Increased cell seeding efficiency in bioplotted three-dimensional PEOT/PBT scaffolds

JOURNAL OF TISSUE ENGINEERING AND REGENERATIVE MEDICINE
J Tissue Eng Regen Med 2016; **10**: 679–689.
Published online 8 November 2013 in Wiley Online Library (wileyonlinelibrary.com) **DOI:** 10.1002/term.1842

RESEARCH ARTICLE

Increased cell seeding efficiency in bioplotted three-dimensional PEOT/PBT scaffolds

A. M. Leferink[1], W. J. Hendrikson[1], J. Rouwkema[2], M. Karperien[1,3], C. A. van Blitterswijk[1] and L. Moroni[1]*

[1]*Department of Tissue Regeneration, MIRA Institute for Biomedical Technology and Technical Medicine, University of Twente, Enschede, the Netherlands*
[2]*Laboratory of Biomechanical Engineering, MIRA Institute for Biomedical Technology and Technical Medicine, University of Twente, Enschede, the Netherlands*
[3]*Department of Developmental Bioengineering, MIRA Institute for Biomedical Technology and Technical Medicine, University of Twente, Enschede, the Netherlands*

Abstract

In regenerative medicine studies, cell seeding efficiency is not only optimized by changing the chemistry of the biomaterials used as cell culture substrates, but also by altering scaffold geometry, culture and seeding conditions. In this study, the importance of seeding parameters, such as initial cell number, seeding volume, seeding concentration and seeding condition is shown. Human mesenchymal stem cells (hMSCs) were seeded into cylindrically shaped 4 × 3 mm polymeric scaffolds, fabricated by fused deposition modelling. The initial cell number ranged from 5×10^4 to 8×10^5 cells, in volumes varying from 50 μl to 400 μl. To study the effect of seeding conditions, a dynamic system, by means of an agitation plate, was compared with static culture for both scaffolds placed in a well plate or in a confined agarose moulded well. Cell seeding efficiency decreased when seeded with high initial cell numbers, whereas 2×10^5 cells seemed to be an optimal initial cell number in the scaffolds used here. The influence of seeding volume was shown to be dependent on the initial cell number used. By optimizing seeding parameters for each specific culture system, a more efficient use of donor cells can be achieved. Copyright © 2013 John Wiley & Sons, Ltd.

Received 25 February 2013; Revised 20 August 2013; Accepted 13 September 2013

Additional supporting information may be found in the online version of this article at the publisher's web site.

Keywords **cell seeding efficiency; mesenchymal stromal cells; rapid prototyping**

1. Introduction

Tissue engineering aims at applying the principles of biology and engineering to develop functional substitutes to replace damaged or lost tissue (Langer and Vacanti 1993). A typical tissue engineering approach consists of combining autologous cells with a synthetic or biological material, which provides a mechanically stable environment to culture a substitute graft *in vitro* before implantation. Many researchers have already managed to control chemical, mechanical and physical properties of these biomaterials by various fabrication methods. One biomaterial and method showing high potential in tissue engineering applications is poly(ethylene oxide terephthalate)/poly(butylene terephthalate) (PEOT/PBT) copolymer scaffolds processed by fused deposition modelling (Malda *et al*., 2005; Moroni *et al*., 2006; Woodfield *et al*., 2004, 2009). PEOT/PBT copolymers have been extensively studied and proved to be biocompatible both *in vitro* and *in vivo* (Bakker *et al*., 1988; Beumer *et al*., 1994a,1994b). These biomaterials have reached clinical applications (PolyActive™) as dermal substitutes (Mensik *et al*., 2002) and bone fillers (Meijer *et al*., 1996; Du *et al*., 2002). More recently, rapid prototyped scaffolds from PEOT/PBT copolymers have been investigated for bone and cartilage regeneration therapies and showed potential to be translated into tissue-engineered clinical treatments (Woodfield

*Correspondence to: L. Moroni, Department of Tissue Regeneration, MIRA Institute, University of Twente, Drienerlolaan 5, ZH134, PO-box 217, 7500 AE Enschede, the Netherlands. E-mail: L.Moroni@utwente.nl

et al., 2004; Moroni *et al*., 2007; Jansen *et al*., 2009; Nandakumar *et al*., 2013). Although the *in vitro* and *in vivo* results with these scaffolds were promising, in most cases mature differentiated cells were used. These cell types tend to lose their phenotype upon expansion by de-differentiation, resulting in a decreased functionality of the cells, which lowers their potential to secrete functional natural tissue within the scaffold.

To overcome this problem, the use of multipotent cell types from autologous source is seen to be promising. One of the cell types that show high potential in this approach are adult progenitor cells from bone marrow stroma, often referred to as mesenchymal stem cells or stromal cells (MSCs). These are a heterogeneous population of plastic-adherent, fibroblast-like cells, from which the progenitor cells in culture are able to self-renew and differentiate into multiple lineages (Mackay *et al*., 1998; Pittenger *et al*., 1999). Recent studies showed that combining human MSCs and biomaterials with controlled properties, or by adding certain growth factors, differentiation towards chondrogenic (Hu *et al*., 2009; Abrahamsson *et al*., 2010), osteogenic (Jaiswal *et al*., 1997; Nguyen *et al*., 2012), myogenic (Tian *et al*., 2010), adipogenic (Li *et al*., 2005; Lee *et al*., 2006), endothelial (Janeczek Portalska *et al*., 2012), and neurogenic (Shakhbazau *et al*., 2011) lineages can be achieved. The distribution and adherence of cells in scaffolds plays a crucial role in the efficiency of tissue engineering approaches. To achieve proper cell penetration into a porous scaffold, the formation of large cell aggregates should be prevented. Unfortunately, even in the absence of cell aggregate formation, cell penetration and thus a homogeneous spatial distribution of cells throughout the scaffold is often limited.

Another challenge is that the availability of autologous donor cells and their expansion capacity without loss of functionality is limited (Sekiya *et al*., 2002). For human MSCs expanded on tissue culture-treated polystyrene, the influence of passage number on the multipotency is well studied. For three-dimensional (3D) culture systems, the relation between cell density, metabolism and growth kinetics are well-determined, but less is known about the maintenance of differentiation capacity upon expansion (Schop *et al*., 2010). We assume that trends found in two-dimensions (2D) with respect to the correlation between plating density and differentiation potential can also be translated to some 3D carriers. This is because some 3D scaffolds could, on the cellular level, still be considered as 2D when their characteristic dimension (e.g. fibre diameter, strut thickness) is typically an order of magnitude larger than cell dimensions. To ensure that cells retain multipotency, the expansion culture time and passage number should be as low as possible. Therefore, it is of vital importance to reduce the loss of cells during seeding on the scaffold and *in vitro* culture (Alvarez-Barreto *et al*., 2007).

