Bioreactor based engineering of large-scale cartilage grafts for joint resurfacing

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Abstract
Apart from partial or total joint replacement, no surgical procedure is currently available to treat large and deep cartilage defects associated with advanced diseases such as osteoarthritis. In this work, we developed a perfusion bioreactor system to engineer human cartilage grafts in a size with clinical relevance for unicompartmental resurfacing of a human knee joint (i.e., 50mm diameter x 3mm thick). Computational fluid dynamics (CFD) models were developed to optimize the flow profile when designing the bioreactor. Using the developed bioreactor, cells could be seeded throughout the large 50mm diameter scaffold with a uniform distribution. Following two weeks of culture, constructs engineered in the bioreactor were viable, homogeneous, and cartilaginous, with biomechanical properties approaching those of native cartilage. In contrast, constructs generated by conventional manual production procedures were highly inhomogeneous and contained large necrotic regions. This work is the first report of engineered cartilage tissues generated in this large scale. Ongoing efforts are aimed at integrating the up-scaled bioreactor within a fully automated and closed manufacturing system for safe, standardized, and GMP compliant production of large-scale engineered cartilage grafts.
Introduction

While Carticel® and Hyalograft-C® have been well established in the clinic for the treatment of traumatic focal cartilage defects (discussed in Brittberg 2008 and Nehrer 2009), no tissue engineered product is currently available to treat large defects or those associated with advanced diseases such as osteoarthritis. Beyond the biological challenges that must be addressed to treat such joint disorders, it remains a significant engineering challenge to generate up-scaled cartilage grafts with dimensions that would be sufficient for the repair of large, advanced, and deep defects.

We previously developed a perfusion bioreactor for seeding and culturing constructs with a clinically relevant thickness (≈4mm) and demonstrated that highly viable and homogeneous tissue constructs could be generated (Wendt 2006). However, the diameter of the engineered constructs, highly representative of those typically found in the literature for research purposes (i.e., ≈8mm diameter), would not be applicable for the treatment of large defects unless multiple plugs were generated and implanted in a surgical procedure resembling mosaicplasty. Therefore, in this work, we scaled-up our perfusion bioreactor system to engineer large-sized human cartilage grafts, in dimensions that would be sufficient for unicompartmental resurfacing of a human knee joint (50mm diameter x 3mm thick).

Computational fluid dynamics (CFD) models were developed to assist in the design of a bioreactor that could generate a uniform velocity profile over the surface of the large diameter scaffold. Experimental validations, using clinically relevant culture conditions, demonstrated that the developed bioreactor system seeded cells uniformly throughout the large-scale scaffold, and following prolonged culture, supported the generation of viable, homogeneous, and cartilaginous tissue constructs with biomechanical properties approaching those of native cartilage. In comparison to conventional manual production procedures, the quality of the large-scale cartilage tissues that were generated critically depended on the use of the scaled-up perfusion bioreactor system.
Methods

Perfusion bioreactor system

The bioreactor system was designed to first perfuse a cell suspension directly through the pores of a 3D scaffold, to seed cells uniformly throughout the entire scaffold (Wendt 2003), and subsequently perfuse culture media, to maintain cell viability within the seeded constructs throughout culture (Wendt 2006). The bioreactor chamber was fabricated from electropolished 316L stainless steel and included four inlet and four outlet ports (Figure 1a) in order to disperse the cell suspension and the culture media uniformly over the large scaffold surface area. Meshes were clamped around the outer 2mm periphery by a stainless steel ring (Figure 1b) to ensure flow through the scaffold pores. To provide mechanical support to the non-woven mesh and prevent deformation against flow induced drag forces, scaffolds were sandwiched between two stainless steel grids (Bopp AG, Switzerland). The chamber was integrated within the bioreactor system shown in Figure 1c, which included a fluid flow pathway for the phase of cell seeding and one pathway for prolonged culture.

Figure 1. (a) Scaled-up perfusion bioreactor for engineering human cartilage grafts in a size with clinical relevance for resurfacing a human knee joint. (b) Hyaff-11 non-woven mesh scaffolds (50mm diameter x 3 mm thick) were clamped at the periphery to ensure flow through the scaffold pores. Stainless steel grids provided mechanical support to cell-scaffold constructs during seeding and perfusion culture. (c) The bioreactor system was designed to first perfuse a cell suspension directly through the pores of a 3D scaffold, to seed cells uniformly throughout the entire scaffold (Wendt 2003), and subsequently perfuse culture media, to maintain cell viability within the seeded constructs throughout culture (Wendt 2006).

