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A Rapid Seeding Technique for the Assembly of Large Cell/ Scaffold Composite Constructs*

LUIS A. SOLCHAGA, Ph.D.¹, ENRICO TOGNANA, Ph.D.⁴, KITSIE PENICK, A.Sc.², HARIHARA BASKARAN, Ph.D.³, VICTOR M. GOLDBERG, M.D.¹, ARNOLD I. CAPLAN, Ph.D.², and JEAN F. WELTER, M.D., Ph.D.²

¹Department of Orthopaedics, Case Western Reserve University Cleveland, Ohio.

²Department of Biology (Skeletal Research Center), Case Western Reserve University Cleveland, Ohio.

³Department of Chemical Engineering, Case Western Reserve University Cleveland, Ohio.

⁴Department of Fidia Advanced Biopolymers, Abano Terme, Italy.

Abstract

These studies address critical technical issues involved in creating human mesenchymal stem cell (hMSC)/scaffold implants for cartilage repair. These issues include obtaining a high cell density and uniform spatial cell distribution within the scaffold, factors that are critical in the initiation and homogeneity of chondrogenic differentiation. For any given scaffold, the initial seeding influences cell density, retention, and spatial distribution within the scaffold, which eventually will affect the function of the construct. Here, we discuss the development of a vacuum-aided seeding technique for HYAFF®-11 sponges which we compared to passive infiltration. Our results show that, under the conditions tested, hMSCs were quantitatively and homogeneously loaded into the scaffolds with 90+% retention rates after 24 h in perfusion culture with no negative effect on cell viability or chondrogenic potential. The retention rates of the vacuum-seeded constructs were at least 2 times greater than those of passively seeded constructs at 72 h. Histomorphometric analysis revealed that the core of the vacuum-seeded constructs contained 240% more cells than the core of passively infiltrated scaffolds. The vacuum seeding technique is safe, rapid, reproducible, and results in controlled quantitative cell loading, high retention, and uniform distribution.

INTRODUCTION

IN THE PAST DECADE, tissue engineering has received increasing attention as a means of creating bioartificial tissues and organs (both for implantation) and as model systems for *in vitro* biological studies.¹⁻⁵ Tissue-engineered cartilage appears promising as an alternative to the currently available therapies for cartilage lesions.^{6,7} Among musculoskeletal tissues, cartilage provides a good candidate for practical tissue engineering applications, as cartilage is relatively simple, avascular, and contains a single cell type.

In most tissue engineering approaches, cells are typically isolated from a small biopsy and then expanded in culture. These culture-expanded cells are then seeded onto a suitable 3-dimensional scaffold, induced to develop into a tissue-like structure *in vitro*,⁸⁻¹² and/or are implanted *in vivo* into an orthotopic site to repair or replace a damaged tissue.¹³ Ultimately,

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Correspondence to: JEAN F. WELTER.

Address reprint requests to: Jean F. Welter, M.D., Ph.D. Skeletal Research Center Case Western Reserve University 2080 Adelbert Road, Millis Hall Cleveland, OH 44106-7080 E-mail: jean.welter@case.edu.

the phenotype of a cell-based tissue-engineered construct depends on a number of factors, such as the composition, shape, size, porosity, and biodegradability of the scaffold, as well as the type, density, and spatial distribution of the cells within the construct.¹⁴ For example, the cellularity of the engineered construct depends on the number of cells initially introduced into the construct, on the retention of these cells during the first few hours to days, until cell–cell and cell–scaffold interactions have been established, and on the mitotic activity of the cells incorporated into the construct. If the construct is intended to develop into a load-bearing tissue such as articular cartilage, a homogeneous composite, with an even spatial distribution of cells within the construct, is also important.