Optimization of cell seeding procedures for various tissue engineering approaches is well studied. Cell seeding efficiency (CSE) of mouse-derived mesenchymal cells was compared on several types of scaffolds using dynamic culture systems, where differences in CSE seemed to be dependent on the chemistry of the carrier material (Griffon *et al*., 2011; Papadimitropoulos *et al*., 2011). In recent studies, CSE was not only optimized by changing chemistry; it can also be dependent on scaffold geometry, and on culture (Schop *et al*., 2010) and seeding conditions (Solchaga *et al*., 2006; Alvarez-Barreto *et al*., 2007; Grayson *et al*., 2008), which was shown for human MSCs (hMSCs) and osteoblast precursor (MC3T3) cells. In a study of Wang *et al*. (2009), hMSCs derived from umbilical cord were seeded at various densities on non-woven polyglycolic acid meshes and cultured in an orbital shaker for fibrocartilage tissue engineering. Within the first week of culture, higher seeding densities did not lead to higher CSE, but did result in significantly higher cell numbers after 4 weeks of culture. Wendt *et al*. (2003) and Grayson *et al*. (2008) reported an increase in CSE as well as a more homogeneous cellular distribution after seeding in a perfusion bioreactor. The scaffolds used were non-woven meshes and ceramic disks, and decellularized bone tissue, respectively, which are very distinct architectures compared with regular 3D fused deposition modelled scaffolds (Wendt *et al*., 2003; Grayson *et al*., 2008). Previous studies from our group have shown that spinner flasks are a simple solution to dynamically seed cells homogeneously throughout 3D fused deposition modelled PEOT/PBT scaffolds. However, these studies were performed with bovine and human chondrocytes, which are much more broadly available than hMSCs. Therefore, cell loss did not cause concern and was compensated for by high seeding densities of 3×10^6 cells per scaffold (Woodfield *et al*., 2004).

Despite these efforts, there are no studies reporting on the influence of basic seeding parameters on seeding efficiency of hMSCs when seeded on 3D fused deposition modelled scaffolds of synthetic polymers. The present study shows the importance of optimizing seeding parameters, such as cell number and seeding volume, and methods, such as dynamic vs. static seeding, to reduce cell loss during seeding. This will ultimately result in more efficient protocols for stem cell-based regenerative medicine applications combining a clinically relevant cell source such as hMSCs with 3D scaffolds, which have shown promising results because of their versatility in structural and architectural customization (Cipitria *et al*., 2012; Reichert *et al*., 2012). Optimization and control of these seeding parameters will lead to an improvement of good manufacturing practice production, and thereby enable the culture of clinically relevant implants.

2. Materials and methods

2.1. Isolation of human bone marrow derived stromal cells

Bone marrow aspirates were obtained from patients who had given written informed consent. The hMSCs, referred to as donor 1, 2 and 3, were isolated and proliferated as

DOI: 10.1002/term

described previously (de Bruijn *et al*., 1999) unless stated otherwise. Briefly, aspirates from three donors (1, male age 73 years; 2, female age 77 years; and 3, female age 55 years;) were resuspended using a 20-gauge needle, plated at a density of 5×10^5 cells/cm^2 and cultured in proliferation medium, which contains minimal essential medium (alpha-MEM; Life Technologies, Gaithersburg, MD, USA), 10% heat-inactivated fetal bovine serum (FBS; Lonza, Breda, the Netherlands), 0.2 mM L-ascorbic acid 2-phosphate magnesium salt (ASAP; Sigma Aldrich, Zwijndrecht, the Netherlands), 2 mM L-glutamine (L-glut; Invitrogen, Bleiswijk, the Netherlands), 100 U/ml penicillin (Life Technologies), 100 μg/ml streptomycin (Life Technologies) and 1 ng/ml basic fibroblast growth factor (bFGF; Instruchemie, Delfzijl, the Netherlands). Cells were grown at 37°C in a humidified atmosphere with 5% CO_2. Medium was refreshed twice per week and cells were used for further subculturing or cryopreservation on reaching near-confluence.

As a control group representing a less heterogeneous cell population, colony-picked hMSCs (male age 22 years) were purchased from the Institute of Regenerative Medicine (Temple, TX, USA) (DiGirolamo *et al*., 1999; Phinney *et al*., 1999). Briefly, a bone marrow aspirate is drawn and mononuclear cells are separated using density centrifugation. The cells are plated to obtain adherent human marrow stromal cells, which are harvested when cells reach 60–80% confluence. These are considered passage zero (P0) cells. These P0 cells are expanded, harvested and frozen at passage 1 (P1) for distribution.

2.2. Scaffold fabrication

The PEOT/PBT block copolymer was obtained from PolyVation BV (Groningen, the Netherlands). For this study, a 300/55/45 PEOT/PBT composition with a weight mass of 55 PEOT to 45 PBT was used, and a molecular mass of 300 Da for the starting poly(ethylene glycol) (PEG) segments used in the co-polymerization process.

The 3D regular grids were fabricated as described previously (Moroni *et al*., 2005) by fused deposition modelling with a bioscaffolder (SysENG, Salzgitter, Germany). For CSE studies, grids were fabricated with a fibre diameter of approximately 250 μm (d_1), a fibre to fibre distance (fibre spacing) of 800 μm (d_2), and a layer thickness of 150 μm (d_3). Cylindrical porous scaffolds (4 mm in diameter by 3 mm in height) were punched out of the 3D regular grids produced. The porosity of these scaffolds is given by the following equation from a theoretical approach (Landers *et al*., 2002):

$$p = 1 - \frac{\pi}{4} * \frac{1}{\frac{d_2}{d_1}} * \frac{1}{\frac{d_3}{d_1}}$$

This results in scaffolds with porosity of 62%, corresponding to a total pore volume of approximately 23 μl per scaffold.

To investigate the effect of scaffolds porosity, scaffolds were fabricated with changing parameters. The scaffold 'standard' refers to the scaffolds with the previous mentioned parameters. Scaffolds with 'thin' or 'thick' fibres refer to changing d_1 to 127 ± 65 μm and 264 ± 85 μm resulting in porosities of 90% and 48%, respectively. Scaffolds with small fibre to fibre distance refer to changing d_1 and d_2 to 208 ± 16 μm and 650 ± 12 μm, respectively, resulting in a porosity of 65%.

All scaffolds were sterilized in 70% ethanol two times for 30 min each, washed in phosphate-buffered saline (PBS) first for 5 min and then for other 30 min two times, and finally incubated in culture medium overnight before cell culture.

2.3. Cell seeding and culture on scaffolds

To study CSE, the pre-wetted scaffolds were dried and transferred to non-treated 48-well plates (Nunc, Sigma-Aldrich, Zwijndrecht, the Netherlands). hMSCs (donors 1–3 at passage 3; donor 4 at passage 4), were harvested from monolayer expansion by trypsinization, seeded in proliferation medium on top of the scaffolds and resuspended gently to fill all the pores of the scaffold. Small volumes up to 100 μl retained mostly inside the scaffolds' pores and on top of the scaffolds. Volumes greater than 100 μl resided around the scaffolds on the bottom of the culture well. The number of cells varied from 5×10^4 to 8×10^5 cells and the seeding volume was varied from 50 μl up to 400 μl for the different experiments, thereby changing seeding concentration from 1.25×10^5 to 8.3×10^6 cells/ml. After 1.5 h of incubation in the given volume, the medium was filled to 500 μl and culture was continued for a total of 24 h. From these conditions, the influence of three parameters on CSE can be determined. First, the influence of initial cell number was investigated by seeding a range of cell numbers in a specific volume. This was carried out for different volumes to determine the optimal cell number in a given volume to maximize CSE. Second, the influence of initial cell number on CSE was determined by using a fixed seeding concentration of 2×10^6 cell/ml. Finally, the influence of seeding volume was investigated for different initial cell numbers.

