Imaging and Evaluation of Regenerative Tissue Implantation Technique for Myocardial Repair

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Abstract: The exigent need for a clinical heart repair system motivated us to develop a multifaceted method using human cardiomyocytes seeded on poly(hydroxyethyl methacrylate) (pHEMA) scaffolding to be implanted directly into cardiac infarct tissue. To facilitate this novel repair approach, an implantation and imaging technique was developed and qualitatively examined in vitro to assess its potential for in vivo use. For implantation, degradable and non-degradable pHEMA scaffolds (ca. 400 x 2000 μm rods) were injected by a catheter needle into an excised chicken heart. After fixation, tissue containing implanted pHEMA was stained en bloc with acridine orange and eosin and embedded for Digital Volumetric Imaging (DVI), a technique which combines ultramicrotome sectioning, fluorescence microscopy, and digital imaging and processing to visualize tissues in three dimensions. The implantation technique showed that both degradable and non-degradable pHEMA were successfully injected into the heart tissue; however, the adhesive and fragile characteristics of non-degradable pHEMA indicated that it was unreliable for implantation use. Cell nuclei stained well with acridine orange, and cardiomyocyte nuclear orientation (which may serve as a proxy to indicate muscle fiber orientation in relation to the implanted scaffold), was observed in 3D images. Optimization of the staining protocol will be necessary to minimize over-saturation by acridine orange. Visualization of this scaffold implantation technique brings the field of tissue engineering one step closer to cardiac muscle repair.

1. INTRODUCTION

Cardiovascular disease introduces a significant mortality rate and cost to society [1]. Consequently, heart muscle repair is a vital area of research in the field of tissue engineering. Extant strategies have shown promising results by introducing different types of stem cells and polymer scaffolding into damaged tissue; however, these methods provide minimal functional restoration as well as adverse side effects [2,3]. Despite these emergent technologies in myocardial regeneration and engineered biomaterials, there is no clinically reliable heart repair system available.

One proposed solution is to engineer micro-rods of biodegradable porous scaffolds seeded with human cardiomyocytes and implant them into an infarct area intending to integrate the cells into the native heart tissue to restore structural and contractile function [5]. The success of this new heart repair system relies on the design of the implanted materials in order to optimize their biocompatibility and function, as well as a dependable way to insert these materials into the heart muscle. The novelty of implanting a porous cell-seeded scaffold directly into a beating heart necessitates the development of a minimally invasive clinical implantation technique.

Furthermore, it is necessary to assess the quality of the different parameters comprising an implantation technique throughout its development process, such as the efficiency (e.g., how many implantation attempts are successful, how much of the implanted material remains implanted) and the artifacts acquired by the implanted material or surrounding tissue. Three dimensional imaging analysis provides an evaluation of the implantation technique by allowing navigation through a 3D digital reconstruction of a sample. Thus, 3D imaging analysis, once optimized to observe and assess in vitro implantations, could be used with in vivo testing to observe the organization of cells seeded within the scaffolding as well as their integration with the surrounding native tissue.

For this purpose, there are three objectives of the study: 1) to develop a reliable in vitro technique to implant a scaffold into myocardium, 2) to establish a method of visualizing the implants within the tissue in order 3) to assess the implantation and visualization protocols to optimize for use in vivo.
2. MATERIALS AND METHODS

2.1 Scaffold Preparation

This study used two polymer compositions as a scaffold cut from disks 15 mm in diameter and 0.63 mm thick. One scaffold type was composed of poly(hydroxyethyl methacrylate) crosslinked with polycaprolactone (degradable pHEMA) and a second scaffold type composed of pHEMA crosslinked with tetraethyleneglycol dimethacrylate (non-degradable pHEMA). The purpose of non-degradable pHEMA was to allow us to become familiar with new techniques using a material we have previous experience in handling before extending the novel techniques to a new material. In further studies only the degradable pHEMA will be used as it is specifically engineered to be degraded by enzymes that target the polycaprolactone crosslinkers. Scaffolds were stored in a 90% acetone, 10% water solution until use. Some degradable and non-degradable pHEMA disks were prestained in 5 ml 0.1% eosin Y at pH 5.0 overnight before use to improve visualization during sectioning.

Scaffolds were sectioned for implantation as follows. Prestained pHEMA: one stained degradable and one stained non-degradable pHEMA disk each were removed from eosin and placed in separate dishes containing phosphate buffered saline (PBS) for sectioning. Unstained pHEMA: one unstained degradable and non-degradable pHEMA disk each were removed from stock and placed in separate dishes with 90% acetone, 10% water solution for sectioning. All disks were sectioned into 400 x 2000 μm rods using an industrial razor blade.