Currently, there are 2 categories of methods for seeding cells in a 3-dimensional matrix with the aim of achieving uniform distribution: passive and active.^{15,16} Typically, in the passive seeding methods, cells are laid on top of the scaffold and allowed to infiltrate the scaffold over time. The primary advantage of passive seeding is that it is a simple process in which cells are not subjected to potentially damaging large mechanical forces, for example, high shear stresses,^{17,18} resulting in a greater viability. The main disadvantage, particularly in the case of thick scaffolds with high degrees of tortuosity, is that the infiltration rates can be very low, resulting in poor loading, and uneven distribution of cells within the scaffolds. In the active seeding methodologies, an external force is applied to enable the cells to infiltrate the scaffold at a faster rate. The nature of the force varies from centrifugal¹⁵ to external pressure gradients.¹⁶ The primary advantage of these methods is that, with good control, one can achieve quantitative loading and a more homogeneous distribution of cells. There are some disadvantages that vary from method to method, primarily that the seeding protocol can become rather complex¹⁶ and time-consuming.¹⁹

The goal of this project was to establish a simple, rapid, and reproducible protocol for uniform seeding of cells in preformed porous scaffolds. The application of this protocol results in fine control of cell seeding density, coupled with high cell viability and retention, and uniform cell distribution in large-scale (14-mm diameter by 6-mm thick) carrier matrices.

MATERIALS AND METHODS

Materials

Cell culture medium and trypsin were obtained from Invitrogen (Carlsbad, CA) or Mediatech (Herndon, VA). Several lots of fetal bovine serum (FBS) were obtained from Invitrogen, and screened as described by Lennon *et al.*²⁰ Cell culture plasticware was from BD Biosciences (Franklin Lakes, NJ). Gas-permeable 0.005-in. Teflon[®] bags were custom-made by the American Fluoroseal Corporation (AFC, Gaithersburg, MD), and were used as bioreactors. Live/dead assay kits and carboxyfluorescein diacetate-succinimidyl ester (CFDA-SE) were from Molecular Probes (Eugene, OR). Percoll, Hoechst 33258 dye, dexamethasone, and Tyrode's salt solution were from Sigma-Aldrich Chemical Co. (St. Louis, MO). HYAFF[®]-11 sponges were generously supplied by Fidia Advanced Biopolymers (Abano Terme, Italy). Growth factors (epidermal growth factor [EGF], fibroblast growth factor [FGF], transforming growth factor β 1 [TGF- β 1]) were from R&D Systems (Minneapolis, MN). ITS⁺ Premix[™] was from Collaborative Biomedical Products (Collaborative Research, Inc. Bedford, MA), and ascorbate-2-phosphate was from WAKO (Richmond, VA). Syringe pumps were from Harvard Instruments (Holliston, MA).

Scaffolds

HYAFF[®]-11 sponges (Fig. 1) were 7 or 14 mm in diameter by 3 to 5 mm thick. HYAFF[®]-11 is a linear derivative of hyaluronic acid (HA) modified by complete esterification of the carboxylic function of the glucuronic acid with benzyl groups. The benzyl derivatization

converts the hydrophilic HA into a hydrophobic material. The sponge was made by mixing HYAFF®-11 dissolved in dimethyl sulfoxide with salt particles, followed by phase inversion, salt leaching, and lyophilization.²¹ The porosity of the sponge material is above 80%, with an average pore size of 83 μm (range 4–345 μm) and a surface area to volume ratio of 0.039 m^2/cm^3 , and high pore interconnectivity.^{22,23}

Cell culture media

Growth media—Human bone marrow-derived mesenchymal stem cells (hMSCs) were expanded in Dulbecco's Modified Eagle's Medium with 1 g of glucose/L (DMEM-LG) supplemented with 10% FBS. Chondrocytes were expanded in DMEM:F12 1:1 supplemented with 10% FBS, 5 $\mu\text{g}/\text{mL}$ of insulin, 10 ng/mL of EGF, 10 ng/mL of FGF, and 1 ng/mL of TGF- β 1.