To assess the influence of well plate adherence on CSE, agarose wells were prepared by casting 3% agarose (UltraPure©; Invitrogen) on a mould with a cylindrical pillar with a diameter of 8 mm and a height of 3 mm in a six-well plate (Figure 1). After the agarose gel was solidified for 30 min at 5°C, the mould was carefully removed from the gel, and a cylinder with an 8 mm-well in the middle was punched out of the gel and transferred to a 12-well plate. Scaffolds were placed in the confined agarose well or in a non-treated 12-well plate (negative control). Two hundred thousand (2×10^5) hMSCs (donor 3, passage 3), were seeded in quintuplicate in 50 μl of proliferation medium on top of the scaffolds and resuspended gently to fill all the pores of the scaffold. After 1.5 hour the medium was filled up to 500 μl and culture was continued for 24 hours.

As a second group, scaffolds were placed in an agarose coated 96-well plate and on non-treated tissue culture

DOI: 10.1002/term

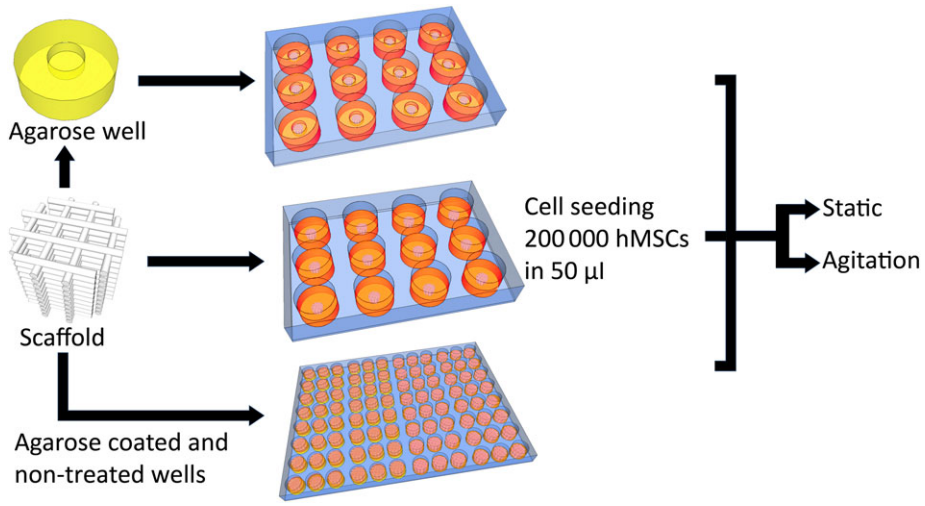

Figure 1. Schematic overview of experimental set-up to compare cell seeding efficiency on scaffolds in confined agarose wells and on scaffolds in a polystyrene well-plate. The influence of agitation during the first 24 hours of culture was also compared with static culture. hMSC, human mesenchymal stem cell

polystyrene (PS) 96-well plate (negative control). Two hundred thousand (2×10^5) hMSCs (donor 2, passage 3), were seeded in octuplicate in 50 µl of proliferation medium on top of the scaffolds and resuspended gently to fill all the pores of the scaffold. After 1.5 h the medium was filled up to 500 µl and culture was also continued for 24 h.

As a third group, scaffolds were placed in a 12-well plate and seeded as described for the first group, but in this case in 400 µl instead of 50 µl of proliferation medium. After 1.5 h the medium was filled up to 500 µl and culture was continued for 24 h. All the seeded scaffolds from the three groups were placed at 37°C in a humidified atmosphere with 5% CO_2 and cultured either on an *x*–*y* rotating agitation plate (20 r.p.m.) or statically on a shelf in an incubator.

2.4. CSE by DNA assay

After culture, all scaffolds were washed gently in PBS, dried by aspirating the PBS, cut in pieces and stored at −80°C for at least 24 h. After thawing, the constructs were digested for 16 h at 56°C with 1 mg/ml proteinase K (Sigma-Aldrich, Zwijndrecht, the Netherlands) in Tris/ethylenediaminetetraacetic acid (EDTA) buffer (pH 7.6). This solution contained 18.5 µg/ml iodoacetamine (Sigma Aldrich) and 1 µg/ml Pepstatin A (Sigma Aldrich). Quantification of total DNA was done using the CyQuant DNA assay (Molecular Probes, Bleiswijk, the Netherlands) and a spectrophotometer (excitation 480 nm, emission 520 nm) (Victor 3; Perkin Elmer, Waltham, Massachussetts, USA). CSE is given by $DNA_{scaffold}/DNA_{initial} \times 100\%$, where the $DNA_{initial}$ is calculated by the initial number of cells seeded × 7.7 pg DNA/cell (Riesle *et al*., 1998).

2.5. Cell loss by DNA assay

hMSCs (donor 4) were seeded with a concentration of 2×10^6 cells/ml on standard scaffolds for different initial cell numbers. After 24 h and after 7 days of culture the media was collected from the wells and stored in −80°C for quantification of DNA. The scaffolds were washed gently with PBS, transferred to Eppendorf tubes and stored for further processing. The well-plate was also stored for DNA analysis. The DNA content in the scaffolds, media and wells was determined as described earlier. Briefly, the tubes containing the media from the scaffolds were centrifuged at 11000 ***g*** to pellet the protein content. The supernatant was removed and 125 µl of Proteinase K solution was added to incubate at 56°C for approximately 16 h. The wells were also incubated with 125 µl of PROTEINASE K solution and after incubation the lysates of the media samples and the wells were pooled per sample. Quantification of total DNA was done using the CyQuant DNA assay (Molecular Probes) and a spectrophotometer (excitation 480 nm, emission 520 nm) (Victor 3; Perkin Elmer).

2.6. Methylene blue staining

The scaffolds were washed gently with PBS and fixed in 10% formalin for 30 min. Then the samples were washed with water and stained for 30 s using a 1% methylene blue solution in 0.1 M borax buffer (pH = 8.5). Scaffolds were subsequently washed with demineralized (DI) water until the water was clear. The scaffolds were imaged with a Nikon SMZ800 stereomicroscope equipped with a QImaginge Retiga 1300 camera (Nikon Instruments, Melville, New York, USA).

2.7. Scanning electron microscopy

The scaffolds were dehydrated after methylene blue staining by the use of an ethanol gradient series of 60%, 70%, 80%, 90%, 96% and 100% v/v ethanol in DI-water. The scaffolds were further processed by critical point drying from liquid carbon dioxide using a Balzers CPD 030 critical point dryer (Bal-tec AG, Balzers, Switzerland).

The dried samples were gold sputter coated (Cressington, Watford, UK) and imaged with scanning electron microscopy (SEM) with a Philips XL 30 ESEM-FEG (Philips, Eindhoven, the Netherlands).

2.8. Statistical analysis

The CSE data was compared using a one-way ANOVA followed by a Tukey's *post hoc* test; $p < 0.05$ was considered statistically significant. All data were expressed as mean with standard deviation as error bar. Data were compared between volumes with equal cell number, between cell numbers with equal volume and between various cell numbers with a constant seeding density for four donors in triplicate or sextuplicate. For the seeding method studies, the data from agarose wells were compared with the data from non-treated PS wells and dynamic seeding was compared with static seeding for two donors in quintuplicate and octuplicate, respectively.

3. Results

3.1. CSE vs. initial cell number per seeding volume

Figure 2 shows the results of CSE, from three hMSC donors separately and as an average. The initial seeding of 2×10^5 cells per scaffold showed the highest efficiency in all volumes. The absolute number of adhered cells increased with increasing the initial number of cells. However, the loss of non-adhered cells also increased. Therefore, the high initial cell numbers of 4×10^5 and 8×10^5 cells showed a slightly lower CSE than 2×10^5 cells. Lower initial cell number also showed slightly lower efficiencies compared with 2×10^5 cells.

