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RESEARCH ARTICLE

A comparative study of seeding techniques and three-dimensional matrices for mesenchymal cell attachment

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Abstract

Mesenchymal stem cells (MSCs) offer significant potential as a cell source in tissue-engineering applications because of their multipotent ability. The objective of this study was to evaluate the behaviour of MSCs during the seeding phase, using four different seeding techniques (spinner flask, custom vacuum system combined with a perfused bioreactor or with an orbital shaker, and orbital shaker) with four different scaffold materials [polyglycolic acid, poly(lactic acid), calcium phosphate and chitosan–hyaluronic acid]. Scaffolds were selected for their structural and/or chemical similarity with bone or cartilage, and characterized via scanning electron microscopy (SEM) and measurement of fluid retention. Cell attachment was compared between seeding techniques and scaffolds via cell-binding kinetics, cell viability and DNA quantification. SEM was used to evaluate cell distribution throughout the constructs. We discovered from cell suspension kinetics and DNA data that the type of loading (i.e. direct or indirect) mainly influences the delivery of cells to their respective scaffolds, and that dynamic seeding in a spinner flask tended to improve the cellularity of polymer constructs, especially mesh. Regardless of the seeding method, bone marrow-derived MSCs displayed a superior affinity for calcium phosphate scaffolds, which may be related to their hydrophobicity. MSCs tended to aggregate into flat sheets, occluding the external pores of matrices and affecting cell distribution, regardless of seeding technique or scaffold. Taken together, these results provide insight into the design of future experiments using MSCs to engineer functional tissue.

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Keywords **mesenchymal stem cells; 3D matrices; seeding techniques; polymers; chitosan; ceramics**

1. Introduction

According to the Centre for Disease Control (CDC), one of every three adults in the USA are affected by arthritis or chronic joint symptoms (Helmick *et al*., 2008). Because of decreased mortality, the prevalence of these debilitating diseases is expected to increase as our population ages (Hootman and Helmick, 2006). From its inception, osteochondral tissue engineering has been heralded as a promising solution in the treatment of articular disease, and has consequently developed into a rapidly growing field. This paradigm relies on the use of scaffolds to grow cells in a physiologically relevant three-dimensional (3D) space, rather than the two-dimensional (2D) monolayers typically encountered during standard laboratory culture. Thus, the goal is to encourage cells to adhere to the 3D lattice and form a clinically functional tissue. However, the cell source, 3D matrix and culture conditions matching these goals remain unclear (Coutts *et al*., 201; Chung and Burdick, 2008).

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The use of mesenchymal stem cells (MSCs) as a cell source for osteochondral tissue engineering is attractive because these cells can differentiate into several cell types (Caplan, 2005), and can be isolated from bone marrow aspirates, adipose and other tissues (Fortier, 2005; Helder *et al.*, 2007). Techniques based on needle re-infusion of a cell suspension in a cartilage defect carry the risk of rapid dissemination of cells away from the lesion (Radice *et al.*, 2000). Delivering MSCs cultured on biodegradable carriers improves their retention within the damaged area and provides local biomechanical support. In addition, recent literature supports the use of scaffolds for their synergistic action to growth factor-mediated events to modulate the differentiation of MSCs (Bosnakovski *et al.*, 2006; Wollenweber *et al.*, 2006; Varghese *et al.*, 2008; Li *et al.*, 2006).

The structural and chemical characteristics of 3D matrices offer opportunities for lineage-specific biochemical and biophysical cues to enhance selective differentiation of MSCs and their subsequent production of matrix (Li *et al.*, 2006; Moroni *et al.*, 2006; Raghunath *et al.*, 2007). Hybrid scaffolds are therefore attractive to create a biomimetic environment promoting the formation of complex tissues such as the osteochondral junction. The purpose of this study was to provide insight into the influence of structural and chemical characteristics of biomaterials relevant to osteochondral tissue enginnering. Among these, calcium phosphate constructs are established as biocompatible and osteoconductive bone graft substitutes, undergoing resorption and replacement by new bone within 6 weeks of implantation in bone defects (Kon *et al.*, 2000). Although, this agent has not been evaluated as a substrate for osteochondral composites, these properties make it attractive as a matrix interacting with subchondral bone. OPLA, an orientated polymer synthesized from D,D-L,L-polylactic acid, has a porous architecture similar to a sponge. This agent has been approved for maxillofacial reconstruction and has been found to support mesenchymal progenitor cells (Tuli *et al.*, 2004). Chondrogenesis has also been documented when chondrocytes are cultured on sponges composed of chitosan, a natural amino-polysaccharide sharing structural similarity with glycosaminoglycans (GAG) naturally present in the extracellular matrix of cartilage (Chenite *et al.*, 2000; Griffon *et al.*, 2005, 2006). Chitosan can be combined with hyaluronic acid, the main component of proteoglycans in cartilage, allowing evaluation of hybrid materials composed of these two agents for cartilage engineering (Nettles *et al.*, 2002; Yamane *et al.*, 2005, 2006; Suh and Matthew, 2000).