Chondrogenic medium—The defined chondrogenic medium was composed of DMEM base with 4.5 g of glucose/L (DMEM-HG). It was supplemented with 1% ITS + Premix™ (625 $\mu\text{g}/\text{mL}$ of insulin, 625 $\mu\text{g}/\text{mL}$ of transferrin, 625 ng/mL of selenious acid, 125 mg/mL of serum albumin, and 535 $\mu\text{g}/\text{mL}$ of linoleic acid), 100 μM ascorbate-2-phosphate, 10^{-7} M dexamethasone, and 10 ng/mL of TGF- β 1.²⁴

Cells

Human bone marrow-derived MSCs were used in these experiments. The cells were harvested as described by Haynesworth *et al.*²⁵ Briefly stated, 10 to 15 mL of human bone marrow was harvested from the posterior iliac crest of healthy adult volunteer donors through a Jamshidi biopsy needle. The marrow aspiration procedure was reviewed and approved by the University Hospitals of Cleveland Institutional Review Board; informed consent was obtained from all donors. The marrow sample was combined with DMEM-LG supplemented with 10% FBS and centrifuged at 500 g for 5 min. The resulting pellet was resuspended in 5 mL of DMEM-LG with 10% of a selected batch of FBS,²⁰ and layered over 35 mL of 63% (v/v) Percoll in Tyrode's salt solution, adjusted to the final NaCl concentration of 0.1 M. After centrifugation at 460 g the top 25% of the gradient (pooled density 1.03 g/mL) was transferred to a 50-mL tube. The volume was increased to 50 mL with serum-containing medium, the tube was centrifuged at 500 g, and the resulting pellet was resuspended in 7 mL of serum-supplemented medium. These cells were seeded onto 10-cm plates at 1.8×10^5 nucleated cells/ cm^2 . After 4 days, nonadherent cells were removed by changing the medium. The medium was changed every 3 days thereafter. After approximately 2 weeks in primary culture, and prior to reaching confluence, the cells were passaged using trypsin, and seeded at 5×10^3 cells/ cm^2 .

Vacuum chamber

A SpeedVac rotary evaporator (Savant Thermo, Marietta, OH) was adapted for use as the vacuum chamber by removing the rotor. Several multiwell plates can be stacked simultaneously within the chamber. The large-bore bleed valve allows for a rapid return of the chamber to atmospheric pressure, and the clear acrylic lid permits observation of the process. A vacuum pump (Welch DuoSeal, Welch Rietschle Thomas, Skokie, IL) was used as the vacuum source, and a calibrated vacuum gauge was placed in line with the chamber to monitor the chamber pressure.

Seeding

The scaffolds were placed in a low-attachment 24-well plate and the cell suspensions were then overlaid on the scaffold at concentrations ranging from 2 to 100×10^6 cells/mL (Fig. 2A) in a final volume of chondrogenic medium equivalent to the fluid retention volume of the matrices. The retention volume of the scaffolds had previously been estimated by weighing a

duplicate set of test scaffolds, first dry and then again following exhaustive vacuum hydration. The difference between these two measurements was used as the retention volume. The cells were then either allowed to passively infiltrate the matrices, or vacuum was applied to draw the cell suspension into the construct. Six cycles of vacuum up to -90 kPa (85 mm Hg absolute), followed by rapid release to atmospheric pressure, were applied (Fig. 2C). Each cycle lasts approximately 30 s. The constructs were inverted after 3 cycles. The entire procedure takes less than 10 min. The constructs were then incubated at 37°C in a humidified atmosphere of 7.5% carbon dioxide (CO_2) in an air environment for 3 h to allow attachment, during which time they were inverted every hour to reduce gravity-induced settling of the cells within the scaffolds (Fig. 2D). After the incubation period, the constructs were placed in the bioreactor.

Bioreactor culture

To provide adequate nutrient supply and gas exchange to the composite constructs, we used a perfusion bioreactor system. Our system utilizes gas permeable Teflon bags, an approach previously established for leukocyte culture.²⁶ The bags shown in Fig. 3 were custom-made for this project by AFC. After the 3-h static incubation period described in the preceding text, the constructs were sealed in the bioreactor bags using clips. The bags were perfused with chondrogenic medium at $250\ \mu\text{L/h}$ via a remote microprocessor-controlled 10-channel syringe pump, which results in an average residence time of 12 h in the bag for the medium. The effluent culture medium was collected in waste containers and was not recycled. The bags were placed on a rocking tray to minimize gradients of nutrients and byproducts throughout the volume of the bag. With respect to achieving uniform conditions within the bioreactor, this approach provides similar advantages to the rotating vessel reactors, but with a simplified liquid handling system. The whole system was housed in a Forma water-jacketed incubator at 37°C with a 7.5% CO_2 atmosphere.