3.2. CSE vs. initial cell number in fixed seeding concentration

As can be seen in Figure 3, the highest CSE for all donors was found when initially 1×10^5 or 2×10^5 cells were seeded. When the initial cell number was increased the CSE decreased, even though the concentration of the cell suspension was kept similar. Donor 3 showed a slight increased seeding efficiency for 8×10^5 cells with respect to 4×10^5 or 6×10^5 cells. Donor 4, which represents a less heterogeneous cell population than the other three donors, showed similar trends. However, lower variations were found between the replicates. For donor 4 the number of cells after 7 days of culture was also determined. The number of cells on the scaffold did not change significantly. However, the number of cells detected outside the scaffold by quantification of DNA in the culture medium and the well-plate bottom was higher after 7 days compared with 24 h (see the Supporting Information, Figure S3).

3.3. CSE vs. seeding volume per initial cell number

In Figure 4 a change in trends with respect to initial seeded cell number was observed. At low cell number, large volumes showed lower efficiencies. Conversely, at high cell numbers, large volumes showed a slight increase in CSE. At high cell numbers (4×10^5 and 8×10^5 cells) seeded in 50 µl, cell suspension nearly dried out after 1.5 h of incubation. Changes in CSE were in most conditions not significant because of the high donor-to-donor experimental variability.

3.4. CSE on scaffolds with various designs

Figure 5 shows the DNA quantification results for hMSCs on scaffolds with different designs. All scaffolds had the same outer dimensions but were altered with respect to fibre thickness or fibre to fibre distance, resulting in changing porosities and pore sizes. Standard scaffolds were composed with a fibre diameter (d_1) of 248 ± 12.8 µm, a fibre to fibre distance (d_2) of 800 ± 11.8 µm and a layer thickness of 159 ± 35.3 µm (d_3) resulting in a porosity of 62%. Scaffolds with 'thin' fibres had a d_1 of 127 ± 65.9 µm, a d_2 of 800 ± 14.3 µm and a d_3 of 154.2 ± 27.9 µm, resulting in a porosity of 90%. Scaffolds with 'thick' fibres had a d_1 of 285 ± 21.8 µm, a d_2 of 800 ± 25 µm and a d_3 of 151.8 ± 24.2 µm, resulting in a porosity of 48%. Scaffolds with a smaller d_2 of 650 ± 12 µm, had a d_1 of 207.7 ± 16.6 µm and a d_3 of 148.5 ± 17.9 µm resulting in a porosity of 65%.

The scaffolds were seeded with 2×10^5 hMSCs from donor 4 in 100 µl of medium. No differences were found in CSE within this range of fibre and pore dimensions.

3.5. CSE in static versus agitation with scaffolds on PS plate or in an agarose well

As can be seen from DNA analysis shown in Figure 6, a significant increase was found in CSE when cells were seeded on scaffolds placed in smaller, more confined 96-well plate wells compared with a 12-well plate well. There were no significant differences between the different conditions seeded in a 96-well plate. However, seeding cells on a scaffold placed in agarose wells (12-well plate) significantly decreased CSE with respect to seeding on scaffolds placed on non-treated PS. Agitation of seeded scaffolds placed on a PS 12-well plate resulted in increased CSE compared with seeded scaffolds placed on a PS 12-well plate in static culture. Again, when using the smaller and more confined 96-well plate this increase was not

DOI: 10.1002/term

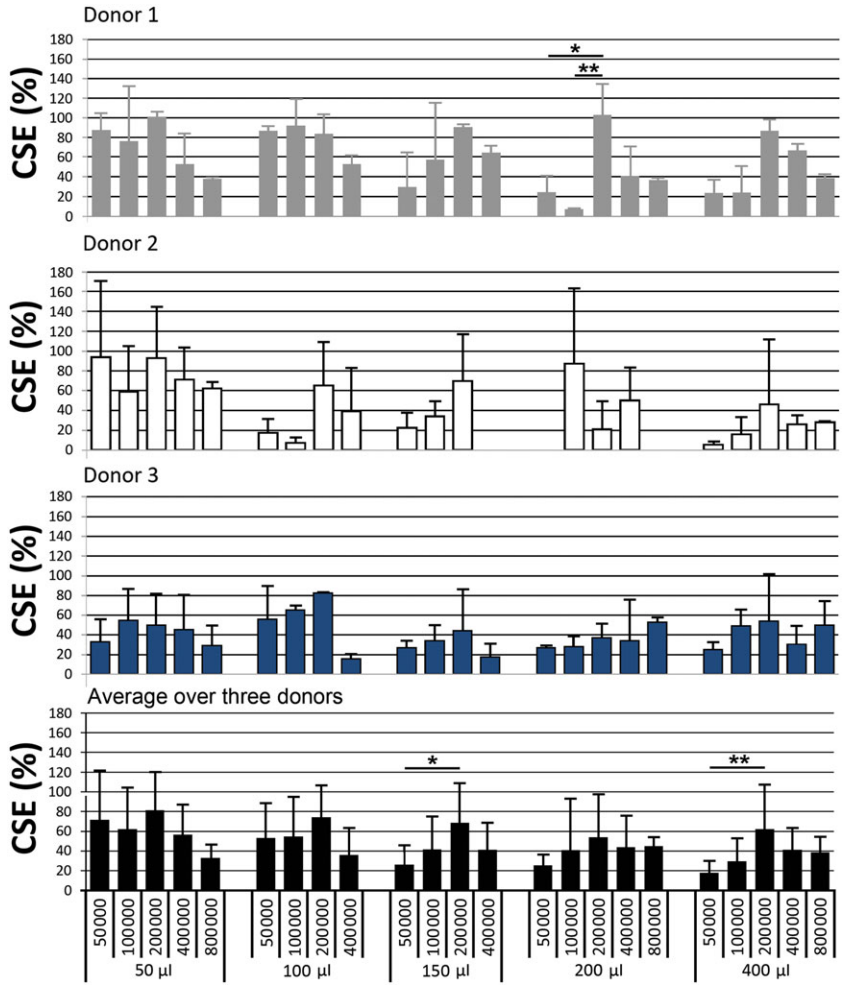

Figure 2. Percentage of initial cell number adhered after 24 h. The labels on the *x*-axis correspond to the initial cell numbers and the cell seeding volumes used in the first 1.5 h of culture. The initial seeding of 2×10^5 cells per scaffold shows the highest seeding efficiency. In absolute numbers, the higher initial cell numbers also show a higher amount of adhered cells; however, the loss of non-adhered cells also increases. The lowest initial cell number shows lower efficiencies when the seeding volume is increased. (n = 3 per donor. Error bar represents standard deviation. Statistical analysis on the average of three donors with $*p < 0.05$, $**p < 0.01$). CSE, cell seeding efficiency

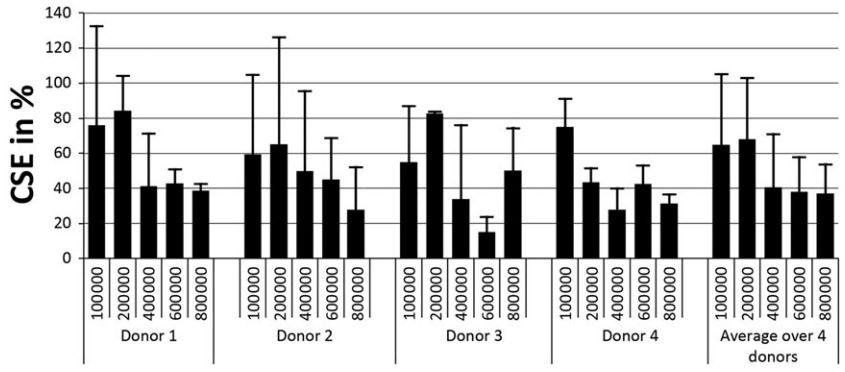

Figure 3. High cell seeding efficiency (CSE) is achieved for 200 000 cells when the concentration of the cell suspension is 2×10^6cells/ml for the different cell numbers. At higher cell numbers the CSE decreased ($n = 6$ for donor 2; $n = 3$ for other donors). Error bar represents the standard deviation

so apparent. An effect of seeding volume was found upon agitation when 2×10^5 cells were seeded in 400 µl (Figure S1). In the case of static culture on PS no difference in CSE was found between seeding in a 50 µl volume or in a 400 µl volume (shown in Figure 4). However, when agitation was applied a small increase in CSE was found in a 50 µl volume, whereas a small decrease was observed for a 400 µl volume.

