Introduction

One of the most powerful properties of stem cells is their ability to differentiate into a variety of cell lineages [1-4]. Accordingly, regenerative medicine strategies have taken advantage of this property when attempting to produce replacements for diseased and damaged tissue [5]. A number of studies have focused on regenerating tissue similar to that found in bone by combining stem cells with biomaterial scaffolds [6-8]. Currently, the standard bone replacement strategies after illness or injury rely on the transplantation of autografts and allografts, requiring harvest of the patient’s own tissue or the use of donor tissue [9]. The use of autografts can result in additional pain to patient as well as cause morbidity at the donor site. When using allografts, there is a possibility of disease transmission and potential immune rejection. The use of engineered tissues can avoid some of these issues, such as the need for donor tissue, by transplanting biomaterial scaffolds seeded with stem cells into the injury site. Currently, mesenchymal stem (MS) cells are the most commonly used stem cell type when engineering bone tissue [6]. MS cells can be derived from a variety of tissues, most commonly bone marrow, and possess the capability to differentiate into osteoblasts as well as chondrocytes and adipocytes [10]. As MS cells are passaged in culture, they begin to proliferate less and differentiate into mature osteoblasts at a slower rate than early passage MS cells [6]. Directing embryonic stem (ES) cells to differentiate into osteoblasts avoids this issue as the two defining features of these cells are their proliferative capacity and pluripotency [1]. We have chosen to use ES cells for testing the biological properties of these composite scaffolds as their use addresses some of the limitations associated with MS cells.
Artificial tissues offer the advantage of being tailored specifically to fit into the injury site as they can be fabricated into a variety of shapes. As these approaches begin to achieve success, the next step to widespread acceptance will require high throughput manufacture of these biomaterial scaffolds. This study examines the steps in the process of fabricating biomaterial scaffolds to identify areas for improvement in terms of automation. We have selected chitosan-calcium phosphate scaffolds as our biomaterial for a variety of reasons, including that such composites can capture the properties of both inorganic and organic features found in naturally occurring bone tissue [11]. Calcium phosphate serves as one of the major biologically active components found in bone tissue, making it a logical choice for this study [12]. Chitosan, a linear polysaccharide derived from the shells of crustaceans, has been used to form biomaterial scaffolds for a variety of applications, including bone tissue fabrication [13, 14]. It also can enhance the ability of cells to adhere to the scaffold. Both of these materials are naturally derived and biocompatible and chitosan-calcium phosphate scaffolds have been used to engineer bone tissue as detailed in numerous studies [15-20]. An additional benefit of using this particular type of composite scaffold is that this biomaterial can be molded into a variety of shapes while providing a suitable microenvironment for cell survival and promoting osteogenic differentiation of stem cells.

In terms of scaffold fabrication, computer numerical control (CNC) machining techniques can be used to manufacture different shapes by pouring the biomaterial mixture into a mold followed by a freeze drying (FD) step to obtain porosity [21]. Thus, scaffold fabrication with these materials is generally carried out at frozen state. However, the FD process takes a long time (2-3 days) to complete, which would cause a bottleneck point in a high throughput manufacturing process. Consequently, the scaffold manufacturing process would be considered relatively slow compared to traditional manufacturing processes. Also, the scaffold fabrication processes are not completely automated with manipulation and transportation still being manual procedures [22]. The EnWave company has developed a new microwave drying technique called radiant energy vacuum (REV) drying, which results in significantly decreased times associated with the drying process and could potentially be automated into the context of the scaffold fabrication workflow [23]. The microwave energy is absorbed directly into the material, and rapid drying is achieved along with generation of pores in the material, which is a desirable feature when designing biomaterial scaffolds for cell culture applications [23]. This REV drying is rapid and uniform and increasingly being used as a commercial drying technology in food science research. With the REV drying method, removal of moisture take place under low pressure with the help of microwave energy as the medium of heat transfer, such that rapid drying can occur even under freezing temperatures [24]. After fabrication of these scaffolds using this new technology, the next major step was to test them to ensure they would support stem cell culture and differentiation.

To our knowledge, this study is the first to use chitosan-calcium phosphate scaffolds with ES cell culture for bone tissue engineering applications. Thus this study combines a novel method of fabricating chitosan-calcium phosphate composite scaffolds for high-throughput bone tissue engineering and it serves as the first report of using this specific composite material as a method of supporting ES cell culture and differentiation. This report demonstrates that these scaffolds support both undifferentiated ES cell culture as well as osteogenic differentiation of ES cells.