Cell viability after vacuum treatment

To determine whether the cells were negatively affected by the vacuum treatment, replicate aliquots of cells were either vacuum treated or left untreated while suspended in the same kind of medium as used for seeding. We plated the cells on 60-mm diameter dishes, and then harvested and counted them on 4 consecutive days following seeding (Fig. 4A). The Molecular Probes live/dead assay was also utilized, following the manufacturer's recommendation, on similarly treated cells.²⁷ In this assay, cell viability is assessed by intracellular esterase activity (cleavage of the AM-ester group) to label live cells. Dead cells are identified by the failure of the membrane barrier to exclude the DNA-labeling dye, ethidium homodimers (Fig. 4B and C).

Harvest and analysis

The constructs were incubated for 0 to 3 days to assess the initial cell distribution and cell retention, or for up to 5 weeks to assess chondrogenic differentiation. After the incubation period, the constructs were removed from the bags, and cut into halves or quarters.

One piece was fixed with 10% neutral buffered formalin and processed for standard histology. In some cases, the cells were metabolically labeled with the amine-reactive, membrane-permeant fluorescent dye CFDA-SE, according to the manufacturer's instructions, before harvesting, to identify live cells. Alternately, some sections were stained with 4', 6'-diamidino-2-phenylindole hydrochloride (DAPI) nuclear stain for image analysis. Sections were then either stained with toluidine blue O or left unstained for fluorescence microscopy. To evaluate cell density and homogeneity of the cell distribution, either DAPI or CFDA fluorescent images were obtained using an inverted microscope (IX-71, Olympus Corporation, Japan) through a $\times 10$ objective and a SPOT-RT camera (Diagnostic Instruments, Sterling Heights, MI). A montage of the entire construct was made from individual images (Fig. 5A

and B).²⁸ The montage was analyzed using image analysis software (Image Pro-Plus, Media Cybernetics, Silver Spring, MD). The images were thresholded and particles were identified as cells and quantified after filtering based on shape and size (Fig. 5C and D). To quantify distribution, the outlines of sections were traced and the geometric center of the section was obtained by image analysis. Horizontal regions of interest (ROI) of 250 μm width were obtained. The number of cells in each ROI was then normalized to the area of the ROI to obtain local cell density. Local cell densities were plotted for each ROI as a function of the normalized distance from the geometric center of the construct section. In addition, cell density at the center of the scaffold was calculated by averaging local cell densities of ROIs that are within a 25% distance from the center of the construct section. For cell retention analysis, all the cells were counted and the number of cells per section was normalized to the surface area of the entire tissue section, yielding the construct cell density. The remaining pieces of the construct were used to determine cell density by evaluation of the total DNA content. Each quarter was dissolved in 1 mL of 0.1 N NaOH and the lysate was neutralized with 1 mL of 0.1 N HCl in 5 M NaCl, and 100 mM NaH_2PO_4 (pH 7.4). One mL of the neutralized lysate was combined with 1 mL of 0.7 $\mu\text{g/mL}$ Hoechst 33258 dye in water. Fluorescence intensity was measured relative to a DNA standard using a DyNAQuant 200 fluorimeter (Hoefer, San Francisco, CA).

Statistical analyses were by analysis of variance, or *t*-tests on paired sample comparisons using SigmaStat (Systat Software, Inc., Point Richmond, CA) or Minitab (MINITAB, State College, PA) software.

RESULTS

Scaffold retention volume

For a nominally 14-mm diameter by 5-mm thick HYAFF[®]-11 sponge, the retention volume was measured to be approximately 750 μL , equivalent to a void fraction of 97%.