DOI: 10.1002/term

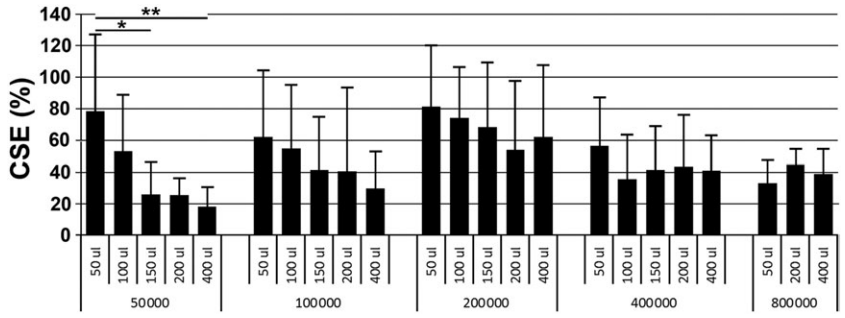

Figure 4. Influence of initial seeding volume on the seeding efficiency per cell number. As it can be seen, there is a significance influence of the seeding volume in the first 1.5 h at low initial cell numbers. For high cell numbers it was observed that when seeding in only 50 µl the cell suspension tends to dry out. Average of three donors, $n = 9$; $^{*}p < 0.05$, $^{**}p < 0.01$. CSE, cell seeding efficiency

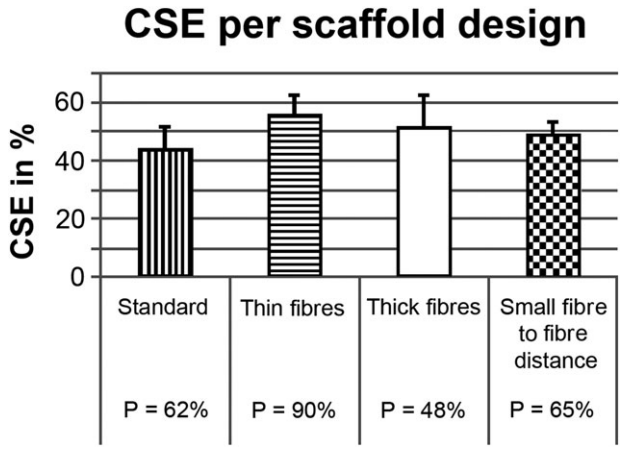

Figure 5. Cell seeding efficiency (CSE) seems not to be dependent on the porosity of the 3D fused deposition modelled scaffolds within the range of scaffold parameters used in this study. Cells were seeded at a density of 200 000 cells in 100 µl (donor 4, $n = 3$). P, porosity

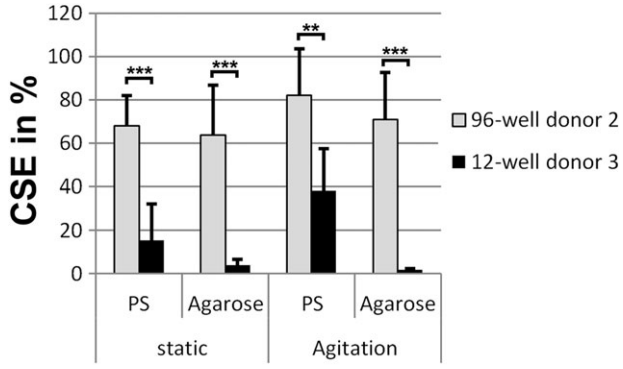

Figure 6. Influence of agitation and confinement of the cell suspension. When the scaffolds are seeded with 200 000 hMSCs in a more confined environment such as in an agarose moulded well this does not necessarily result in higher seeding efficiencies. In contrast there is a drop in cell number. From light microscopy, it was observed that the cells on agarose formed aggregates. It was also observed that the hydrophilicity of the gel allows the cell suspension to spread over the surface of the well outside the scaffold ($n = 8$ donor 2, $n = 5$ donor 3; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). PS, polystyrene

3.6. Cell adherence and morphology characterization

Figure 7 shows bright field microscopy images of scaffolds seeded with 100 000, 400 000 and 800 000 cells in a fixed concentration of 2×10^6 cells/ml. The cells resided in the longitudinal pores of the scaffold and tended to form aggregates in the pore volume. The SEM and methylene blue-staining results for the same conditions confirmed a spread cell morphology for those cells adhered on the fibres of the scaffolds and the formation of cell aggregates across the pores (Figure S2).

4. Discussion

The use of multipotent cell types from autologous source, such as hMSCs, is perceived as promising in tissue engineering approaches. A challenge to overcome is that the availability of autologous donor cells and their capacity for expansion without loss of functionality is limited (Sekiya *et al*., 2002). Although several studies on CSE have been reported, there are no other studies that have assessed the influence of the seeding parameters on CSE when combining hMSCs and 3D fused deposition modelled polymeric scaffolds in tissue engineering approaches.

In a study reported earlier (Chen *et al*., 2011b) no influence of the initial cell number on CSE was found when comparing a seeding density of 6×10^5 cells with 1.2×10^7 cells for both human periosteum-derived cells (hPDC) and a human osteosarcoma cell line (SaOS-2) on 3D fused deposition modelled titanium (Ti) scaffolds. In contrast to those results, the present study shows that in the case of hMSCs there are differences in CSE when varying the initial cell number. Although in the study of Chen *et al*. (2011b) human donor derived cells were used, these cells were already differentiated and therefore possibly possessed different cell adherence properties. Moreover, cell adherence is known to be remarkably different in different biomaterials, thus limiting the comparative analysis between Ti and PEOT/PBT scaffolds.

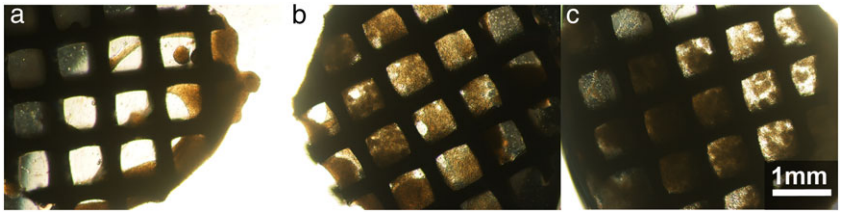

Figure 7. Bright field microscopy images of (a) 100 000, (b) 400 000 and (c) 800 000 human mesenchymal stem cells (hMSCs) (donor 2) in a fixed concentration of 2×10^6 cells/ml after 24 h of culture. Cells aggregate in the pores or form layers at the bottom of the scaffolds

In the case of hMSCs, donor variability can be caused by heterogeneity of the cell population and can result in differences in cell adherence and in cell functionality. Furthermore, the number of human MSCs that can be isolated from a bone marrow aspirate is limited. Therefore, it is of clinical importance and relevance to optimize the CSE to obtain sufficient numbers of cells in the scaffolds to be able to study cell functionality and to use the knowledge gained to further improve stem cell-based regenerative medicine therapies.