The initial attachment of cells to a scaffold is a prerequisite for a successful tissue engineering outcome, as it is the clincher of cell–matrix interactions (Mahmood *et al.*, 2004). The ideal criteria for seeding techniques of 3D scaffolds have been described as: (a) a high yield, or percentage of seeded cells attaching to the matrix, to optimize the use of donor cells; (b) a high kinetic rate, to minimize the time during which shear-sensitive cells will be suspended freely in the medium; and (c) a spatially uniform attachment of cells throughout the matrix, to provide a basis for uniform tissue regeneration (Vunjak-Novakovic *et al.*, 1998). Seeding techniques have traditionally been classified as static (direct) or dynamic (indirect). Direct loading of cells consists of injecting a small volume of cell suspension at the surface or in the centre of the scaffold, prior to incubation in Petri dishes (Fromstein *et al.*, 2008; Tsai *et al.*, 2006). Static seeding often result in a low yield and concentration of cells at the site of injection. These limitations have prompted the search for alternative techniques. Indirect seeding techniques rely on the attachment of cells suspended in a dynamic environment to improve their diffusion throughout the matrices and have been shown to improve the final construct (Mahmoudifar and Doran, 2006; Kim *et al.*, 1998). Several devices and strategies have been employed for dynamic seeding in the fabrication of engineered tissue. The classical example is the spinner flask technique, where scaffolds are immobilized and exposed to suspended cells in turbulent convective motion (Vunjak-Navikovic *et al.*, 1998; Chastain *et al.*, 2006). In contrast, a rotating-wall vessel bioreactor minimizes shear and turbulence around suspended cells, while providing mechanical stimulation (Hammond and Hammond, 2001; Wendt *et al.*, 2003). Orbital shakers have also been commonly used for different cell types (Brown *et al.*, 2000). Beyond these conventional methodologies, our laboratory has previously developed a custom-designed vacuum chamber, found to improve the attachment and distribution of chondrocytes within a polymer mesh (Griffon *et al.*, 2005). However, while the list of seeding techniques tested for tissue engineering is extensive, the ideal technique has yet to be determined. Previous studies have yielded contradictory results. For instance, the spinner flask was found to be more efficient than static seeding by some authors (Xiao *et al.*, 1999), whereas others found the opposite (Kim *et al.*, 1998). Although optimization of cell attachment and distribution is essential for *in vitro* cultivation of clinically relevant constructs, the ideal technique for seeding mesenchymal stem cells on 3D matrices remains unclear.

The present investigation focuses on the initial attachment of MSCs seeded with four dynamic techniques (i.e. spinner flask, vacuum/perfused bioreactor, static orbital shaker, and vacuum orbital shaker) on four scaffolds (PGA, OPLA, calcium phosphate, and chitosan–HA) selected for their chemical and/or structural similarity with bone or cartilage. Our goal was to identify the parameters influencing the recruitment of MSCs onto these candidate scaffolds and to offer insight on their preferences for attachment.

2. Materials and methods

2.1. Mesenchymal stem cells

A mesenchymal cell line (D1 ORL UVA, ATCC) derived from a multipotent mouse bone marrow stromal precursor

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was used for this study. Complete culture medium consisted of Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose, supplemented with 10% fetal bovine serum, 1.5 g/l sodium bicarbonate, 1.0 mM sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were harvested and used at their sixth passage. For each seeding technique, 2×10^6 cells were seeded on each scaffold. The experiment was repeated three times successively.

2.2. Scaffolds

Chitosan–hyaluronic acid scaffolds (Chi–HA, $n = 48$) were prepared using a freeze-drying technique. Briefly, chitosan flakes (MW 500 kDa; Chitoclear, Vanson) and sodium hyaluronate (MW 3000 kDa; Hylartin®, Pharmacia) were dissolved and stirred for 48 h in a solution of acetic acid to obtain a 2% chitosan −0.01% sodium hyaluronate solution. The solution was poured into a 96-well mould and cooled at a controlled rate of −0.3 °C for 72 h. The resulting solid product was then lyophilized for 48 h. The scaffolds measured 5 mm diameter ×, 3 mm height (Figure 1). The minimum porosity of these scaffolds was maintained above 80%, with interconnective pores measuring 70–120 µm on scanning electron microscopy (SEM; Figure 1). The scaffold was selected for its chemical similarity with native cartilage and to allow comparison of all seeding techniques.

A poly-glycolic acid mesh (PGA; $n = 24$; Synthecon, Houston, TX, USA) was cut to obtain scaffolds of 5 × 3 mm (Figure 1). This non-woven mesh was made of 13 µm diameter fibres with a void volume of 97%, and has been previously tested for cartilage engineering, therefore acting as a reference material in the study reported here (Griffon *et al*., 2005, 2006; Seddighi *et al*., 2007).

Ultraporous β-tricalcium phosphate scaffolds (CP; $n = 24$; BD Biosciences) and poly(lactic acid) sponges (OPLA; $n = 24$; BD Biosciences) were also cut to the same size (Figure 1). These are FDA-approved materials that were selected based on their high porosities (around 90%), interconnected pores similar to those of chitosan sponges, and previous use for tissue engineering (Hee *et al*., 2006).

Prior to each experiment, scaffolds ($n = 16$ for Chi–HA; $n = 8$ for each of PGA, CP and OPLA) were rehydrated through a series of ethanol/phosphate buffered saline (PBS) solutions (100, 95, 75, 50, 0% ethanol). Subsequently, the scaffolds were incubated at 37 °C in a shaker incubator in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin for 24 h before cell seeding.