**Materials and methods**

All cell culture products were purchased from Invitrogen except where indicated. All chemicals were purchased from Sigma except where indicated.

**Fabrication of chitosan-calcium phosphate composite scaffolds and characterization using scanning electron microscopy (SEM)**

**Figure 1** summarizes the scaffold fabrication process including the two ways of drying these scaffolds (microwave and freeze). To fabricate chitosan-calcium phosphate scaffolds, chitosan solutions (2% by weight) were made by dissolving the chitosan into acetic acid. A range of β-Calcium phosphate tribasic concentrations from 0% to 40% by weight was added to the chitosan solutions. These solutions were then poured into a variety of molds followed by a freezing step and either microwave (EnWave Corpora-
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Figure 1. Schematic of the chitosan-calcium phosphate scaffold fabrication process. First, chitosan (2% by weight) is mixed with acetic acid followed by addition of calcium phosphate (30% by weight). This mixture is then added to molds, which can vary in shape and size, which are then frozen. These frozen scaffolds then undergo a microwave or freeze drying process to remove excess water and enhance porosity, resulting in the production of microwave and freeze dried scaffolds.

For these studies, we used two different mouse ES cell lines: the R1 cell line obtained from the Nagy Lab [25] and the CE3 cell line which expresses green fluorescent protein (GFP) under a β-actin promoter obtained from the American Type Culture Collection (ATCC) [26]. The R1 cells were cultured upon mouse embryonic fibroblast feeder layers obtained from Global Stem in the presence of Dubecco’s modified eagle medium containing 15% ES-qualified fetal bovine serum, 2 mM glutamine, 1 x 10^{-4} M nonessential amino acids, 1 x 10^{-4} M β-mercaptoethanol, 10³ U/ml
leukemia inhibitory factor (Chemicon), 50 U/ml penicillin and 50 mg /ml streptomycin. The CE3 cells were cultured upon gelatin coated flasks (Becton Dickinson) using the same media as the R1 cells.

To show that the composite scaffolds could support ES cell culture, undifferentiated CE3 cells were seeded at a density of 75,000 cells per well into 24 well plates (Corning) containing one composite scaffold per well (n=3) for each type of scaffold (freeze dried and microwave dried). As a control, the same number of cells were seeded onto gelatin coated wells (n=3). Representative fluorescent images were taken with an Olympus IX81 motorized research microscope equipped with a Photometrics CoolSnap HQ2 CCD camera and an X-Cite® exacte microscope fluorescent light source after 3 days of culture. Images were processed using Adobe Photoshop CS5 Extended software - version 12.0.

To promote osteogenic differentiation of R1 ES cells, embryoid bodies (EBs) were formed by culturing undifferentiated cells on non-adhesive plates (BD) for 5 days with the media being changed every other day. These cells were then plated on the composite scaffolds using two different methods (n=3 for each condition). The first method involved dissociating the EBs by treating them with 0.25% trypsin-EDTA for 20 minutes and then seeding these cells onto the scaffolds at density of 50,000 cells per well. The second method involved seeding single EBs onto each composite scaffold. These cells were grown in ES cell media described previously without the presence of LIF (which maintains ES cells in their undifferentiated state) and with the presence of osteogenic factors, including 50 µg/mL ascorbic acid, 50 mM β-glycerophosphate, and 1 µM dexamethasone as previously described by Buttery et al. [27]. These cultures were then analyzed after 8 days of culture using the methods described below.

Assessment of viability and differentiation

To assess cell viability after seeding onto the composite scaffolds, the LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells was used. The cells were incubated with 0.5 mL of 2 µM calcein AM and 4 µM EthD-1 solution for 20 minutes as previously described [28]. The calcein AM solution is catalyzed by the intracellular esterase activity present in live cells to form calcein, which emits green fluorescence. The EthD-1 solution produces a bright red fluorescent signal when it binds to DNA, but it cannot penetrate intact cell membranes. The staining was then imaged using a fluorescent light source as previously described. Images were processed using Adobe Photoshop CS5 Extended software - version 12.0.

Immunohistochemistry

Immunohistochemistry was used to assess the differentiation state of the stem cells after 8 days of culture in media containing osteogenic factors. The cells were first washed with phosphate buffered saline (PBS, pH 7.4) and then fixed for 1 hour in 3.7% formaldehyde solution at room temperature. The cells were then treated with 0.1% Triton-X solution for 45 minutes to permeabilize their membrane followed by treatment with 5% normal goat serum solution to serve as a blocking step. The cells were then incubated with a primary antibody that detects the isoform of alkaline phosphatase expressed in bone, liver and kidney (Millipore, mouse anti-TRA-2-49) overnight at 4˚C. The next day the cells were rinsed thoroughly with PBS followed by incubating with an Alexa Fluor 488 goat anti-mouse IgG secondary antibody (Millipore) for 4 hours at room temperature. The cells were then washed and the resulting staining imaged as previously described.