In the present study, the highest efficiency was found with an initial cell number of 2×10^5 cells for all seeding volumes analysed (Figure 2). A similar trend was found in the study of Alvarez-Barreto *et al*. (2007), where the seeding of a low number of MC3T3-E1 cells (2.5×10^5cells) showed a higher CSE than the seeding of a high number of cells (5×10^5 and 1×10^6cells). This was shown for two scaffold architectures – fibrous meshes and porous foams – with similar outer dimensions and porosity but different pore shapes and sizes. The lower CSE measured for high initial cell numbers when compared with 2×10^5cells, in the study reported here, could be caused by aggregation of the cells, which is observed by microscopy (Figure 7 and Figure S1) and has been reported before (Grayson *et al*., 2008). These non-adhered aggregates could have been lost during the washing step with PBS. Alternatively, limited availability of scaffold surface area could result in a decrease in CSE because of a plateau in absolute cell number when reaching near-confluence (Holy *et al*., 2000). The low CSE observed with low initial cell numbers could be explained by the low density of the cells in the suspension jeopardizing cell–cell contact. Recent studies reported on a possible effect of macromolecular crowding on cell culture. Here, cell culture is mentioned as a typical example, where cells anchored to a culture plate find themselves bathed in an ocean of medium that is hardly representative of their *in vivo* microenvironment (Chen *et al*., 2011a). It can also be observed that some conditions showed high variation between the replicates. Seeding the cells for the experiments in this study was carried out by one experimenter, thus excluding variation in results from handling. The high deviations could be possibly caused by donor-to-donor variance, which may result from the heterogeneity of each cell population during isolation. Therefore, cells from a fourth donor, which were colony-picked and known to represent a more homogeneous population, were also included in this study. Indeed, lower variations were observed between replicates for this more homogeneous hMSC population.

To investigate whether the density of the cells or only the absolute cell number of cells has an influence on CSE, the concentration of the cell suspension was kept constant (Figure 3). In this way, the nutrient availability per cell and cell to cell distance was also constant. As can be seen in Figure 3, the highest CSE was found when initially 1×10^5 or 2×10^5 cells were seeded for all donors. When the initial cell number was increased, the CSE decreased. This could still be caused by the formation of cell aggregates, as mentioned before, or by the limited scaffold surface available with respect to the number of seeded cells, leading to a decreased probability that one cell finds unoccupied scaffold surface to adhere to. An explanation for the decrease in CSE when increasing the cell number could be that the seeding volume for this high initial cell number was larger than for the lower cell numbers, which results in a fraction of the cell suspension flowing outside the scaffold. For one out of the four donors analysed, an increase in CSE was found when 8×10^5 cells were seeded compared with 6×10^5 cells. This singular case could be explained by the fact that some of the aggregates that were formed stayed entrapped within the scaffold. Chen *et al*. (2011b), also reported a negative influence of larger seeding volume on CSE. However, this was only reported for a low cell number (60 000 cells). When comparing the results on cell number in the scaffolds after 1 day and 7 days it can be seen that the number of cells did not increase significantly (Figure S3). The number of cells outside the scaffolds showed an increase after 7 days of culture. This could be caused by part of the cells populating the pores without firmly attaching to the scaffolds. As the material has shown good biocompatibility before, no severe cell death was expected. The difference between the total number of cells detected in the scaffold, medium and well plate together compared with the initial seeded cell number could result from cell loss during processing for analysis. A number of cells could have been entrapped within the scaffold but without adhering to the scaffold within 24 h. After washing the scaffolds with PBS, these cells could have been partly lost.

In Figure 4, a change in trends can be observed, where at low cell number low seeding volumes showed the highest efficiencies, whereas at high cell numbers higher seeding volumes did not affect CSE. It was observed by

DOI: 10.1002/term

bright field microscopy that high initial cell numbers in 50 μl nearly dried out within 1.5 h of incubation. Although this drying of the cell suspension could lead to an increase in cell death, it does not seem to explain the low CSE for these high cell numbers, as at higher seeding volumes similar low cell numbers were detected after 24 h of culture. This shows that the optimal cell seeding volume is not only related to the pore volume and surface area of the scaffold, but is also dependent on the initial number of cells seeded.

To test if the porosity of the scaffold influences the number of cells adhered three other scaffold designs were introduced with changing fibre dimensions and fibre to fibre distance, giving various porosities ranging from 48% to 90%. No influence was found on CSE within these range of variations in scaffold design. Although it cannot be excluded that significant differences could have been found for scaffold designs with larger variations, efforts in this study were focused on examining optimal CSE conditions for scaffolds with porosities and pore sizes that had already been shown to be promising for musculoskeletal tissue engineering applications (Yilgor *et al*., 2008; Kemppainen and Hollister 2010; Sobral *et al*., 2011).

Another method that could improve CSE is by placing the scaffolds in a confined environment. Agarose wells have shown to be useful to culture cells within a defined area and to prevent the cells from adhering to the bottom of a culture plate (Rivron *et al*., 2009). To improve the homogeneity of the cellular distribution throughout the construct and therewith the CSE, gentle agitation could be applied. To test if the use of these two methods results in higher seeding efficiencies within the scaffolds, 2×10^5 cells were seeded in 50 μl or 400 μl volumes on scaffolds with the same culture parameters as the 'standard scaffold' in the previous experiments. Two different sized culture wells were used. DNA quantification (Figure 6) showed that the use of agarose as a non-adherent layer did not significantly improve CSE for either a small well from a 96-well plate coated with agarose or for a confined moulded agarose well placed in a 12-well plate. In the 12-well plate the use of an agarose well even seemed to decrease the CSE. From light microscopy, it could be seen that the cells in the agarose well started to aggregate resulting in non-adhered cell clumps outside the scaffold. It was observed that, directly upon seeding, the cell suspension easily flowed outside the scaffold and spread on the agarose gel completely covering the bottom of the agarose well. This agarose well had a diameter of 8 mm, whereas the scaffold was only 4 mm in diameter, resulting in some cell aggregates being separate from the scaffold. This spreading behaviour of the cell suspension could be caused by the high hydrophilicity of the agarose well bottom and sidewalls, as this behaviour was not observed on non-tissue culture treated PS and was also not so apparent in an agarose-coated well of a 96-well plate. In the case of smaller, confined wells, the CSE was significantly higher than for scaffolds placed in a 12-well plate. Although the two experiments with different well-plates were carried out for different donors, donor 3 showed higher CSE than donor 2 in previous experiments. Therefore, it is fair to conclude that seeding scaffolds in small, confined wells improves the CSE.

To improve both cell distribution and CSE, scaffolds were placed on an *x*–*y*-agitation plate. A difference in the effect of agitation on CSE was found when the seeding volume was changed from 50 μl to 400 μl for scaffolds placed in a PS 12-well plate. In the case of the 50 μl volume a small increase in CSE was found upon agitation, whereas seeding in a 400 μl volume resulted in a decrease in CSE upon agitation. This could be caused by the fact that 400 μl was a too high volume to fit into the pores of the scaffold. Agitation promoted the flow of cells outside the scaffold. When seeded in only 50 μl, the cell suspension resided within the pores of the scaffold during agitation.

