 (EnvisionTec) was chosen for both the perfusion network, and the connectors. Vascular networks were fabricated based upon the designs developed in COMSOL and SolidWorks. Briefly, these designs were imported as STLs into Magics (Materialize) software and supports were automatically generated. The support structure and the corresponding vascular network were then imported into the Perfactory RP (EnvisionTEC) software to build the build file based upon manufacture specifications with a step size of 50 microns. Once the build file was generated, this was then transferred to the printer where the parts were fabricated. The support structure was carefully removed using a razor blade and sanded down. Parts were cured using an Otofash (EnvisionTEC) light polymerization chamber, according to the manufacturers post-processing specifications. The structures were then sterilized under UV light in solutions of 100% ethanol, 25%–75% PBS-ethanol, 50%–50% PBS-ethanol, 75%–25% PBS-ethanol, and 100% PBS (5 minutes per solution). Lastly, parts were rehydrated in PBS until use.

Viability Analysis

Beads were harvested at each of six harvest sites for each experimental group (Figure 1d). The six locations were categorized as bottom inner, bottom outer, middle inner (core), middle outer, top inner, and top outer. Here bottom, middle, and top refer to vertical distance from the inlet, while inner and outer refer to radial distance from the centerline.

Three beads were harvested from each site after 24 hours (n=3). Additionally, 3 beads were removed from the static culture group as controls. After this initial harvest, the constructs were aggregated and media was changed. After 24 hours of aggregation (48 hours from experiment start), beads from each harvest site were once again harvested (n=3). At this time point, harvest sites did apply to the static culture, and were therefore taken into account. In addition to the six harvest sites, beads were harvested for dead controls from each group (n=3). Dead control beads were incubated in 70% methanol for 15 minutes before aspirating the methanol.

Viability Assessment

Harvested beads were immersed in a solution of 1mM calcein AM (CAM), 2mM ethidium homodimer-1 (EH) (Fisher) in PBS for 30 minutes before imaging with a fluorescent microscope. Each bead was imaged at both $2.5 \times$ and $10 \times$ magnifications and analyzed using ImageJ Software (NIH).

Statistical Analysis

Each analysis was performed in triplicate (n=3). Statistical significance was determined by one-way analysis of variance and Tukey's multiple-comparison test. A confidence interval of 95% ($\alpha = 0.05$) was used for all analyses. Mean values of triplicates and standard deviation error bars are reported on each figure as well as relevant statistical relationships.

Results

COMSOL Diffusion Studies

In silico modeling provides many advantages, most notably the ability to generate accurate models without requiring the time or cost associated with a full culture of hMSCs. COMSOL Multiphysics was chosen to investigate the diffusion of oxygen throughout aggregated constructs in an effort to determine the optimal network porosity. COMSOL diffusion studies focused on the middle of the three vertical branches, as this branch featured the largest separation between pores and alginate scaffolds due to the presence of only 2 horizontal branches. Initially, the maximum porosity network, represented by a center-center pore distance of 1.5 mm was compared to the minimum (within reason) porosity network, represented by a 9 mm center-center pore spacing. These results, displayed in Figure 3, demonstrate a clear difference in oxygen concentrations between these two groups as seen by the blue to red spectrum of oxygen oxygens. However, it is important to note that, when modeled with platinum-cured silicone tubing, neither group falls below the 0.04 mM threshold at any point. In fact, the lowest value in the 9 mm spacing group is about 7 mM oxygen.

Effect Of Non-Gas Permeable Bioreactor Wall On Oxygen Concentration

Due to the prevalence of suitable oxygen values throughout when using highly-permeable bioreactor growth chambers, the effect of using a less permeable bioreactor wall was examined by the use of FEP tubing, which has a gas permeability three orders of magnitude lower than that of silicone tubing. As demonstrated here, in a simulated *in vivo* environment, oxygen concentration nears 0 mM throughout the construct in the 9 mm pore spacing group. Oxygen concentration was then examined in the symmetrical upper and lower branches. These groups were similarly run using both silicone and FEP tubing, but as before, the silicone tubing provided so much oxygen content that the entire system contained healthy levels of oxygen. Therefore, only the FEP results are displayed in Figures 3c and 3d. These models validate the assumption that middle branch analyses can serve to model oxygen concentrations in the interior due to their larger interior void space. Here, the exact same minimum oxygen concentrations are observed as in the corresponding middle group models. Once again, the 9 mm pore spacing concentration nears 0 mM.