Cell viability and proliferation after vacuum treatment

Growth curves developed from cells subjected to a sham vacuum seeding procedure or left as untreated control cells did not reveal any negative impact of the vacuum treatment (Fig. 4A). Similar to the proliferation assays, the live/dead assay revealed no statistically significant differences in the number of viable cells in vacuum-treated constructs compared to that in control constructs 24 h after treatment (Fig. 4B and C).

Static (passive) seeding

Passive seeding of the cells onto sponge-type matrices resulted in poor penetration of the cells into the interior of the scaffold (Figs. 5B and 6). This is due in part to the hydrophobicity of the material and, to some extent, to the plugging of the porous scaffold by cells trapped at the outer surface of the construct.

Vacuum seeding

Evacuation of the chamber by the application of vacuum greater than -80 kPa caused the air trapped within the porous structure to expand sufficiently that it escaped from the sponges as bubbles. It took on average 35 s for the pressure to drop by approximately 90 kPa from atmospheric (~ 100 kPa). When the vacuum was released, a substantial portion of the surrounding cell suspension was drawn into the sponges by the contracting air bubbles still trapped in the sponge (Fig. 2D). In preliminary experiments (not shown), the complete volume of cell suspension was usually drawn into the scaffolds after 3–4 vacuum–release cycles. Sometimes, 1 or 2 additional cycles increased the volume of suspension infiltrated into the scaffold slightly; we therefore standardized our protocol to use 6 cycles in all cases, so that all the samples would be treated the same. Thus, 6 cycles were used in all the experiments.

presented here. Occasionally a small patch of unwetted material would remain where the bottom surface of the scaffold contacted the bottom of the well. We resolved this issue by inverting the scaffolds after 3 vacuum cycles. Matching the total cell suspension volume to the available void space in the scaffold (measured as described above for each lot of scaffold material) resulted in essentially all of the cell suspension being drawn into the scaffolds. The process can be completed in less than 10 min. Cells were seeded (Fig. 7) using low (13×10^6 cells/mL, Fig. 7A), medium (33×10^6 cells/mL, Fig. 7B), and high (53×10^6 cells/mL, Fig. 7C) seeding densities. At low cell densities, cells preferentially line the interior surfaces of the carrier scaffold, leaving the pore space empty (Fig. 7D). At higher ($60\text{--}70 \times 10^6$ cells/mL) densities, scaffold void spaces are saturated with cells (Fig. 7E).

Initial cell retention

Cell retention was quantified both by measuring the DNA content in the cell-scaffold constructs, and by image analysis of histological sections. After 24 or 72 h of incubation under perfusion culture, the vast majority (80–90%) of the cells vacuum loaded onto sponges were retained within the matrices, even when cells were loaded at very high densities (Fig. 8A). In contrast, 24-h cell retention was lower (70%) in passively seeded constructs ($p < 0.02$), and fell to about 40% at 72 h ($p < 2 \times 10^{-6}$). Microphotographs of constructs after vacuum loading and incubation with CFDA-SE-labeled cells (Fig. 7) show that increasing seeding density yielded an increased filling of the pores of the scaffold until the space available was completely filled with cells at densities of about 70×10^6 cells/mL.

Cell distribution

Passive infiltration of the cells resulted in an unsatisfactory, nonhomogeneous distribution of the cells both across the surface and thickness of the construct. In contrast, cyclic vacuum loading at up to 90 kPa below atmospheric, followed by a rapid return to atmospheric pressure, for 6 cycles resulted in a uniform loading profile.

Histomorphometric analysis of constructs harvested 24 h after seeding, measuring area-normalized cell number as a function of the depth of the scaffold, shows that the vacuum seeding process results in a greater overall cell density ($p < 0.01$). The biggest differences in cell density are found at the center of the scaffold, where scaffolds loaded by cyclic vacuum contain 240% more cells per unit area (675 vs. 285 cells/mm², $p < 0.01$) scaffolds loaded by passive infiltration (Figs. 5 and 6). There is also a qualitative difference in the distribution as seen in the contour plot of cells; the vacuum-seeded constructs have more uniform cell distribution than the control constructs (Fig. 6). Analysis of variance of cell density data in vacuum-treated and control constructs shows that there is a significant difference in the distributions; the cell density of vacuum-treated constructs does not depend on depth from the construct surface ($p = 0.787$), whereas the cell density of control constructs depends on depth ($p = 0.017$).