Computational fluid dynamics (CFD) simulations

A fluid finite element analysis of the fluid flow within the bioreactor was developed using MSC Marc, applying no slip boundary conditions and outlet fluid pressure = 0. Both the phases of cell seeding and subsequent cell culture were simulated. The Hyaff-11 mesh was modeled as a viscous fluid, calculated from Darcy and Poiseuille equations, using experimentally measured values for scaffold porosity and permeability.

Cell seeding & construct culture

Human Articular Chondrocytes (HAC) were isolated from cartilage biopsies obtained from three individuals (age: 26-60 years) after informed consent and in accordance with the local Ethical Commission. HAC were expanded for 2 passages (8-10 doublings) in basic culture medium (DMEM, 10% fetal bovine serum, 1mM sodium pyruvate, 100mM HEPES buffer, 100U/ml penicillin, 100µg/ml...
streptomyacin, and 0.29mg/ml L-glutamine) further supplemented with transforming growth factor-β1 (TGF-β1, 1 ng/ml) and fibroblast growth factor-2 (FGF-2, 5 ng/ml), factors previously shown to increase the proliferation rate and post-expansion redifferentiation capacity of human chondrocytes (Barbero Osteoarthritis Cartilage 2004). A clinically relevant cell seeding density was determined based on the number of chondrocytes that can be obtained from an average cartilage biopsy size obtained for autologous chondrocyte implantation (ACI) procedures (≈280mg) (Brittberg Injury 2008), the average chondrocyte yield from a human cartilage digest (2.5E+06 cells/g tissue) (Jacob 2003), and the number of cell doublings during expansion with two passages (≈8 doublings). Based on these calculations, 2.0E+08 HAC were resuspended in basic culture media (90ml) and perfusion seeded into a Hyaff-11 non-woven mesh (50mm diameter x 3mm thick; Fidia Advanced Biopolymers, Italy) at a superficial velocity of 250µm/s for 16 hours within two independent bioreactor systems. Cell-seeded meshes were then either harvested and stained with MTT to assess the distribution of cells seeded throughout the scaffold or were further perfusion cultured in the bioreactor (superficial velocity of 100µm/s) for two weeks to generate a cartilaginous graft. Constructs were cultured in 300ml chondrogenic media (basic culture media supplemented with 10µg/mL Insulin, 0.1mM ascorbic acid 2-phosphate, and 10ng/mL Transforming Growth Factor-β3 (Moretti 2005)) under hypoxic oxygen levels (5%O₂) (Barbero 2010). In parallel, constructs (n=2 per experiment) were also engineered in our research-scale bioreactor system (Wendt 2006) (2.0E+06 chondrocytes seeded and cultured on 6mm diameter x 3mm thick Hyaff-11) to serve as controls for the scaled-up bioreactor system. In one experiment, large-scale engineered constructs were also generated by static cell seeding and static culture to compare the bioreactor based approach to conventional manual manufacturing techniques. Engineered constructs were assessed biochemically, histologically, and biomechanically.

**Histological analyses**
Cell-seeded meshes (n=2) were cut into four sections, rinsed in PBS, and incubated at 37°C for 2 hours with 0.12mM MTT (3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma, St. Louis, MO) to assess the distribution of cells throughout the scaffold. Following two weeks of culture, sections of engineered constructs were rinsed in phosphate buffered saline, fixed in 4% formalin, embedded in paraffin, cross-sectioned (10µm thick) and stained with Safranin-O for glycosaminoglycans (GAG).

**Biochemical quantification**
Engineered constructs were digested with protease K solution (1mg/ml protease K in 50mM Tris with 1mM EDTA, 1mM iodoacetamide, and 10µg/ml pepstatin-A for 15 hours at 56°C) as previously described (Hollander et al, 1994). DNA was quantified with the CyQUANT® Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR), with calf thymus DNA as a standard. GAG was quantified with the dimethylmethylene blue colorimetric assay, with chondroitin sulfate as a standard (Farndale et al, 1986).