Effect Of Pore Spacing On Oxygen Concentration In Non-Permeable Bioreactor Wall

With the extremes of network porosity measured, the next series of models was undertaken to determine the minimum pore distance at which oxygen concentration remains above 4 mM. Figure S3a represents a 6 mm center-center pore spacing, while Figure S3b represents a 3 mm center-center spacing. Here, oxygen concentration in the 6 mm model falls below 4 mM in areas where the distance from each bead to the nearest pore is at a maximum, whereas the 3 mm model maintains oxygen levels above 4 mM at all locations. Finally, the effect of utilizing a single tube, representing low media flow, was examined. A single tube allowed for the system to continue to be housed in the bioreactor (the backpressure would cause system rupture if no network were included), while the low permeability of aggregated alginate allowed for low flow levels, as the media followed the path of least resistance straight out the downstream end of the tube. Here, Figure 3g clearly demonstrates that a single tube with 9 mm pore spacing will not support cell viability.

In Vitro TEB Construct Viability

TEB construct viability was further examined *in vitro* through the use of viability assays after 24 hours of pre-aggregation flow (Day 1), and after 24 hours of post-aggregation flow (Day 2). Five live/dead viability images were taken per magnification (2.5 × and 10 ×), per harvest site, per experimental group and processed to obtain percent live cell counts. The percentage of live cells in each experimental group were not statistically different ($p < 0.05$) at the Day 1 time point, as evidenced by the averaged values displayed in Figure 4h. Values for all groups are displayed in Figure S4, demonstrating the lack of statistical difference ($p < 0.05$) between any experimental groups after Day 1. Day 2 analyses displayed more varying results between groups. In general, a trend of decreasing viability with increasing pore spacing was noted. Figures 4c–4f provide representative images of samples taken from the core of Static Control and Silicone experimental groups. These results are graphed in Figure 4i, where groups that do not share a letter are statistically different ($p < 0.05$). It is worth noting that every group, with the exception of the Distant Pore group, is statistically different ($p < 0.05$) from the Static Control, with the Silicone group achieving an average 3.8 times

the percentage of live cells in static groups. Figure 4j is a graphical representation of the composite averages of each harvest site, which displays the general trend of decreasing viability with increasing pore spacing. Graphs of the percentage of live cells for each harvest site can be found in Figure S5, which further illustrate the general viability trend.

Discussion

Tissue engineering alternatives to traditional bone grafting techniques provide many benefits over conventional techniques, including the ability to customize the construct to meet patient needs, to rapidly and consistently reproduce TEB constructs, and a greatly expanded supply. Unfortunately, current TEB alternatives are limited by a lack of inherent vasculature, which restricts maximum construct size. Ideally, a TEB construct would be developed under optimal *in vitro* conditions, but remain viable when implanted *in vivo*. To this end, we first modeled non-permeable reactor walls to mimic an *in vivo* environment, while highly permeable reactor walls were chosen to demonstrate the enhanced efficacy of the *in vitro* environment. Second, both wall types were examined through COMSOL modeling to determine the optimal porosity and geometry of the printed macroscale vascular networks. Lastly, the printed grafts were tested in an *in vitro* setting to investigate the effects of porosity on the viability of hMSCs in comparison to the *in silico* results.

By comparing different COMSOL diffusion models, it was observed that the use of platinum-cured silicone tubing for the reaction chamber caused the diffusion of oxygen from the periphery to dominate the diffusion of oxygen from the 3D printed vascular networks. This resulted in all groups achieving oxygen levels above the chosen floor of 4 mM. For this reason, FEP tubing was utilized to mimic an *in vivo* environment, in which oxygen would only be supplied from nearby the 3D printed vascular systems.

It was shown that while a 1.5 mm pore spacing will achieve O₂ levels above 4 mM, a 3 mm pore spacing will also achieve this same result. However, at a spacing of 6 mm O₂ levels begin to fall below 4 mM, and at a spacing of 9 mm, a significant portion of the construct nears 0 mM oxygen. In single tube networks with 9 mm pore spacing, the entire construct fell well below 4 mM O₂. Finally, it was shown that while a single tube with pores every 1.5 mm results in O₂ levels above 4mM throughout the construct, this architecture results in significant gradients, where O₂ levels drop with respect to radial distance from the center. Due to the dependence of proliferation and differentiation on O₂ concentration, this architecture would result in non-uniform differentiation, and likely non-functionality of the construct. Therefore, the developed construct must make an effort to equalize the distance of each bead to its nearest pore, a challenge which is best met through the use of a symmetric, branched network.