2.2 Implantation

One chicken heart (commercially available) was frozen at 20°C for storage and thawed for implantation. A 2-0 monofilament polypropylene suture (Ethicon, Inc., Somerville, NJ) was used to aid in orientation of the sample. The suture was inserted with one pass through the tissue for each of eight implantation sites and knotted outside the tissue after each pass to hold the suture in place throughout processing. One pHEMA rod was pushed into the needle tip of a 21 gauge catheter needle with spring-wire guide (Arrow International, Inc., Reading, PA) and then the pHEMA rod was injected into the tissue with a known orientation to the suture. Tissue containing implanted pHEMA rods and suture was excised and trimmed if the size exceeded 4 mm on any side. Specimens were then loaded into histology cassettes and placed in 10% neutral buffered Formalin overnight.

2.3 Staining

The excised tissue was stained en bloc according to a protocol developed in order to produce staining with fluorescent dyes similar in appearance to that obtained with hematoxylin and eosin, using acridine orange to visualize cell nuclei and matrix and eosin Y to visualize the implanted pHEMA. Cassettes were rinsed three times in 50 ml PBS for 10 min each then placed in 100 ml 0.02 M citrate buffer at pH 5.0 for 1 hr. Cassettes were stained in 100 ml 0.1% eosin Y at pH 5.0 overnight and rinsed two times in 200 ml 0.02 M citrate buffer at pH 5.0 for 3 hr each. Cassettes were then stained in 100 ml 0.1% acridine orange in 0.01 M citrate buffer at pH 5.0 overnight and rinsed two times in 200 mL 0.02 M citrate buffer at pH 5.0 for one hr each. Tissue was dehydrated in graded ethanol starting at 50%, 70%, and twice at 100%, each gradation in 200 ml for one hr. Tissue was prepared for embedding by washing two times in 100 ml xylene for 1 hr each.

2.4 Embedding

Spurr’s Low Viscosity Embedding Resin was prepared in advance at 50% opacity as follows: 10.0 g vinylcyclohexane di(ep)oxide, 6.0 g diglycidyl ether of polypropylene glycol, and 26.0 g nonenyl succinic anhydride were mixed until homogeneous, then 4.8 g sudan black was stirred in for 1 hr, after which 448 μl dimethylaminoethanol was added and mixture continued stirring for 20 min. Spurr’s Resin was stored at -20°C until thawed to room temperature for use.

Specimens were removed from cassettes and individually placed into a xylene/Spurr’s Resin in gradation from 2:1 to 1:1 to 1:3 for 30-60 min each. After the excess suture was cut off specimens were individually placed into sample mold tubes (Microscience Group, Inc., Corte
Madera, CA). The tips of sample mold tubes were filled with enough Spurr’s Resin to cover the specimen. Partially-filled sample mold tubes were centrifuged at 2356 g for 5 min at 20°C then heated at 65°C for about 2 hr until Spurr’s Resin began to thicken. The empty upper part of the sample mold tube was then filled with Spurr’s Resin and placed back in the oven to continue polymerization overnight. Once polymerized, the tip of the sample mold tube was cut off of the embedded specimen.

2.5 Imaging

Samples were analyzed using a Digital Volumetric System (DVI) imager and associated RESView 3.2 rendering software (Microscience Group, Inc., Corte Madera, CA) as described previously [4]. During image acquisition, gain and exposure were adjusted to allow enough signal from the cells so that the full dynamic range of the camera was used without experiencing signal saturation. DVI is a technique which combines ultramicrotome sectioning, fluorescence microscopy and digital imaging and processing to visualize specimens in a 3D rendering that can be rotated around any axis, sectioned to view the specimen internally, and segmented based upon shape or color.

2.6 Artifact Assessment

To observe the effects of fixation and staining on the implanted pHEMA, degradable and non-degradable pHEMA scaffolding were prepared and implanted as described above. In these experiments, pHEMA was not prestained before implantation and no suture was used. Six pieces of tissue 10 mm x 3 mm x 4mm were excised from a chicken heart prior to implantation. In each of three tissue pieces, four degradable pHEMA rods were injected in parallel into one end of the tissue. Similarly, in each of three more tissue pieces, four non-degradable pHEMA rods were injected in parallel into one end of the tissue. Following implantation, one specimen containing degradable pHEMA and one specimen containing non-degradable pHEMA implants were refrigerated unfixed until use. A second degradable pHEMA and a non-degradable pHEMA specimen were fixed in 10% neutral buffered Formalin overnight, and then refrigerated in PBS until use. The remaining degradable pHEMA and non-degradable pHEMA specimens were fixed in 10% neutral buffered Formalin overnight then stained as described above. Stained specimens were held in xylene. All specimens were then embedded in Optimal Culture Temperature Compound (Tissue-Tek, Sakura Finetek USA, Inc., Torrance, CA) for cryosectioning.