Chondrogenesis

The high cell retention persisted at 1 week, the last time point at which it was practical to make DNA content measurements. As early as 2 weeks after seeding as described here, hMSCs and chondrocytes seeded underwent chondrogenic differentiation producing cartilaginous matrix, as shown by metachromatic staining with toluidine blue and by immunostaining with antibodies against type II collagen. By 3 weeks, a cartilaginous ECM was even more developed (Fig. 9).

DISCUSSION

For many cell types, cell density and cell–cell contact are potent modulators of differentiation and proliferation. Success at engineering tissues such as tendon²⁹ and heart muscle³⁰ has been shown to depend on fine control of the initial seeding density. At low seeding densities, the cells tend to form a fibrous connective tissue without the desired structural properties,³¹ while excessively high seeding densities increase the risk of cell death due to nutrient limitations. Previous studies have defined conditions under which the chondrocyte phenotype can be induced in hMSCs, or restored in culture-expanded chondrocytes.^{24,32} The requirements include not only a defined chondrogenic medium, but also the need for very high cell densities and intimate cell–cell contact in a 3-dimensional environment. No precise definition of a threshold cell density has been made, although the work of Lennon *et al.* suggests that even minor inhomogeneity of the cell population diminishes or impedes chondrogenesis.³³ While high-density conditions are readily achievable using cells alone in small-scale cell culture using micromass or pellet culture systems,³² the development of larger specimens suitable for preclinical models and, ultimately, clinical applications, requires the use of a supportive substrate or scaffold to provide structural integrity and support for the cellular component. Because of the demonstrated importance of cell–cell interaction and paracrine factors in chondrogenic differentiation, we have adopted the working hypothesis that, for cartilage tissue engineering, maximizing the initial cell density is the optimal approach.

Combining cells with a carrier scaffold is one of the first, and arguably most important, steps in tissue engineering. An intuitive solution to this step would be to form the scaffold around the cells; however, this severely limits the choice of physical and chemical properties of the scaffold material, as many useful solvents and manufacturing processes would be cytotoxic. We have, therefore, focused on preformed scaffolds, because this theoretically allows us the future option to control scaffold properties.

The goal of this project was therefore to establish an optimized and reproducible protocol to seed such pre-formed scaffolds with cells. Scaffolds such as HYAFF®-11 sponges are attractive for cartilage tissue engineering because of their chemistry, but, although highly (>80%) porous, they are fairly difficult to seed. This is due to a combination of the hydrophobic surface and the pore structure, which has a high rate of connectivity, but small inter-pore openings (Fig. 1, inset). However, once seeded, these same properties lead to outstanding cell retention, and the scaffolds retain a rigidity that makes them easy to manipulate. The pore structure ensures intimate cell–cell contact, which should enhance chondrogenic differentiation.^{32,33}

Static seeding (i.e., the cells are layered on to the scaffold) has historically been the most commonly used seeding method.^{14,34–37} Drawbacks of this method are that, as in our study, it usually results in a relatively low initial cell density.³⁸ It also tends to result in inhomogeneity owing to cell trapping at the surface of the implant. It is thus less than optimal for developing tissue equivalents with high cell density, in particular those in which tissue-specific development requires intimate cell–cell interactions. In our hands, static seeding failed to provide the kind of control of cell density and cell distribution that we require.

In contrast, our experimental results show that, in pre-formed scaffolds, our vacuum loading technique allows us to achieve:

- Fine control of the seeding density: As long as the final volume of the cell suspension was equal to the retention volume of the scaffold, seeding was essentially quantitative. The seeding density can thus be adjusted by varying the cell concentration in the inoculum.