After performing the *in vitro* study, Day 1 viability analyses at each harvest site yielded no statistically different results across all experimental groups, as expected due to the presence of forced convection on the surface of each bead, providing sufficient oxygen throughout the bead construct. The lack of statistically different results removed any dependence of Day 2 results on Day 1 viability. Rather, Day 2 results could be attributed solely to network and bioreactor wall type.

Day 2 viability results displayed a trend of decreasing viability with decreasing porosity, as evidenced by the Day 2 composite averages represented in Figure 4j. This trend validates the hypothesis that oxygen concentrations, and therefore cell viability, would increase with increasing porosity. It is hypothesized that while Single Tube and Distant Pore viabilities were high, these groups would experience reduced osteogenic differentiation due to the reduced oxygen levels as shown in *in silico* modeling.

Perhaps the most important Day 2 results are obtained from the Middle Inner (core) harvest sites (Figure 4i). Here, the need for inherent vasculature is obvious, with only about 20% of Static Control cells still living after just 24 hours. At this harvest site, viability was statistically different from all groups except for the Distant Pore group.

On the subject of the Static Control group, it is worth noting that it is not surprising that the percentage of live cells was often higher than in the Distant Pore and Single Tube groups at all harvest sites except for the core. This is because in the Static Control group, all harvest sites except for the middle inner site were directly on the periphery of the construct, and thus directly exposed to static media (Figure 3h). On the other hand, the outer harvest sites in the bioreactor groups were adjacent to the non-gas permeable bioreactor wall, and thus saw little direct exposure to media and oxygen. It is for this reason that the Static Core viability is as high as it is in the composite average, and the Middle Inner results are so important. Further, as overall construct size increases, the surface area to volume ration will decrease. Therefore, a larger percentage of cells will be contained in the core than on the periphery. Similarly, as construct size increases, the time required for complete host-invasion vascularization increases, resulting in long term hypoxic conditions in the core region. Therefore, while the composite average is necessary to demonstrate cell viability at all construct locations, the Middle Inner, or core, harvest site is perhaps the most important, and contains the largest percentage of cells when dealing with large constructs.

As for the viability in the Single Tube and Distant Pore groups, it is hypothesized that imperfect aggregation led to higher viability levels than expected from the COMSOL modeling, as some degree of convective flow was observed throughout these constructs. While the permeability of alginate prevents any convective nutrient transfer, its diffusivity is comparable to that of the growth media, making it an ideal material to allow for diffusive transport while preventing hemorrhaging when fully aggregated.

Conclusions

The vascular network designed and printed in this study demonstrates great promise for use high-volume TEBs cultured in in perfusion bioreactors. The thorough combination of computer-aided design, *in silico* modeling, and *in vitro* experiments presented here resulted in the development of a 3D printed network which provided adequate levels of oxygen throughout a TEB construct of more than 20 cm³, or twice the size of any current TEB constructs⁷ to support the viability of differentiating human mesenchymal stem cells. Furthermore, the potential efficacy of tissue engineered bone constructs as implants that can be utilized for direct anastomosis was demonstrated by perfusing fully aggregated constructs for the first time. Analysis of these results determined that post-aggregation viability was a

function of oxygen concentration throughout the construct, and therefore porosity and distance of each bead from the nearest pore. The incorporation of large-diameter inlet and outlet channels allows for the potential direct anastomosis of these constructs to existing host vasculature. In conjunction with this, the major vascular axis featured in these constructs could allow for immediate perfusion upon implantation, while the network of pores would sustain cell life until the network can be fully integrated with the body via spontaneous host vascular ingrowth. While long-term studies will be necessary, this study provides early evidence that 3D printed macroscale vascular networks might find utility as a method to increase viability while providing vasculature in tissue engineered bone constructs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was funded by the National Institutes of Health (R01 AR061460). Additionally, this work was supported by funds appropriated to the Voluntary Graduate Education Program from the U.S. Navy in support of O.B., as well as a National Science Foundation Graduate Fellowship to B.N.B.N.