Previous work has shown the beneficial effect of perfusion or convection flow on cell attachment and distribution (Alvarez-Barreto *et al*., 2007). In the present study, the culture plate was only agitated in the *x*–*y* plane, which caused less convection throughout the scaffold than, for example, a spinner flask or a perfusion bioreactor. It was observed that cells seeded on PS 12-well plates without scaffolds formed aggregates in the centre of the well when *x*–*y*-agitation was applied. Adherence to the PS was lower upon agitation than when cells were cultured statically. It may be that in the experiment with the scaffolds placed in a 12-well plate, on both PS and on agarose, lower cell numbers were found because of this cell aggregation. Aggregates have shown to better retain multipotency when culturing hMSCs (Baraniak and McDevitt, 2012), but in this study the aggregates may have been lost upon sample processing because of low adherence to the scaffold (Figure 7 and Figure S2). Future studies will further investigate the influence of convection or perfusion culture on CSE and cell differentiation. In summary, this study has shown that the optimal seeding volume is dependent on the initial cell number seeded. In absolute cell numbers, a higher initial seeded number of cells ($> 2 \times 10^5$ cells) results in most cases in a higher number of cells adhered to the scaffold. However, a low number of cells (1×10^5 and 2×10^5) generally resulted in a higher CSE.

5. Conclusion

Seeding cells into a scaffold is a critical step in a tissue engineering process. Here, optimizing seeding parameters is shown to be valuable to reduce cell losses. Initial cell numbers, seeding concentration, seeding volume and seeding condition were varied over a broad range. CSE decreased for the highest initial cell number owing to aggregation, while the lowest initial cell number also showed a decrease in CSE possibly owing to limited cell–cell contact. The influence of seeding volume is highly dependent on the initial cell number used. In the case of seeding 5×10^4 cells, a small seeding volume of

DOI: 10.1002/term

50 μl shows a significantly better CSE than seeding in 150 μl or 400 μl. For higher cell numbers, this effect of seeding volume on CSE was not apparent. When the concentration of the cell suspension is constant, the available pore volume of the scaffold will also influence the CSE. No difference was found in CSE for different scaffold designs with different porosities or pore sizes, which resulted in pore volumes ranging from 18 μl to 34 μl. However, when seeding volumes are greater than the available pore volume, the cell suspension will flow partly outside the scaffold, thereby resulting in a higher cell loss. Agitation upon seeding did not improve CSE when using human MSCs on this type of scaffold. However, a significant increase in CSE was found both upon static and agitation seeding when scaffolds were place in a more confined smaller wells of a 96-well plate compared with scaffolds placed in a 12-well plate. It can be concluded that by optimizing the seeding parameters for each specific culture system, a more efficient use of donor cells is achieved.

Conflict of interest

The authors have declared that there is no conflict of interest.

Acknowledgements

The authors gratefully acknowledge the funding from the Netherlands Institute for Regenerative Medicine (NIRM) through the grant number FES0908. Some of the materials employed in this work were provided by the Texas A&M Health Science Center College of Medicine Institute for Regenerative Medicine at Scott & White through a grant from NCRR of the NIH (Grant #P40RR017447).

References

Abrahamsson CK, Yang F, Park H, *et al*. 2010; Chondrogenesis and mineralization during *in vitro* culture of human mesenchymal stem cells on three-dimensional woven scaffolds. *Tissue Eng Part A* **16**: 3709–3718.

Alvarez-Barreto JF, Linehan SM, Shambaugh RL, *et al*. 2007; Flow perfusion improves seeding of tissue engineering scaffolds with different architectures. *Ann Biomed Eng* **35**: 429–442.

Bakker D, van Blitterswijk CA, Hesseling SC, *et al*. 1988; Effect of implantation site on phagocyte/polymer interaction and fibrous capsule formation. *Biomaterials* **9**: 14–23.

Baraniak PR, McDevitt TC. 2012; Scaffold-free culture of mesenchymal stem cell spheroids in suspension preserves multilineage potential. *Cell Tissue Res* **347**: 701–711.

Beumer GJ, van Blitterswijk CA, Ponec M. 1994a; Biocompatibility of a biodegradable matrix used as a skin substitute: an *in vivo* evaluation. *J Biomed Mater Res* **28**: 545–552.

Beumer GJ, van Blitterswijk CA, Ponec M. 1994b; Degradative behaviour of polymeric matrices in (sub)dermal and muscle tissue of the rat a quantitative study. *Biomaterials* **15**: 551–559.

Chen C, Loe F, Blocki A, *et al*. 2011a. Applying macromolecular crowding to enhance extracellular matrix deposition and its remodeling *in vitro* for tissue engineering and cell-based therapies. *Adv Drug Deliv Rev* **63**: 277–290.

Chen YT, Bloemen V, Impens S, *et al*. 2011b. Characterization and optimization of cell seeding in scaffolds by factorial design: quality by design approach for skeletal tissue engineering. *Tissue Eng Part C Methods* **17**: 1211–1221.

Cipitria A, Lange C, Schell H, *et al*. 2012; Porous scaffold architecture guides tissue formation. *J Bone Min Res* **27**: 1275–1288.

de Bruijn JD, van den Brink I, Mendes S, *et al*. 1999; Bone induction by implants coated with cultured osteogenic bone marrow cells. *Adv Dent Res* **13**: 74–81.

DiGirolamo CM, Stokes D, Colter D, *et al*. 1999; Propagation and senescence of human marrow stromal cells in culture a simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate. *Br J Haematol* **107**: 275–281.

Du C, Meijer GJ, van de Valk C, *et al*. 2002; Bone growth in biomimetic apatite coated porous Polyactive((R)) 1000PEGT70PBT30 implants. *Biomaterials* **23**: 4649–4656.

Grayson WL, Bhumiratana S, Cannizzaro C, *et al*. 2008; Effects of initial seeding density and fluid perfusion rate on formation of tissue-engineered bone. *Tissue Eng Part A* **14**: 1809–1820.

Griffon DJ, Abulencia JP, Ragetly GR, *et al*. 2011; A comparative study of seeding techniques and three-dimensional matrices for mesenchymal cell attachment. *J Tissue Eng Regen Med* **5**: 169–179.

Holy CE, Shoichet MS, Davies JE. 2000; Engineering three-dimensional bone tissue *in vitro* using biodegradable scaffolds: investigating initial cell-seeding density and culture period. *J Biomed Mater Res* **51**: 376–382.

Hu J, Feng K, Liu X, *et al*. 2009; Chondrogenic and osteogenic differentiations of human bone marrow-derived mesenchymal stem cells on a nanofibrous scaffold with designed pore network. *Biomaterials* **30**: 5061–5067.

Jaiswal N, Haynesworth SE, Caplan AI, Bruder SP. 1997; Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. *J Cell Biochem* **64**: 295–312.

Janeczek Portalska K, Leferink A, Groen N, *et al*. 2012; Endothelial differentiation of mesenchymal stromal cells. *PLoS One* **7**: e46842.

Jansen EJ, Pieper J, Gijbels MJ, *et al*. 2009; PEOT/PBT based scaffolds with low mechanical properties improve cartilage repair tissue formation in osteochondral defects. *J Biomed Mater Res A* **89**: 444–452.