2.3. Seeding techniques

2.3.1. Seeding in a spinner flask

Four Kirshner wires (supporting 12 scaffolds: four PGA, four OPLA and four Chi–HA) were fixed to a silicone stopper placed in the mouth of a spinner flask containing 120 ml culture medium with 24×10^6 cells (i.e. 2×10^6 cells/scaffold) and a magnetic bar 25 mm long rotating at 60 rpm (Figure 2) (Vunjak-Novakovic *et al*., 1998). Three scaffolds were threaded on each Kirshner wire, their position being randomized among the three scaffold types. Silicone tubing (3 mm long) was placed in between the scaffolds to prevent slippage. Gas exchange was facilitated by diffusion through a barbed connector on the flask. Ceramic scaffolds could not be seeded in a spinner flask because of their brittleness and tendency to break when skewered on wires.

2.3.2. Seeding in a vacuum chamber and perfused bioreactor (vacuum/perfused bioreactor)

Twelve scaffolds (four PGA, four OPLA and four Chi–HA) were seeded, based on a modification of a vacuum dynamic seeding technique previously described (Figure 2) (Griffon *et al*., 2005). Seeding chambers were made from untreated blood collection tubes (Monoject, Sherwood Medical, St. Louis, MO, USA), needles and injection caps. The tubes were cut to create glass cylinders measuring 1.4 cm in length, including rubber stoppers that occluded each end. A needle, 12 mm long and 0.45 mm in diameter (26-gauge hypodermic needle; Sur-Vet, Terumo Medical Corp, Elkton, MD, USA) was

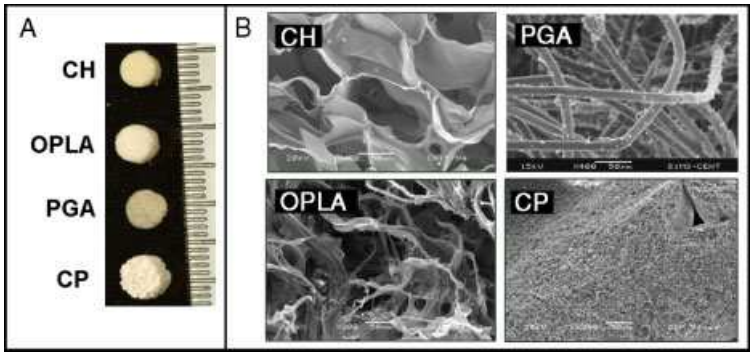

Figure 1. Macroscopic and SEM examination of chitosan–hyaluronic acid (CH), polylactic acid (OPLA), polyglycolic acid (PGA) and tricalcium phosphate (CP) scaffolds. (A) Scaffolds placed next to a metric ruler. (B) SEM of scaffolds at ×300 (OPLA, CP) and ×400 (CH, PGA)

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Seeding technique	Spinner flask	Vacuum / Perfused Bioreactor	Vacuum / orbital shaker	Orbital shaker
PGA	n= 12	n= 12		
OPLA	n= 12	n= 12		
Chit-HA	n= 12	n= 12	n= 12	n= 12
CP			n= 12	n= 12

Figure 2. Dynamic seeding techniques. (A) Scaffolds were threaded through K-wires and placed in a spinner flask filled with complete medium including suspended cells. (B) Vacuum seeding was used for two groups (vacuum/perfused bioreactor and vacuum orbital shaker) to load the MSCs onto the scaffolds. (C) The orbital shaker was used for dynamic culture after cell seeding (direct or vacuum method). (D) In the perfused bioreactor technique, a rotating vessel containing complete medium and scaffolds allowed for exposure of the contents to microgravity conditions. The constructs were placed in the bioreactor after vacuum seeding. The table shows the number of each scaffold type seeded using the various seeding techniques

implanted in the bottom stopper, with the tip barely emerging from the surface of the stopper. A needle 25 mm long and 0.5 mm in diameter (25-gauge hypodermic needle; Sur-Vet) was inserted in the opposite stopper. The scaffold was then placed in the tube, on the surface of the bottom stopper. An injection cap was placed on each of the needles to create a closed chamber. Patency of the needle was checked prior to closing the chamber and applying 3 ml negative pressure with a syringe (Monoject, Kendall, Tyco Healthcare Group LP, Mansfield, MA, USA). A tuberculin syringe (Sur-Vet) was used to slowly inject 250 µl cell suspension (8×10^6 cells/ml, i.e. 2×10^6 cells/scaffold) at the upper surface of each scaffold. The syringe was then placed through the other injection cap to retrieve the cell suspension at the bottom of the chamber. The medium was re-injected at the upper surface of the matrix through the longer needle. The cell suspension was passed three times through the chamber prior to incubation at 37 °C for 1 h. Twelve seeded scaffolds (four PGA, four OPLA and four Chi–HA) were then transferred into the rotating bioreactor (Rotary Cell Culture System, Synthecon Inc., Houston, TX, USA), filled with 120 ml medium and rotated at 17 rpm (Figure 1D). The vacuum chambers were flushed with fresh medium and transferred to the rotating bioreactor to ensure cell carry-over.