References

1. Liu Y, Lim J, Teoh SH. Review: Development of clinically relevant scaffolds for vascularised bone tissue engineering. *Biotechnol Adv.* 2013; 31:688–705. [PubMed: 23142624]
2. Laurencin CT, Ambrosio AMA, Borden MD, Cooper JA. Tissue Engineering: Orthopedic Applications. *Annu Rev Biomed Eng.* 1999; 1:19–46. [PubMed: 11701481]
3. Ziegler-Graham K, MacKenzie EJ, Ephraim PL, Trivison TG, Brookmeyer R. Estimating the Prevalence of Limb Loss in the United States: 2005 to 2050. *Arch Phys Med Rehabil.* 2008; 89:422–429. [PubMed: 18295618]
4. Giannoudis PV, Dinopoulos H, Tsiridis E. Bone substitutes: an update. *Injury.* 2005; 36(Suppl 3):S20–S27. [PubMed: 16188545]
5. Sen MK, Miclau T. Autologous iliac crest bone graft: should it still be the gold standard for treating nonunions? *Injury.* 2007; 38(Suppl 1):S75–80. [PubMed: 17383488]
6. Calori GM, et al. Incidence of donor site morbidity following harvesting from iliac crest or RIA graft. *Injury.* 2014; 45:S116–S120. [PubMed: 25457330]
7. Sladkova M, de Peppo G. Bioreactor Systems for Human Bone Tissue Engineering. *Processes.* 2014; 2:494–525.
8. Yeatts AB, Fisher JP. Tubular Perfusion System for the Long-Term Dynamic Culture of Human Mesenchymal Stem Cells. *Tissue Eng Part C Methods.* 2011; 17:337–348. [PubMed: 20929287]
9. Yeatts AB, et al. In Vivo Bone Regeneration Using Tubular Perfusion System Bioreactor Cultured Nanofibrous Scaffolds. *Tissue Eng Part A.* 2014; 20:139–146. [PubMed: 23865551]
10. Abbah, Sa, et al. Osteogenic behavior of alginate encapsulated bone marrow stromal cells: An in vitro study. *J Mater Sci Mater Med.* 2008; 19:2113–2119. [PubMed: 17136608]
11. Nguyen Bao-Ngoc, B.; Henry, Ko; Moriarty Rebecca, A.; Etheridge Julie, M.; Fisher John, P. *Tissue Engineering Part A.* Jan. 2016 ahead of print
12. Bertassoni LE, et al. Hydrogel bioprinted microchannel networks for vascularization of tissue engineering constructs. *Lab Chip.* 2014; 14:2202–2211. [PubMed: 24860845]
13. Bettahalli NMS, Steg H, Wessling M, Stamatialis D. Development of poly(l-lactic acid) hollow fiber membranes for artificial vasculature in tissue engineering scaffolds. *J Memb Sci.* 2011; 371:117–126.

14. Kolesky DB, et al. 3D bioprinting of vascularized, heterogeneous cell-laden tissue constructs. *Adv Mater*. 2014; 26:3124–30. [PubMed: 24550124]
15. Tocchio A, et al. Versatile fabrication of vascularizable scaffolds for large tissue engineering in bioreactor. *Biomaterials*. 2015; 45:124–31. [PubMed: 25662502]
16. Rouwkema J, Rivron NC, van Blitterswijk Ca. Vascularization in tissue engineering. *Trends Biotechnol*. 2008; 26:434–441. [PubMed: 18585808]
17. Liu J, Zheng H, Poh P, Machens HG, Schilling A. Hydrogels for Engineering of Perfusable Vascular Networks. *Int J Mol Sci*. 2015; 16:15997–16016. [PubMed: 26184185]
18. Yeatts AB, Gordon CN, Fisher JP. Formation of an aggregated alginate construct in a tubular perfusion system. *Tissue Eng Part C Methods*. 2011; 17:1171–8. [PubMed: 21895493]
19. Robeson MJ. Model of a tubular perfusion bioreactor using computational fluid dynamics. *Electron Theses Diss*. 2015
20. Wang XM, Waite TD. Gel layer formation and hollow fiber membrane filterability of polysaccharide dispersions. *J Memb Sci*. 2008; 322:204–213.
21. Hwang CM, et al. Fabrication of three-dimensional porous cell-laden hydrogel for tissue engineering. *Biofabrication*. 2010; 2:035003. [PubMed: 20823504]
22. Wang XM, Waite TD. Impact of gel layer formation on colloid retention in membrane filtration processes. *J Memb Sci*. 2008; 325:486–494.
23. Yeatts AB, Geibel EM, Fears FF, Fisher JP. Human mesenchymal stem cell position within scaffolds influences cell fate during dynamic culture. *Biotechnol Bioeng*. 2012; 109:2381–2391. [PubMed: 22422570]
24. Utting JC, et al. Hypoxia inhibits the growth, differentiation and bone-forming capacity of rat osteoblasts. *Exp Cell Res*. 2006; 312:1693–1702. [PubMed: 16529738]
25. Mohyeldin A, Garzón-Muvdi T, Quiñones-Hinojosa A. Oxygen in Stem Cell Biology: A Critical Component of the Stem Cell Niche. *Cell Stem Cell*. 2010; 7:150–161. [PubMed: 20682444]
26. Lennon DP, Edmison JM, Caplan, et al. Cultivation of rat marrow-derived mesenchymal stem cells in reduced oxygen tension: effects on in vitro and in vivo osteochondrogenesis. *J Cell Physiol*. 2001; 187:345–355. [PubMed: 11319758]
27. Krinner, a; Zscharnack, M.; Bader, a; Drasdo, D.; Galle, J. Impact of oxygen environment on mesenchymal stem cell expansion and chondrogenic differentiation. *Cell Prolif*. 2009; 42:471–484. [PubMed: 19594581]