Kemppainen JM, Hollister SJ. 2010; Differential effects of designed scaffold permeability on chondrogenesis by chondrocytes and bone marrow stromal cells. *Biomaterials* **31**: 279–287.

Landers R, Hubner U, Schmelzeisen R, *et al*. 2002; Rapid prototyping of scaffolds derived from thermoreversible hydrogels and tailored for applications in tissue engineering. *Biomaterials* **23**: 4437–4447.

Langer R, Vacanti JP. 1993; Tissue engineering. *Science* **260**: 920–926.

Lee HK, Lee BH, Park SA, *et al*. 2006; The proteomic analysis of an adipocyte differentiated from human mesenchymal stem cells using two-dimensional gel electrophoresis. *Proteomics* **6**: 1223–1229.

Li WJ, Tuli R, Huang XX, *et al*. 2005; Multilineage differentiation of human mesenchymal stem cells in a three-dimensional nanofibrous scaffold. *Biomaterials* **26**: 5158–5166.

Mackay AM, Beck SC, Murphy JM, *et al*. 1998; Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. *Tissue Eng* **4**: 415–428.

Malda J, Woodfield TBF, van der Vloodt F, *et al*. 2005; The effect of PEGT/PBT scaffold architecture on the composition of tissue engineered cartilage. *Biomaterials* **26**: 63–72.

Meijer GJ, van Dooren A, Gaillard ML, *et al*. 1996; Polyactive as a bone-filler in a beagle dog model. *Int J Oral Maxillofac Surg* **25**: 210–216.

Mensik I, Lamme EN, Riesle J, *et al*. 2002; Effectiveness and safety of the PEGT/PBT copolymer scaffold as dermal substitute in scar reconstruction wounds (feasibility trial). *Cell Tissue Bank* **3**: 245–253.

Moroni L, de Wijn JR, van Blitterswijk CA. 2005; Three-dimensional fiber-deposited PEOT/PBT copolymer scaffolds for tissue engineering: influence of porosity, molecular network mesh size, and swelling in aqueous media on dynamic

mechanical properties. *J Biomed Mater Res A* **75**: 957–965.
Moroni L, de Wijn JR, van Blitterswijk CA. 2006; 3D fiber-deposited scaffolds for tissue engineering: influence of pores geometry and architecture on dynamic mechanical properties. *Biomaterials* **27**: 974–985.
Moroni L, Hendriks JA, Schotel R, *et al*. 2007; Design of biphasic polymeric 3-dimensional fiber deposited scaffolds for cartilage tissue engineering applications. *Tissue Eng* **13**: 361–371.
Nandakumar A, Cruz C, Mentink A, *et al*. 2013; Monolithic and assembled polymer-ceramic composites for bone regeneration. *Acta Biomater* **9**: 5708–5717.
Nguyen LT, Liao S, Chan CK, *et al*. 2012; Enhanced osteogenic differentiation with 3D electrospun nanofibrous scaffolds. *Nanomedicine (Lond)* **7**(10):1561–1575.
Papadimitropoulos A, Riboldi SA, Tonnarelli B, *et al*. 2011; A collagen network phase improves cell seeding of open-pore structure scaffolds under perfusion. *J Tissue Eng Regen Med* **7**: 183–191.
Phinney DG, Kopen G, Righter W, *et al*. 1999; Donor variation in the growth properties and osteogenic potential of human marrow stromal cells. *J Cell Biochem* **75**: 424–436.
Pittenger MF, Mackay AM, Beck SC, *et al*. 1999; Multilineage potential of adult human mesenchymal stem cells. *Science* **284**: 143–147.
Reichert JC, Cipitria A, Epari DR, *et al*. 2012; A tissue engineering solution for segmental defect regeneration in load-bearing long bones. *Sci Transl Med* **4**: 141ra93.
Riesle J, Hollander AP, Langer R, *et al*. 1998; Collagen in tissue-engineered cartilage: types, structure, and crosslinks. *J Cell Biochem* **71**: 313–327.
Rivron NC, Rouwkema J, Truckenmuller R, *et al*. 2009; Tissue assembly and organization: developmental mechanisms in microfabricated tissues. *Biomaterials* **30**: 4851–4858.
Schop D, van Dijkhuizen-Radersma R, Borgart E, *et al*. 2010; Expansion of human mesenchymal stromal cells on microcarriers: growth and metabolism. *J Tissue Eng Regen Med* **4**: 131–140.
Sekiya I, Larson BL, Smith JR, *et al*. 2002; Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality. *Stem Cells* **20**: 530–541.
Shakhbazau AV, Petyovka NV, Kosmacheva SM, *et al*. 2011; Neurogenic induction of human mesenchymal stem cells in fibrin 3D matrix. *Bull Exp Biol Med* **150**: 547–550.
Sobral JM, Caridade SG, Sousa RA, *et al*. 2011; Three-dimensional plotted scaffolds with controlled pore size gradients: effect of scaffold geometry on mechanical performance and cell seeding efficiency. *Acta Biomater* **7**: 1009–1018.
Solchaga LA, Tognana E, Penick K, *et al*. 2006; A rapid seeding technique for the assembly of large cell/scaffold composite constructs. *Tissue Eng* **12**: 1851–1863.
Tian H, Bharadwaj S, Liu Y, *et al*. 2010; Myogenic differentiation of human bone marrow mesenchymal stem cells on a 3D nano fibrous scaffold for bladder tissue engineering. *Biomaterials* **31**: 870–877.
Wang L, Sesharedy K, Weiss ML, *et al*. 2009; Effect of initial seeding density on human umbilical cord mesenchymal stromal cells for fibrocartilage tissue engineering. *Tissue Eng Part A* **15**: 1009–1017.
Wendt D, Marsano A, Jakob M, *et al*. 2003; Oscillating perfusion of cell suspensions through three-dimensional scaffolds enhances cell seeding efficiency and uniformity. *Biotechnol Bioeng* **84**: 205–214.
Woodfield TBF, Malda J, de Wijn J, *et al*. 2004; Design of porous scaffolds for cartilage tissue engineering using a three-dimensional fiber-deposition technique. *Biomaterials* **25**: 4149–4161.
Woodfield TBF, Moroni L, Malda J. 2009; Combinatorial approaches to controlling cell behaviour and tissue formation in 3D via rapid-prototyping and smart scaffold design. *Comb Chem High Throughput Screen* **12**: 562–579.
Yilgor P, Sousa RA, Reis RL, *et al*. 2008; 3D plotted PCL scaffolds for stem cell based bone tissue engineering. *Macromol Symp* **269**: 92–99.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site.

Figure S1. Scanning Electron Microscopy and Stereo Microscopy on Fixated and stained samples. (A and D) 100.000 cells in 50 μL, (B and E) 400.000 cells in 200 μL (C and F) 800.000cells in 400 μL for donor 4 after seven days of culture.(G) magnified view of cell morphology of 200.000 hMSCs in 100 μL after seven days of culture. All other cell seeding volumes showed similar cell morphology. Scale bars A-C: 500 μm, D-F: 1 mm, G: 100 μm.
Figure S2. The influence of agitation on CSE for scaffolds seeded with 200.000 hMSCs in 400 μl. A significant decrease in CSE is found when cells are seeded on scaffolds placed in agarose wells. There was no difference found in CSE when agitation was applied during 24 hours of cell culture.
Figure S3. DNA quantification after 1 and 7 days of culture on scaffolds and on culture media.