2.3.3. Seeding in a vacuum chamber and orbital shaker (vacuum/orbital shaker)

Eight scaffolds (four CP and four Chi–HA) were seeded in vacuum chambers as previously described. Subsequent to the incubation period within the vacuum chambers, the scaffolds were transferred to untreated six-well non-tissue culture plates with 10 ml medium/well. The plates were placed on a platform shaker oscillating at 100 rpm (Figure 2).

2.3.4. Static seeding on an orbital shaker (orbital shaker)

Eight scaffolds (four CP and four Chi–HA) were placed in untreated six-well non-tissue culture plates and directly injected with 20 µl cell suspension (1×10^8 cells/ml, i.e. 2×10^6 cells/scaffold) and allowed to incubate at 37 °C for 1 h. Complete medium (10 ml) was added to each well. The plates were then placed on a platform shaker oscillating at 100 rpm (Figure 2) (Jukes *et al.*, 2008). All constructs were maintained for 48 h at 37 °C in 5% CO_2 in complete culture medium, as used for MSC expansion.

2.4. Evaluation of fluid retention

Each specimen was weighed directly after harvest from the culture vessel to determine its wet weight. The constructs were then lyophilized for 48 h and the dry weights were obtained. The percentage of fluid retention was subsequently calculated as:

$$Fluid\ retention\ (\%) = (weight_{\mathrm{wet}} - weight_{\mathrm{dry}}) \times 100/weight_{\mathrm{dry}}$$

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2.5. Evaluation of cell-binding kinetics

Cell-binding kinetics and viability were evaluated after the initial incubation phase, once the scaffolds had been transferred to their respective culture environments with fresh complete medium (time 0). From this time onward, the number of cells suspended in medium and their viability were evaluated every 2 h for the first 14 h, and at 24, 30, 36, 42 and 48 h, via Trypan blue exclusion. Measurements were taken separately for each scaffold type between the two orbital shaker techniques, because individual scaffolds had their own wells, unlike the spinner flask and perfused bioreactor, where the scaffolds were exposed to the same pool of culture medium. Moreover, the wells were individually inspected, ensuring that there was insignificant adhesion to the culture plate. The cell concentration in the medium was compared to the initial cell concentration for each time point (C/C_0) to obtain the percentage of the initial cell concentration remaining in suspension (Vunjak-Novakovic *et al.*, 1998).

2.6. Evaluation of cell viability

The viability of cells in the constructs was determined using the Live/Dead Viability/Cytotoxicity Kit (Invitrogen), according to the manufacturer's protocol. The constructs were washed three times in sterile PBS for 2 min and sectioned perpendicularly into 1.5 mm slices, using a parallel razor blade. Slices from the periphery and centre of each construct were placed on a glass slide and immersed in 200 µl PBS solution containing 2 mM calcein AM and 4 mM ethidium homodimer 1 reagents, prior to incubation for 40 min at 37 °C. Confocal microscopy (Olympus BX50 Confocal Microscope, Olympus), using Melles Griot argon and krypton lasers at excitation wavelengths of 488 nm and 568 nm, allowed the visualization of calcein AM (labelling live cells = green fluorescence) and ethidium homodimer-1 (labelling dead cells = red fluorescence). The intensities of viable and dead cells were recorded on four slides/construct at a magnification of ×40 and two slides/construct at a magnification of ×10. The slides were analysed using Fluoview software (Olympus) to determine the percentage of viable cells.

2.7. Evaluation of cell content and distribution

Four constructs/group were digested in papain (Sigma) for 16 h at 60 °C and assayed for DNA content via a fluorometric assay with Hoechst 33258 (Kim *et al.*, 1988). The DNA content was normalized to the wet and dry weights of the construct.

Six constructs/group were assessed by SEM analysis. Forty-eight hours after seeding, the constructs were fixed in a 2.5% gluteraldehyde solution with sodium cacodylate buffer for 2 h. After rinsing with buffer, they were submerged in 1% osmium tetroxide in 0.1 M sodium cacodylate for 90 min. Following a buffer rinse, the constructs were dehydrated through an ethanol series. Finally, the constructs were placed in hexamethyldisilazane for 45 min and left under a fume hood until completely dry. Two constructs/group were bisected in order to evaluate the surfaces as well as the central section of the construct. Four constructs/group were kept whole to evaluate the entire periphery (less the underside). Specimens were mounted for each construct and sputter-coated with gold–palladium prior to examination with SEM (Hitachi S4700) at 1.0 kV. The criteria evaluated included cell morphology, attachment to the biomaterial and cell distribution (Griffon *et al.*, 2005, 2006).

Constructs from each group were assessed by histology 48 h after seeding for cell distribution throughout the scaffolds. The constructs were bisected and embedded in either paraffin (PGA, OPLA, Chi–HA constructs) or glycol methacrylate (GMA) (CP constructs). Sections (4 µm thick for PGA, OPLA and Chi–HA or 5 µm thick for CP) were stained with safranin-O/fast green (in paraffin) or toluidine blue (in GMA) to evaluate cell distribution.

2.8. Statistical analysis

Cell-binding kinetic data, DNA content/mg dry weight, DNA content/mg wet weight, and water content were compared between groups, with $p < 0.05$ considered statistically significant. All data were expressed as mean ± standard deviation (SD). Data were compared between scaffold types for each seeding technique and between seeding techniques for each scaffold type. Statistical differences were evaluated with a one-way analysis of variance and Tukey's highly significant differences test, using Systat statistical software (Wilkinson).