- Uniform seeding: No peripheral to central seeding gradients were observed using the vacuum loading technique. Although the pore connectivity in HYAFF®-11 is high, there were occasional void spaces in the cell distribution. These are likely due to noninterconnected pores in the scaffold.
- Speed: The entire seeding process can be accomplished within 10 min.
- Scalability: We have successfully used the process in samples from 3-mm diameter by 3-mm thick up to 14-mm diameter by 6-mm thick. Although we have not yet explored larger sizes, in the above range, the size of the scaffold did not have a negative impact on the number of cells that could be loaded or on the distribution uniformity. The current size range is useful for laboratory experiments and overlaps with clinically useful sizes.
- High viability: The growth curves and live/dead assay results indicate that exposing hMSCs or chondrocyte suspensions to these levels of vacuum does not have a deleterious effect on the cells.
- High retention: A higher percentage of the seeded cells was retained when the vacuum technique was used.

The mechanism we propose for this method is shown in Fig. 10. It is emphasized here that it is the hydrophobic character of the HYAFF®-11 scaffold that both necessitates the use of the vacuum method and accounts for its success. Other dynamic scaffold seeding techniques are described in the tissue engineering literature.³⁸⁻⁴⁵ In one such approach, the carrier scaffold is suspended in a spinner flask containing culture medium. The medium is then inoculated with cells that attach to the carrier over time. This method works well for a variety of tissues. However, published seeding yields are variable, ranging from 60% for cardiac myocytes to near 100% for bovine chondrocytes.^{30,38} Furthermore, nonuniform distribution of cells^{46, 47} with a higher density of cells lining the scaffold surface³⁹ has been reported, possibly because introducing cells into the interior of the scaffolds by convection alone in the stirred flask is likely inefficient. Another potential downside to this method, in particular when applying it to hMSCs, is that it can take days³⁰ or even up to a week to seed a construct.⁴⁸ Other approaches include variations on filtration seeding, a method in which a cell suspension is drawn through the scaffold either once or repeatedly. Although this approach has been used to prepare high-density constructs, in our hands it resulted in poor cell viability and uniformity and was discontinued (not shown). However, Wendt *et al.* have reported encouraging results (approximately 75% seeding efficiency) with filtration seeding, using an oscillatory filtration seeding method in which the same volume of cell suspension is repeatedly passed through the scaffold.⁴⁷

Beyond the seeding efficiency, the homogeneity of the cell distribution and the retention of the cells will ultimately affect the distribution of newly synthesized ECM as the cells develop the chondrocyte phenotype. These factors thus also influence the final structural properties of the construct. Cell retention and distribution depend on the physical properties of the scaffold, such as surface chemistry, porosity, pore size, and interconnectivity of the pore network. Other factors, such as active exclusion of a subset of the cells, either by migration or apoptosis *in situ*, may then affect the final configuration of the construct. In these studies, the method used for the initial cell-seeding step itself, that is, vacuum or passive seeding, significantly influenced the initial spatial distribution of the cells within the construct, as well as their retention, and ultimately the quality (reflected by, e.g., the amount, homogeneity, and distribution of ECM) of the tissue formed. In the particular case of cartilage tissue engineering, the issues of cell density and uniformity are critically important because, under the medium conditions necessary for chondrogenic differentiation, very little further cell division occurs once terminal differentiation of the cells has been initiated.⁴⁹ Thus, there is no opportunity to

“correct” a suboptimal seeding distribution by cell division. However, the beneficial effect of an even distribution has been shown for other engineered tissue types, for example, smooth muscle, as well.³⁸

We should note that although it was developed for use with hydrophobic sponges, and has the greatest impact in these, we have observed a similar beneficial effect on cell distribution in a range of scaffold materials and presentations not shown here. For example, in preliminary experiments using mesh-type scaffolds (e.g., Fig. 1B), passive seeding of the cells onto the scaffolds resulted in a deeper penetration of the cells into the scaffold than passive seeding in the sponge-type carriers. There was, however, a tendency for the cell suspension to flow through and out of the scaffold material. Nevertheless, vacuum seeding of mesh-type carriers improved the infiltration and distribution of the cells compared to passive seeding. In particular, vacuum-assisted infiltration will be beneficial in the case of thicker scaffolds.