Results

Fabrication of scaffolds

The first set of experiments performed involved fabricating chitosan-calcium phosphate scaffolds over a range of calcium phosphate concentrations, ranging from 0% to 40% per weight while keeping the chitosan concentration constant at 2% per weight. At lower percentages, the scaffolds dissolved when placed in stem cell culture media (data not shown). When a calcium phosphate concentration of 30% or higher was used when fabricating the composite scaffolds, they would remain intact and stable in media for extended time periods of two weeks. Having determined the necessary conditions for generation of these scaffolds, the next step was to use CNC machining to fabricate molds to produce a variety of shapes using both types of drying processes as shown in Figure 2.
folds were produced in the shapes of tensile bars, long cylinders, and small round cylinders that fit the dimensions of standard 24 well tissue culture plates. No macroscopic differences were observed between scaffolds fabricated using the two different methods of drying. The next step was to characterize the properties of these scaffolds at a microscopic level through the use of SEM as shown in Figure 3. At all levels of resolution, the scaffolds show similar topography independent of the drying method. As the resolution increases, the macroporous nature of these scaffolds becomes evident and little difference is observed between the two fabrication methods. Similar SEM studies were performed on autoclaved versions of these scaffolds and the topography of the scaffolds remained intact (data not shown).

Undifferentiated embryonic stem culture on chitosan-calcium phosphate scaffolds

To determine if these composite scaffolds could support stem cell culture, undifferentiated CE3 mouse ES cells were seeded on top of them. CE3 cells were used as these cells express GFP constantly – enabling them to be visualized on the opaque composite scaffolds. Figure 4 shows the results of this experiment as undifferentiated ES cells were able to adhere and proliferate on all three types of sur-

Figure 2. Free form molds were used to fabricate a variety of shapes, including tensile bar, round cylinder, and small round scaffolds suitable for use with standard 24 well tissue culture plates (from left to right). Scale bar is 1 cm. All scaffolds contain 30% calcium phosphate and 70% chitosan. The first and third scaffolds shown were fabricated using the freeze drying method while the second scaffold was fabricated using the microwave drying method.

Figure 3. Scanning Electron Microscopy (SEM) images comparing the features of composite chitosan-calcium phosphate scaffolds at various resolutions. (A) Low (C) Medium and (E) High resolution images of composite scaffold made using the traditional freeze drying method. (B) Low (D) Medium and (F) High resolution images of composite scaffolds made using the faster alternative microwave drying method. For low resolution images, the scale bar is 2.00 mm. For medium resolution, the scale bar is 100 µm. For the high resolution images, the scale bar is 50 µm.
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Figure 4. Composite chitosan-calcium phosphate scaffolds support undifferentiated embryonic stem (ES) cell culture after 3 days of culture. A) Control GFP labelled ES cells seeded on 2D gelatin surfaces. B) GFP labelled ES cells seeded on freeze dried scaffolds. C) GFP labelled ES cells seeded on microwave dried scaffolds. The bright white regions indicate GFP positive ES cells. The grey regions in B and C are the scaffolds. Scale bar is 500 µm.

Figure 5. Cell viability staining after 8 days of culture in the presence of osteogenic factors. A) Dissociated EBs seeded onto 2D gelatin surfaces. B) Dissociated EBs seeded onto freeze dried chitosan-calcium phosphate scaffolds. C) Dissociated EBs seeded onto microwave dried chitosan-calcium phosphate scaffolds. Green fluorescence indicates live cells while red fluorescence indicates dead cells. Scale bar is 500 µm.

faces: 2D gelatin coated wells (serving as a control surface), freeze dried composite scaffolds, and microwave dried composite scaffolds. The scaffolds themselves had some auto-fluorescence, but a much stronger fluorescent signal was observed from the CE3 cells expressing GFP. These images were taken on Day 3 of culture. The undifferentiated ES cells had a high rate of proliferation on both the gelatin and composite scaffolds.