3. Results

3.1. Fluid retention

Fluid retention was first compared between scaffolds, irrespective of the seeding technique. Chi–HA scaffolds absorbed the largest amount of fluid (1040.70 ± 34.77%). Fluid retention was similar between PGA mesh and porous OPLA sponge (793.76 ± 62.80% and 814.40 ± 28.665, respectively) and lowest in CP scaffolds (90.43 ± 4.37%; Figure 3). No difference was found when fluid retention was compared within scaffold type and between seeding techniques. The fluid retention properties of a scaffold reflect the nature of the matrix and are not influenced by the seeding technique.

3.2. Cell binding kinetics and cell viability

Cell death in the medium was negligible throughout the experiment for all seeding techniques, as assessed by Trypan blue exclusion (data not shown). The number

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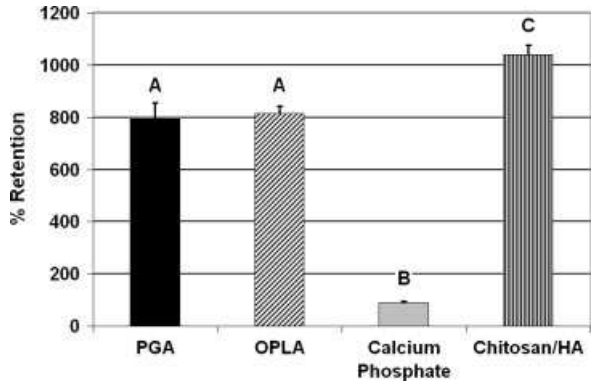

Figure 3. Scaffold water retention was measured after 48 h of seeding, irrespective of technique, and normalized to dry weight. Data represent the mean ± SEM ($n = 16$ for PGA, OPLA and calcium phosphate (CP); $n = 32$ for chitosan/HA). (A–C) Groups with different letters differ statistically ($p < 0.05$)

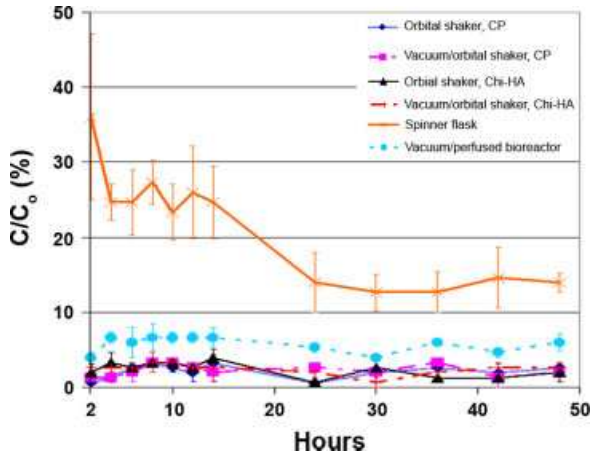

Figure 4. Cell binding kinetics. The number of cells in suspension (C) with respect to initial cell concentration (C_0) was calculated for the different seeding techniques over the duration of the experiment. Data represent mean ± SEM

of cells remaining in suspension within a spinner flask decreased exponentially, reaching a plateau 24 h after scaffold placement in the culture vessel (Figure 4). This plateau occurred at a C/C_0 value of approximately 0.15, implying that 15% of the original cell population remained in suspension for the remainder of the experiment. In contrast, the number of cells in suspension in the two techniques using an orbital shaker remained constant within a C/C_0 range of 1–4%. This indicates that a majority of cells seeded with each of these methods attached within 2 h of introduction into the culture vessel. The viability of cells estimated by a live/dead fluorescent assay at the end of the study did not differ between groups and was >90% in all constructs.

3.3. DNA analysis

Differences in DNA content were found between the various seeding techniques (Figure 5). The cellularity of the Chi–HA constructs was improved when scaffolds were seeded in the spinner flask compared to the three other techniques. Similar results were obtained with the PGA mesh, where constructs seeded in the spinner flask contained more DNA than those loaded with the vacuum/perfused bioreactor technique. In contrast, no significant difference was found for the OPLA constructs between these two seeding techniques. Finally, no significant difference was found between the orbital shaker and vacuum/orbital shaker techniques for the CP constructs.

In addition to these findings, differences in DNA content were found between scaffold types for the same seeding technique (Figure 6). Similar statistical results were found whether DNA content was normalized to the dry or the wet weight of constructs in each group. For each of the four seeding techniques, the Chi–HA constructs contained less DNA than any other construct (CP, orbital shaker and vacuum/orbital shaker; PGA and OPLA, spinner flask and vacuum/perfused bioreactor). PGA constructs were more cellular than OPLA and Chi–HA constructs when seeded in the spinner flask technique. In contrast, DNA content was higher in OPLA constructs compared to both PGA and Chi–HA when the cells were loaded with the vacuum/perfused bioreactor technique.

3.4. Histology

Histological staining confirmed the presence of cells on all constructs (Figure 7). Cells attached to all non-mineralized constructs in a patchy pattern. They were present throughout all these scaffolds, although they were more abundant near the periphery than in the centre of Chi–HA and OPLA scaffolds. Some cells reached the centre of CP scaffolds, but were clearly more abundant on all surfaces of these matrices.