**Suture pull-out test**
Since the large-scale grafts would likely require fixation with sutures to the surrounding tissue when implanted, pull-out tests were performed to measure the maximal force that could be applied on a suture in the axial direction before pulling out from the construct, as previously described (Farhadi 2006). Sections of the engineered constructs (≈20mm length x 10mm wide) were secured at their lower end with a suture (POLYSORB 5-0, coated, braided lactomer 9-1, synthetic absorbable suture with a P-13 needle, Syneture) inserted 1 to 2 mm above the end of the specimen and fixed with a flying triple knot to a holding hook affixed to the base plate of the mechanical testing instrument (MTS Synergie 100, MTS Systems Corporation, Eden Prairie, MN). The knot from the suture was additionally held by a needle holder to reduce the internal sliding from the knot. The upper end of the specimen
was gripped with another suture hung from a rigid metal hook attached to the load cell of the test instrument. The actuator was programmed to apply elongation at a rate of 1 mm/min until the suture pulled out of the specimen. The maximal applied force was normalized to the specimen thickness, measured prior to performing the tests, and is reported as N/mm.

*Indentation tests*

Biomechanical properties of the engineered constructs were assessed by both dynamic and impact indentation tests (Helena’s thesis). Three specimens were punched out of each construct and assessed with five repetitions for each test. Dynamic loading was performed with a Synergie 100 MTS® using an indenter tip of radius 1.585 mm. Repeated single sinusoidal cycles at 0.1 Hz were applied under displacement control to a depth of ≈0.1 mm, separated by 50 s pauses, shown to be sufficient to allow dimensional recovery. Impact loading was performed with a single impact micro-indentation (SIMI) device, mounted in a rigid load frame. The SIMI indenter pendulum (radius = 500 µm) falls freely, with impact force determined by mass (1.7 g) and gravity, which provides indentation depths of ≈0.1 to 0.2 mm in healthy cartilage. The motion of the indenter during indentation and rebound is captured by an electromagnetic coil at a sampling rate of 125 KHz. The aggregate moduli ($E^*$) were calculated using the following equation:

$$E^* = \frac{3}{4} \left(1 - \nu^2\right) \left[\frac{P_{\alpha_{max}}}{R^{1/2} \alpha_{max}^{3/2}}\right]$$

where, $\nu$ is Poisson’s ratio (with $\nu=0.44$ [Korhonen, J Biomechanics 2002]), $\alpha_{max}$ is the maximum displacement, $P_{\alpha_{max}}$ is the force at maximum displacement, and $R$ is the indenter radius.
Results

**CFD simulations**
With the goal of obtaining uniform cell seeding and homogeneous tissue development, we aimed to design a perfusion bioreactor chamber that could generate a uniform velocity profile over the surface of a 50mm diameter scaffold. Initial concepts for the chamber were based on a basic scaling of our “research-sized” bioreactor, previously designed to house a scaffold 6-8mm in diameter (Wendt 2006). As shown in Figure 2a, CFD simulations predicted these initial designs, which were based on coaxial single inlet and outlet ports, to induce significantly higher fluid velocities at the center of the scaffold than towards the periphery. Varying the diameter of the inlet/outlet ports had relatively minor effects on the uniformity of the velocity profiles (data not shown). Alternatively, simulations based on the multiple-port chamber (Figure 1a), with flow rates for both the cell seeding and construct culture phases, showed the flow to quickly disperse within the main region of the chamber, resulting in a rather uniform velocity profile over the surface of the 50mm diameter mesh (Figure 2b).

![Simulation of "simple" scale-up with seeding flow rate](image)

*Simulation of "simple" scale-up with seeding flow rate
I think for the paper it's more clear to show the full bioreactor as above than just the half of an asymmetric view you originally provided in the STEPS report.*

![Simulations with: seeding flow rate, culture flow rate](image)

*Simulations with:
- seeding flow rate
- culture flow rate
I think simulations with the slightly rotated view as in the top image are more clear than the bottom image, if possible. (Of course, without including the stainless steel plates with holes). Could we keep the same range in the scale bar for both simulations, otherwise if each are somehow autoscaled I suspect the seeding & culturing simulations would look the same?*  

**Cell seeding distribution**
Consistent with the CFD simulations, which predicted a uniform velocity profile at the surface of the scaffold, MTT staining of the cell-seeded 50mm meshes showed a homogeneous distribution of cells over the surface and throughout the thickness of the scaffold (Figure 3), similar to previous results of perfusion cell seeding of small-scale scaffolds (Wendt 03, Wendt 06). The absence of staining at the periphery of the scaffold verified that the clamping was effective in ensuring fluid flow (i.e., the cell suspension) through the scaffold pores and preventing flow around the edges of the scaffold.
**Figure 3**: Sections of MTT stained meshes following perfusion cell seeding. Cells were distributed throughout the large-diameter scaffolds. The unstained periphery of the scaffold reflects the outer 2mm region that was clamped in the bioreactor.