Figure 1. Bioreactor Setup

a) Reaction chamber schematic. Media is perfused through inlet connector in direction of red arrow. All media travels through the printed network, shown in blue, where it escapes through the network pores and passes through the void space between beads. Media then passes through outlet connector, back to the reservoir, where the cycle begins anew. b) Overall setup (acellular). Reaction chamber is hung vertically (far right) to allow for clearance of any air bubbles. Here, media reservoir is shown stacked on top of bioreactor pump, which can be digitally adjusted to allow for a wide variety of flow rates. Media shown here is simply water with green dye, to demonstrate more clearly the full perfusion achieved by the network. c) 4 cell-seeded reaction chambers in parallel using the same pump, housed in one incubator, hung vertically to remove air bubbles d) Harvest sites are enumerated on a fully-aggregated, acellular construct as follows: 1) Bottom Inner 2) Bottom Outer 3) Middle Inner (Core) 4) Middle Outer 5) Top Inner 6) Top Outer. Once again, construct has been perfused with green dye to demonstrate complete perfusion achieved by the printed network. Here, reaction chamber outer tubing and connectors have been removed to demonstrate structural integrity post aggregation.

Figure 2. Architecture of vascular network

a) Major dimensions. b) Sectioned parallel to flow direction. c) Network arranged vertically, as in experiments. Pores run entire length of network, bead stop disk prevents beads from falling below the lowest network pore. d) Section view shows interior geometry, notably ridges and overhangs designed to ensure even distribution of flow throughout all channels, as well as vertical pores, angled in direction of flow. e) Final product, printed, trimmed, and cleaned.

Figure 3. 2D COMSOL Modeling

All panels share the scale depicted on the far right (mM). a) Middle branches, 1.5 mm pore spacing, PCS tubing b) middle section, 9 mm pore spacing, PCS tubing c) Top/Bottom branches, 1.5 mm pore spacing, FEP tubing d) Top/Bottom branches, 9 mm pore spacing, FEP tubing e) Middle branches, 1.5 mm pore spacing, FEP tubing f) Middle branches, 9 mm pore spacing, FEP tubing g) Single tube, 9 mm pore spacing, FEP tubing h) Static control. Here, the peripheral alginate is directly exposed to media. In all panels, the circles represent individual, cell-laden alginate beads, while the void space is acellular alginate. The rectangular nodes on each channel represent network pores, modeled as constant surface concentrations.

Figure 4. Viability Analyses

a,b) Day 1 images from Middle Inner harvest site of the Silicone group at $2.5 \times$ and $10 \times$ magnifications. c,d) Day 2 images from Static Core at $2.5 \times$ and $10 \times$ magnifications. e,f) Day 2 images from Middle Inner harvest site of the Silicone group at $2.5 \times$ and $10 \times$ magnifications. g,h) Day 2 Dead Control images at $2.5 \times$ and $10 \times$ magnifications. i) Legend to be used for graphs in h–j. In these graphs, the vertical axis displays percentage of live cells at the time point. Groups that do not share a letter are statistically different ($p < 0.05$). Note there is no statistical difference in any group in panel h. Dead control results are not displayed due to the complete absence of live cells. j) Composite averages across all harvest sites for each group after Day 1. k) Percent live from all Middle Inner harvest sites l) Composite averages across all harvest sites for each group after Day 2. m) COMSOL simulation results, aligned vertically with the percent live graph results for the group that they represent. Units on legend are mM.

Table 1
Experimental Group Parameters

The experimental groups utilized in this study differ from each other in network pore spacing, bioreactor chamber tubing, and the vascular network embedded within each construct. Here, “Pore Spacing” represents the center-to-center distance of each pore. Pores can easily be seen on a full branched network in Figure 1a.

Experimental Group	Pore Spacing (mm)	Reaction Chamber Tubing	3D Printed Network Type
Static Control	N/A	None	None
Single Tube	9.0	FEP	Single Channel
Distant Pore	9.0	FEP	Full Branched
Close Pore	1.5	FEP	Full Branched
Silicone	1.5	PCS	Full Branched