3.5. Scanning electron microscopy analysis

Objective quantification of the cell distribution using SEM was not possible, as the cells formed aggregates on the scaffolds. MSCs attaching to PGA meshes and OPLA sponges formed flat, layered sheets mixed with aggregates of highly elongated cells (Figure 8), while the cells seeded on CP scaffolds formed essentially flat sheets, partially occluding the superficial pores of the matrix (Figure 8). In contrast, MSCs appeared less abundant in Chi–HA constructs, regardless of the seeding technique assessed (Figure 8). The morphology of MSCs was consistent throughout Chi–HA scaffolds and consisted essentially of aggregates of spherical cells. The density of cells in the centre of constructs appeared to decrease from the periphery to the centre of the constructs, regardless of scaffold and seeding technique. Cells tended to agglutinate at the surface of constructs, thereby preventing our ability to count individual cells.

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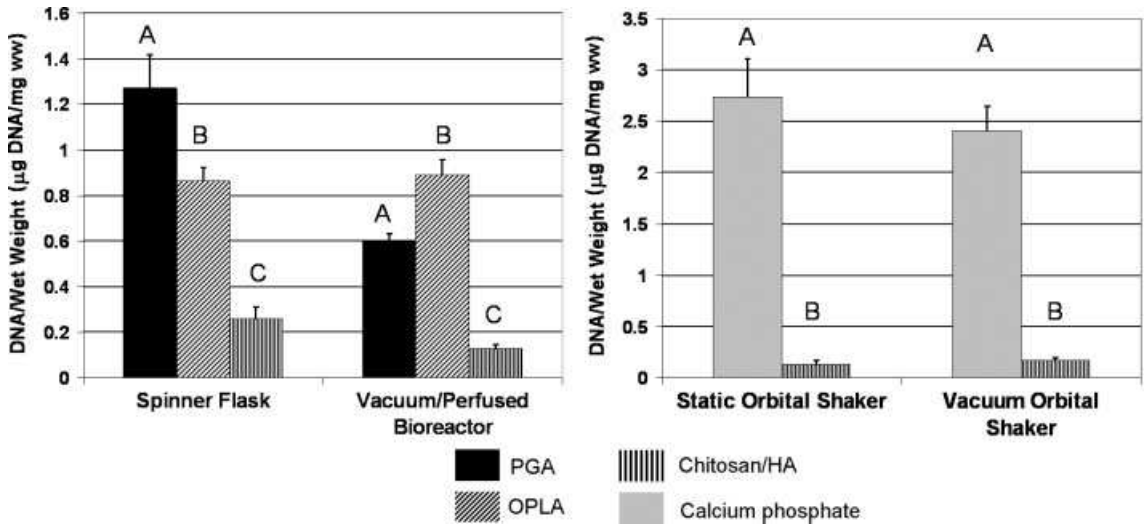

Figure 5. Effect of the seeding technique on the DNA content of constructs at 48 h. DNA content was compared among seeding techniques for each type of scaffold. Data represent mean ± SEM ($n = 8$). (A, B) Groups with different letters differ in DNA content within scaffold type ($p < 0.05$)

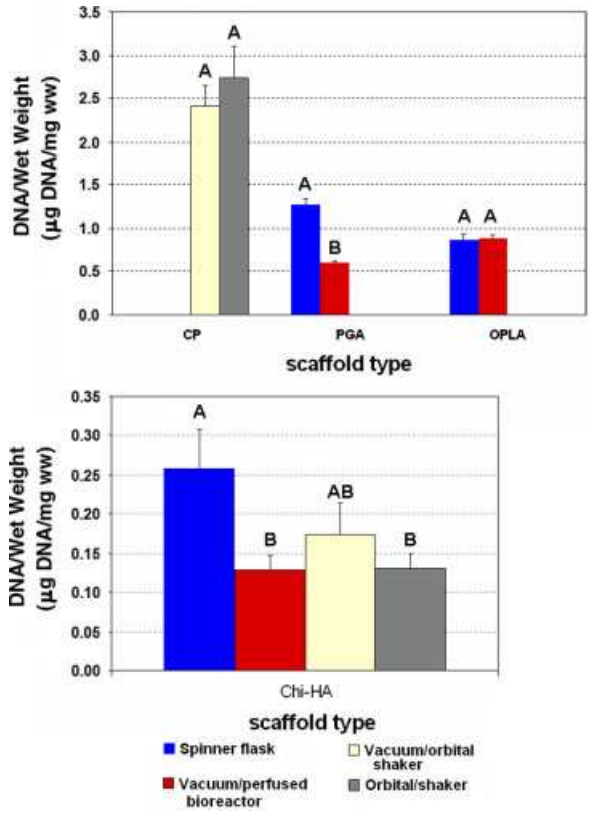

Figure 6. Effect of scaffold on DNA content of constructs at 48 h. DNA content was compared among scaffold types for each seeding technique. Data represent mean ± SEM ($n = 8$). (A–C) Groups with different letters differ statistically within seeding technique ($p < 0.05$)

4. Discussion

Cell attachment to 3D matrices is a prerequisite to the production of clinically relevant engineered tissue. It is therefore crucial to characterize and optimize this process, commonly referred to as 'cell seeding'. The study reported here evaluated several seeding methods and scaffold compositions for the attachment and initial proliferation of MSCs, to determine the parameters that will improve the likelihood of successful osteochondral tissue engineering. The main findings of this study were: (a) cell binding kinetics are improved via direct loading of scaffolds with MSCs compared to an indirect technique relying on cell convection; (b) seeding technique influences not only MSC attachment onto scaffolds but also the initial cell proliferation; and (c) scaffold chemical composition and structural characteristics influence MSC adhesion and proliferation.