**Graft composition and structure**

Statically-seeded/statically-cultured constructs had an average of 0.24±0.07% GAG per wet weight tissue, but histologically were highly heterogeneous. As shown in Figure 4a, statically cultured constructs were encapsulated by a dense layer of cells and were intensely stained for Safranin-O along the outer 0.5-1.0mm periphery, but contained a necrotic internal region that was essentially void of viable cells and extracellular matrix. Due to the highly inhomogeneous structure of the static constructs, additional analyses were not performed. Constructs seeded and cultured in the large-scale perfusion bioreactors contained similar fractions of GAG as those cultured in the research-scale systems (50mm: 0.15±0.03%; 6mm: 0.43±0.05% GAG per wet weight) and were histologically highly homogeneous. Cells were uniformly distributed throughout the entire volume of the scaffolds and embedded within extracellular matrix positively stained for Safranin-O (Figure 4b&c). To quantitatively assess the uniformity of extracellular matrix deposition within a large 50mm construct, samples were punched from eight different radial locations and biochemically assessed for GAG content. Samples along the radius were found to have similar GAG fractions, with a coefficient of variation of only 27.5% among the specimens.
Figure 4. Safranin-O staining for GAG following 2 weeks of culture. (Left) Large-scale constructs generated by conventional production methods (static seeding and static culture) had a dense layer of cells and GAG along the outer periphery but contained a necrotic internal region void of cells and matrix. (Middle) Small-scale constructs (6mm diameter x 3mm thick) generated in our previously described “research-scale” perfusion bioreactor (Wendt 2006) contained cells and Safranin-O stained matrix that were uniformly distributed throughout the scaffold. (Right) Large-scale constructs generated in the up-scaled perfusion bioreactor were histologically similar to small-scale perfused constructs, with cells and GAG homogeneously distributed throughout the cross-sections.

Graft handling properties
Following two weeks of perfusion culture, the large-scale constructs were quite stiff such that they could easily be handled with forceps and did not bend under their own weight (Figure 5). On the other hand, these constructs were flexible enough to readily be bent with applied force, suggesting their potential to be molded to the contours of a condyle when implanted.

Graft biomechanical properties
Following two weeks of culture, the average suture pull-out force for 50mm constructs was 0.83 ± 0.10N/mm, which was 20% of the force previously found for native cartilage (4.5N/mm) (Farhadi 2006). Suture retention test were not performed on 6mm constructs due their small size, which physically precluded their testing. The aggregate moduli under impact testing were similar for 50mm and 6mm constructs (50mm: 1.70 ± 0.19MPa; 6mm: 1.88 ± 0.49MPa), and were approximately 10% of the modulus of native cartilage (LOB abstract, 2008). The aggregate modulus of 50mm constructs under dynamic testing was 30% that of 6mm constructs (50mm: 0.32 ± 0.13MPa; 6mm: 1.12 ± 0.56MPa) and 5% of the modulus for native cartilage (LOB abstract, 2008). The biomechanical properties of cell-free Hyaff-11 meshes were also assessed and found to have a negligible suture pull-out force and negligible aggregate moduli (under both dynamic and impact modes), with values below the sensitivities of the instruments.
Discussion
In this work, we have developed a perfusion bioreactor system to scale-up engineered human cartilage grafts to a size with clinical relevance for unicompartamental resurfacing of a human condyle. CFD modeling was employed during bioreactor development as part of a rational design strategy, leading to a bioreactor chamber that facilitated uniform cell seeding throughout the large scaffold volume and the generation of a homogeneous cartilaginous tissue construct. To our knowledge, this is the first report of engineered cartilage constructs generated in this large-scale.