Differential interference contrast (DIC) microscopy using a Nikon E800 optical microscope at 10x (0.45 N.A.) was used to visualize unstained specimens.

3. RESULTS

3.1 Implantation Technique

The pHEMA rods were successfully implanted and remained in the tissue throughout processing for DVI. One drawback of the implantation technique was that in preparing the pHEMA rods for injection and loading them into the catheter needle, the scaffold residually stuck to surfaces, especially in a non-aqueous environment as the scaffolding became desiccated. As the catheter needle attempted to deposit pHEMA rods into tissue, the rods sometimes remained on the plunger or in the bore of the needle as it was withdrawn. Because of this adhesive characteristic, which was exacerbated in the prestained pHEMA, the efficiency of the implantation technique needs to be improved. Analysis of 3D renderings proved that both degradable and non-degradable pHEMA rods were successfully implanted into chicken heart muscle, though not with optimal efficiency as some did not remain in the tissue after the needle was withdrawn.

The behavior of the degradable pHEMA observed during implantation included a slight tendency to adhere to the needle bore. The scaffold was an appropriate consistency for handling and few tears were observed in the DVI images (Figure 1a). The non-degradable pHEMA was both more fragile and more adhesive. It often affixed to surfaces in handling and tore or crumbled during sectioning and loading into the needle. In 3D renderings, non-degradable pHEMA rods were often cracked and fragmented within the tissue (Figure 1b). We
have not determined if the fragmentation results from loading the rods into the needle or from the processing protocol for DVI.

Another interesting observation was a gap at the tissue-implant interface. This space around the implanted pHEMA was seen by DVI in all specimens that could result from the tip of the needle when the implant was injected into the tissue or from the implant shrinking during the staining and dehydration process. When used *in vivo*, we expect this gap to be minimal due to pressure applied by the surrounding tissue. Also, when the scaffolding is seeded with cells, the gap should close up with proliferating cells, extracellular matrix, and protein so that if it went through the processing for DVI, the gap should be more integrated and hold the scaffolding in the tissue. This is one occurrence where the *in vivo* case will be better with the natural body response than *in vitro*.

### 3.2 Visualization

In DVI images, both the implanted pHEMA and the surrounding tissue were easily visualized by the acridine orange and eosin staining (Figure 2). Prestained pHEMA did not show up better in DVI images than implants that were not prestained. Acridine orange fluoresced in both the tissue and the implanted pHEMA. During DVI, the acridine orange fluoresced very brightly in the pHEMA and caused the dye to over-saturate the implant. Eosin also fluoresced in the implant as expected. Cell nuclei fluoresced with acridine orange and were also visualized in 3D images (Figure 2). Cardiomyocyte nuclear orientation may serve as a proxy to determine muscle fiber orientation. Therefore, we were able to observe the orientation of implanted scaffold in comparison to nuclear orientation in 3D images.

The DVI images allowed us to examine tissue specimens from all angles in two and three dimensions (Figure 2A and 2B). We were able to follow the cell nuclei and pHEMA through the specimen in order to observe muscle fiber contour, implant structure, and the interaction of the tissue with the implant throughout the entire specimen.

### 3.3 Artifact Assessment

There were no conclusive observations in comparing the specimens cryosectioned at different points in the processing fixation and staining procedure. Cryosectioned specimens showed tissue shrinking as well as swelling of the implanted pHEMA. The shrinking resulted from the processing procedure and was attributed mostly to staining and dehydration, but not fixation. When comparing the DVI images with those taken by DIC at different stages in the processing, the pHEMA showed swelling in processing for the cryosectioning. The pHEMA is differentially hydrated based upon the solution it is in. Therefore, despite some tissue shrinking, the
DVI provides a reliable representation of the implanted pHEMA.

![Figure 2](image-url)  
*Figure 2. Two dimensional (A) and three dimensional (B) images of implanted degradable pHEMA captured by DVI. Scale bars are 250 μm (A) and 300 μm (B).*

4. DISCUSSION

In summary, we have developed a technique for implanting pHEMA scaffolding into cardiac tissue. The implantation technique was tested in *vitro* and the general procedure was validated, as the pHEMA rods were able to be both implanted into and remain in the tissue. However, the implantation still needs to be optimized, in regards to adhesion and determining the cause of fragmentation in the pHEMA.

We also established a viable method of visualizing the implants through staining and DVI. While the staining procedure enabled visualization of the implant, the matrix took up too much dye. Further optimization will be necessary for detailed visualization of both the tissue and the implant. The images obtained from DVI indicated the orientation and relationship of the implant to surrounding tissue and lamellar structure of the heart muscle. This will be helpful in determining the orientation most beneficial to integration of new muscle tissue as well as the relationship of that orientation to the optimum restoration of function. Through these innovative implantation and visualization techniques, minimally invasive cardiac muscle repair is within reach of this novel repair system.

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