Cells remaining in suspension were counted not only to track their movement from culture medium to scaffold, but to also provide an indication of detachment and death. A seeding technique resulting in a high kinetic rate is desirable to minimize the time in suspension culture for anchorage-dependent and shear-sensitive cells (Vunjak-Novakovic *et al.*, 1998). The number of cells in suspension within the spinner flask decreased exponentially over the 48 h of the study, which is similar to the behaviour previously reported with chondrocytes (Vunjak-Novakovic *et al.*, 1998). The spinner flask technique relies on fluid convection and collision with the immobilized scaffolds. This technique was designed to improve cell distribution throughout the matrix, but slows down the attachment of cells compared to direct seeding techniques. In contrast, cells attached almost immediately to the scaffolds after direct loading. This kinetic rate was similar whether cells were seeded via direct inoculation into scaffolds or in a vacuum chamber. Both of these techniques delivered a large percentage of the initial cell population in a short period of time, forcing cells to interact with and commit to that material. The high percentage of live cells in constructs after 48 h is expected, as dead cells should detach from the scaffolds. The majority of these cells would then be suspended in

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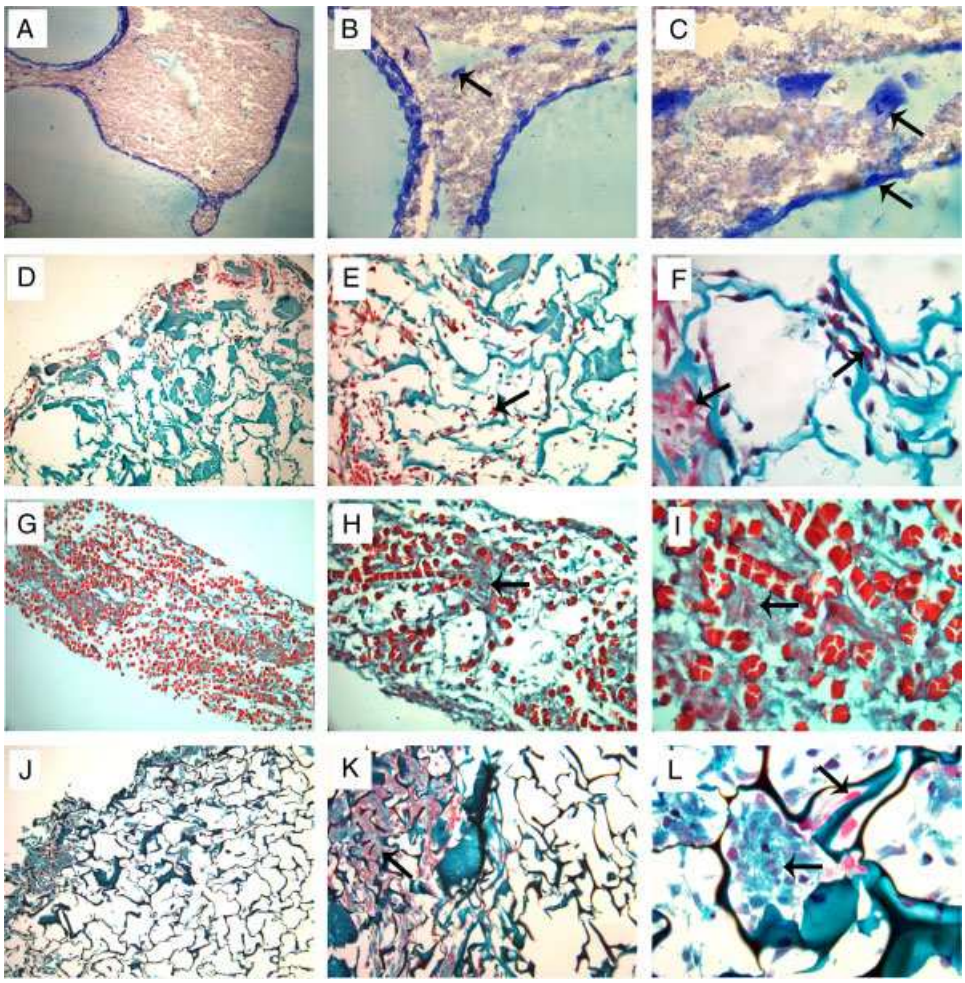

Figure 7. Histological images of constructs after 48 h of incubation, illustrating the structure of scaffolds and cell distribution. (A–C) CP constructs, cells stained blue with toluidine blue. Magnifications: (A) ×10; (B) ×40; (C) ×100. (D–F) OPLA constructs: nuclei are pink, cytoplasm stains blue or pink. Magnifications: (D) ×20; (E) ×40; (F) ×100. (G–I) PGA constructs: nuclei stain pink, cytoplasm grey-blue, PGA fibres red. Magnifications: (G) ×20; (H) ×40; (I) ×100. (J–L) Chi–HA constructs: nuclei stain pink, cytoplasm blue, Chi–HA fibres black or blue. Magnifications: (J) ×20; (K) ×40; (L) ×100. Arrows point to cell nuclei

the medium, as matrix production is not sufficient at this stage to trap them within the construct.