While one seemingly intuitive design to generate uniform flow over the scaffold would be the inclusion of a flow distributor (e.g., perforated plate) within the chamber, it is likely that a significant fraction of the perfused cells would attach/settle on such a device during the cell seeding phase (Wendt 03), thereby dramatically reducing cell utilization. As opposed to scaling-up the bioreactor chamber through a trial and error approach, CFD modeling allowed for the efficient assessment and refinement of various chamber geometries during the initial conceptual design stage. Therefore, the bioreactor could be optimized, in term of flow profile, prior to manufacturing costly prototypes and performing time-consuming experimental tests, saving time and resources.

Hyalograft-C, a 2cm x 2cm x 0.15cm thick Hyaff-11 based cartilage graft, has been shown to provide a clinical improvement to young patients with focal cartilage defects (Nehrer 09). While the current manufacturing process, based on conventional manual static cell/tissue culture techniques (Pavesio 2003), may be sufficient to produce Hyalograft-C in its current size, we speculated that a perfusion bioreactor-based approach could improve the quality of larger sized grafts. Indeed, the data we have presented here are consistent with our previous study (Wendt 2006) showing that the viability, homogeneity, and quality of engineered tissue constructs that are several millimeters in thickness can be dramatically improved when cultured under perfusion vs conventional static culture methods. Moreover, we have also shown that we could...
generate 50mm diameter constructs in the scaled-up bioreactor with similar quality to those generated in our small research-scale bioreactor system, despite the 70-fold upscaling (5.9cm³ vs. 0.085cm³).

Figure 6. Ongoing efforts are aimed to integrate the large-scale bioreactor within a prototype of an automated and closed bioreactor system for graft manufacturing. Bioreactor systems that control, automate, and scale the production of engineered products not only have the potential to improve overall graft quality, but can improve standardization and reduce operating costs, ultimately improving the clinical and commercial success of the engineered product (Martin 2009).

One of the central questions in cartilage tissue engineering that remains to be answered is “How good is good enough?”. In other words, what degree of in vitro maturation is required for a cartilage graft to support or induce a successful repair? Based on ectopic in vivo model systems, Hyaff-11 based constructs that were pre-cultivated in vitro to generate a cartilaginous extracellular matrix had an enhanced capacity to further develop in vivo as compared to those constructs that contained only cells attached to the Hyaff-11 meshes (Moretti 2005, Farhadi 2006). Using a bioreactor-based in vitro model system to simulate joint loading, Demarteau found that mechanical deformation increased the synthesis and accumulation of GAG in engineered cartilage constructs, but only if the tissues were sufficiently developed prior to the time of loading (Demarteau 2003), suggesting that engineered cartilage tissues may need to possess sufficient mechanical integrity and biological responsiveness prior to implantation.

At the time of its implantation, the Hyalograft-C graft contains chondrocytes attached to the Hyaff-11 mesh fibers, but little to no extracellular matrix, and thus, maturation of the implanted construct (biochemically and mechanically) must occur over time within the repair site (Hollader 2006). While a graft with negligible mechanical properties may be sufficient for filling focal defects, in which the surrounding healthy native cartilage may be sufficient to support joint loading, a graft for resurfacing a condyle would likely need to be at a more advanced stage of maturation to provide a higher degree of functionality. Interestingly, recent mid-term follow-up studies have shown poorer clinical outcomes, in terms of defect filling and repair, of larger vs smaller defects treated with Hyalograft-C (Kon 2010, Nehrer 2009). Based on only a two-week culture period, the 50mm constructs engineered in our scaled-up bioreactor had 20% of the suture retention and 10% of the equilibrium moduli of native cartilage tissue. If deemed necessary, the culture time could simply be prolonged or culture conditions further optimized (e.g., culture media supplements, flow rate, oxygen tension) to further increase construct maturity. However, a more developed tissue with higher mechanical properties may not be easily molded to the contours of a joint and may require the engineering of a graft of pre-defined shape. In that case, the design strategy
outlined in this work could serve as a foundation to design a bioreactor for seeding and culturing constructs of anatomically shaped grafts. We have previously reported on the engineering of thin small-scale Hyaff-11 based cartilage constructs, generated by conventional labor-intensive and manual static cell/tissue culture techniques (Candrian 2008). In the current study, we have generated cartilaginous constructs with comparable GAG staining and GAG contents, which were 140-fold larger in size and which were produced by bioreactor-based methods that are amenable to process automation. In this context, ongoing efforts are aimed at integrating the up-scaled bioreactor within a fully automated and closed manufacturing system (Figure 6) for safe, standardized, and GMP compliant production of large-scale engineered cartilage grafts.

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