In summary, the immediate goal of these studies was to address the critical technical issues involved in creating implants designed to repair clinically relevant cartilage defects. Our vacuum seeding method allows us to obtain high density and uniform distribution of cells into porous tissue-engineering scaffolds. The technique is simple, rapid, and highly reproducible. High initial retention and an even spatial distribution of the cells within the construct, which are important for inducing differentiation, are both easily achievable with this method. Our results show that, under the conditions used, hMSCs survived the seeding procedure, colonized the scaffold, and demonstrated chondrogenic differentiation as early as 2 weeks after seeding. Cartilaginous ECM, as revealed by the metachromatic staining with toluidine blue and by staining with antibodies against type II collagen, was produced.

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FIG. 1. SEM photograph of a sample of HYAFF®-11 sponge (A) and nonwoven mesh (B) material. Scale bar = 1 mm. Inset: Higher magnification of a scaffold pore, showing connectivity. Scale bar = 10 μ m.

FIG. 2.

Seeding protocol. (A, B) Cell suspension is added to several scaffolds in a low-attachment multiwell plate. (C) The multiwell plate is placed in the vacuum chamber, which is then evacuated as described in the text. Looking through the lid of the chamber, trapped air is seen bubbling out of the scaffold. The lid of the multiwell plate was weighted with a lead doughnut to keep it in place during vacuum cycling. (D) The cell suspension has been drawn quantitatively into the scaffold after 6 vacuum cycles.

FIG. 3. Bioreactor bags containing 14-mm diameter seeded constructs (arrows). Luer fittings at either end of the bag will be connected to either a medium feed line or a line leading to a waste container.

FIG. 4.
(A) Monolayer growth curves of sham vacuum- and non-vacuum-treated cells. (B, C) Live/dead assays performed on replicate samples. No statistically significant differences were noted. (Color images available online at www.liebertpub.com/ten.)

FIG. 5. (A, B) Raw image of DAPI-stained construct cross section. (A) After vacuum seeding, (B) after passive seeding. (C, D) Digital images of A and B, respectively, after processing as described in Materials and Methods to identify individual cells. Seeding density was 60×10^6 cells/mL. Mean $\pm$ SD, $n = 6$.

Cell Distribution

FIG. 6.

Cell distribution as a function of distance from the center of the construct. Seeding density was 60×10^6 cells/mL. Mean $\pm$ SD, $n = 6$.

FIG. 7.

Control of cell density in the construct. Progressive filling of the scaffold with cells as the cell density of the suspension increases from 13 to 33 to 53×10^6 cells/mL (**A–C**). At low densities (**D**) cells line the scaffold surfaces, whereas complete filling of the scaffold void spaces occurs at about $60\text{--}70 \times 10^6$ cells/mL. Panels **A–C** show composite fluorescent images of 2 cross sections of the entire constructs, taken 24 h after seeding. (**D, E**) High-magnification view showing the local cell distribution. (Color images available online at www.liebertpub.com/ten.)

FIG. 8.

(**A**) Seeding density at 24 and 72 h using vacuum or passive seeding approaches. 10×10^6 cells were seeded per implant. (**B**) Retention of vacuum-seeded cells at 1 and 7 days after seeding. Mean $\pm$ SD, $n = 3$.

FIG. 9.

Tissue-engineered cartilage construct, 3 weeks after seeding a HYAFF®-11 sponge with hMSCs. Cells were seeded onto HYAFF®-11 sponges at a density of 60×10^6 cells/mL. (Color images available online at www.liebertpub.com/ten.)

FIG. 10.

Proposed mechanism of vacuum seeding. The figure represents a single well of a multiwell plate containing a scaffold (black and white jagged area) and the cell suspension (gray) at various stages of the vacuum seeding process. **(A)** Air is trapped within the 3-dimensional fabric of the scaffold by the cell suspension. **(B)** As the chamber containing the multiwell plate is evacuated, the air partially bubbles out of the scaffold (indicated by the arrows). **(C)** When the vacuum is suddenly released, the air remaining in the bubbles and scaffold collapses (again indicated by the arrows), and the cell suspension is forced into the resulting voids. **(D)** After a few vacuum cycles, all the air is expelled and replaced by the cell suspension.