Osteogenic differentiation of embryonic stem cell cultures on chitosan-calcium phosphate scaffolds

After determining that these composite scaffolds could sustain ES cell culture, the next step was to induce the cell to differentiate into osteogenic lineages while seeded on the scaffolds. For this set of studies, R1 ES cells were used as they do not express GFP and could be stained to assess cell viability and extent of differentiation. To induce the ES cells to differentiate, a 5 day embryoid body formation process was used as described in the methods. The first set of experiments seeded dissociated EBs on scaffolds fabricated using both methods. These cells were then cultured in the presence of osteogenic factors (ascorbic acid, β-glycerophosphate, and dexamethasone) for 8 days followed by cell viability staining as shown in Figure 5. Similar levels of viability were observed across cultures both on the gelatin coated surfaces and the composite scaffolds with both viable and dead cells being observed for all conditions. The cells seeded on the gelatin tended to spread out more compared to those cells seeded on the scaffolds.

To increase the fraction of cells that survived, intact EBs were also seeded on top of the composite scaffolds and cell viability was assessed...
When the intact EB culture method was used, much higher levels of cell proliferation and viability were observed. The cells tended to spread out more using this culture method on both the control gelatin surfaces as well as the composite scaffolds compared to the dissociated EB culture method. As seen in Figure 6B, the cells that have migrated away from the EB tend to spread out. In Figure 6C, the cells closer to the EB have a small, rounded morphology. These effects were observed for both sets of scaffolds.

Due to marked improvement in proliferation and viability, this method was then used for the cell seeding studies to analyze the differentiation state of the ES cells. To confirm that these cells were differentiating in osteoblasts, staining for bone specific isoform of alkaline phosphatase (AP) was performed as shown in Figure 7. While diffuse AP was observed when the cells were seeded on the 2D gelatin surfaces, more intense staining for this marker was observed when the cells were seeded on scaffolds fabricated using both methods. The staining was observed on cells that had migrated out from the EB.

**Discussion**

The purpose of this work was to evaluate chitosan-calcium phosphate scaffolds fabricated using a new technique (microwave drying) compared to the traditional fabrication method (freeze drying) to determine if they would support ES cell culture with the end goal of engineering bone tissue. Characterization of these scaffolds demonstrates that both fabrication methods produce structurally similar scaffolds and that microwave drying can provide a faster...
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way of manufacturing these biomaterial scaffolds, which is an important consideration when automating this scaffold fabrication process.

Scaffolds fabricated under both sets of conditions also showed similar characteristics in terms of supporting both undifferentiated and differentiated ES cell cultures. In terms of cell seeding, we found that culturing intact EBs on these scaffolds led to enhanced cell survival compared to the use of dissociated EBs. It is possible that the dissociation process leads to a decrease in viability and this observation was consistent with other studies investigating the best method for seeding ES cells on biomaterial scaffolds [28]. As mentioned previously, the majority of bone tissue engineering studies use MS cells and these studies have focused on seeding dissociated cell cultures onto scaffolds [29, 30]. These previous studies report higher levels of cell viability after seeding onto scaffolds than what we observed when seeding our dissociated ES cell derived cultures. This difference in dissociated cell culture may be due to the differences in gene expression profiles between MS and ES cell lines. However, when we seeded intact EBs on the scaffolds, higher levels of cell viability and proliferation were observed, avoiding this issue.

ES cells tend to spread out as they differentiate, suggesting that the dissociated cultures seeded on the scaffolds that did not exhibit spreading remained undifferentiated. When the intact EBs were seeded on the scaffolds, they did spread and differentiate as indicated by the AP staining that was observed in the cells that had migrated out from the EBs. Another interesting observation is that higher levels of AP staining were observed when the cells were seeded on the scaffolds compared to those seeded on the 2D gelatin surface, suggesting that the scaffold material further enhances osteogenic differentiation beyond what was induced by the soluble factors present in the media.

This study highlights the use of ES cells as an alternative MS cells and it is the first report of using chitosan-calcium phosphate scaffolds combined with ES cells as a strategy for bone tissue engineering. ES cell lines offer the advantages of enhanced proliferation and differentiation capacity compared to MS cells [6]. In terms of future work towards high throughput manufacture of biomaterial scaffolds, the next steps will involve integrating and automating the individual processes of the scaffold fabrication process with the microwave drying procedure. Other areas to further study include evaluating methods of seeding cells onto scaffolds to ensure cell survival and osteogenic differentiation that would be compatible with such an automated tissue engineering process. In terms of the stem cells, this study has only begun to characterize the potential of ES cells combined with chitosan-calcium phosphate scaffolds for bone tissue engineering. Additionally with the recent development of induced pluripotent stem (IPS) cells [4], this study can serve as starting point when developing similar scaffolds for examining the osteogenic potential of this new type of stem cell.

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