The DNA content of PGA and Chi–HA constructs was greater when these scaffolds were seeded in a spinner flask than with a direct loading technique. Haemodynamic environments have previously been reported to improve the proliferation of different cell populations (Malaviya and Nerem, 2002; Mahmoudifar and Doran, 2005; Li *et al.*, 2004). This effect may result from enhanced delivery of nutrients combined with direct mechanical stimulation of cells (Malaviya and Nerem, 2002; Mahmoudifar and Doran, 2005; Li *et al.*, 2004). One of the arguments for using dynamic techniques in tissue engineering is the enhanced delivery of nutrients to cells. However, cells seemed primarily localized to the superficial aspect of the PGA, OPLA and CP scaffolds, regardless of the seeding technique. The tendency of MSCs to form aggregates prevented us from counting individual cells throughout sections of the scaffolds. Direct seeding is known to result in poor penetration of the cells into the scaffolds to a depth of up to 500 μm, depending on scaffold material (Niemeyer *et al.*, 2004). However, application of vacuum during seeding and dynamic culturing in spinner flask or bioreactor have been shown to improve cell penetration into the scaffolds (Solchaga *et al.*, 2006; Zhao and Ma, 2005). The presence of flat sheets of cells occluding the superficial pores may have limited the ability of MSCs to penetrate the inner aspects of the scaffolds in our study. Despite this behaviour, the percentage of cells attaching to the scaffolds reached a level compatible with the optimization of donor cells. Although uniform cell seeding would be ideal, longer culture periods may allow cells to proliferate and migrate toward the centre of constructs.

The structural characteristics and the chemical composition of the matrices tested in our study also influenced the attachment of MSCs. Scaffolds were strategically placed in the spinner flask to provide unbiased exposure to MSCs suspended in the medium, yet cells attached preferentially to the PGA mesh than the hybrid chitosan scaffolds. We have reported similar findings when chondrocytes were seeded on PGA mesh and chitosan sponges (Griffon *et al.*, 2005). We consequently confirmed these findings on chitosan fibres compared to PGA fibres of

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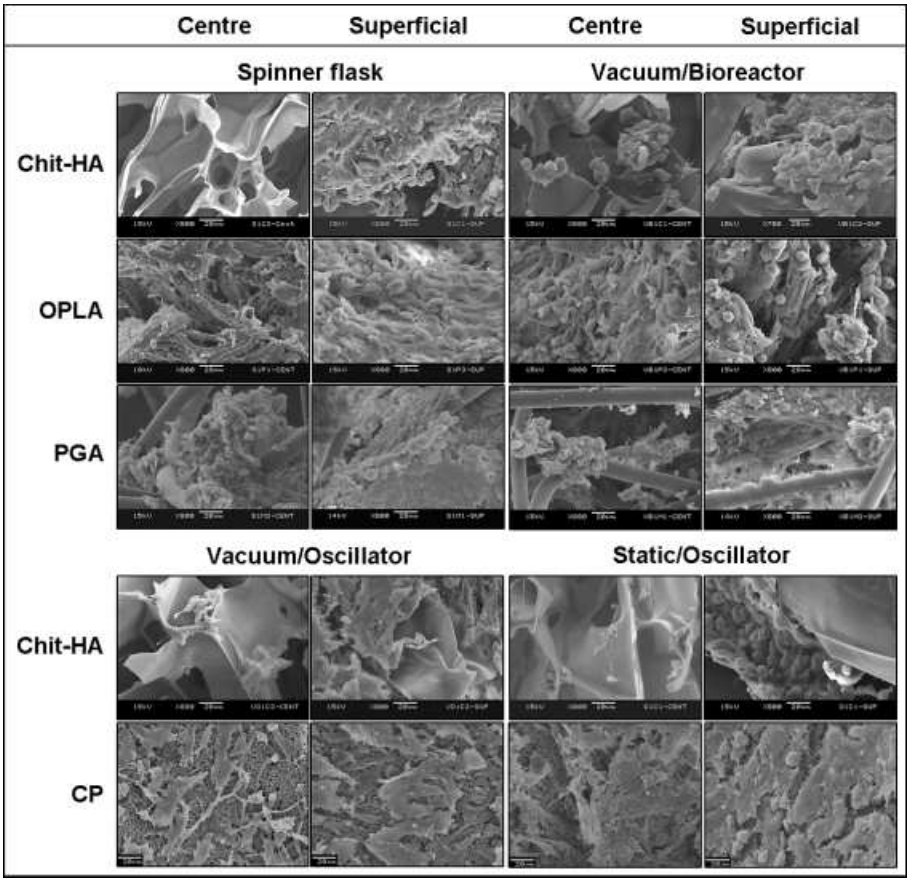

Figure 8. SEM images (×800) of constructs after 48 h of incubation, illustrating the central and superficial regions of each construct type for each seeding technique. Scale bar = 20 μm. The density of cells was greater at the periphery than in the centre, regardless of scaffolds and seeding technique

similar diameter (13 μm). The lack of cell attachment on chitosan compared to PGA was therefore attributed to a difference in chemical composition, rather than the structural properties of the matrices (Ragetly *et al*., 2008). The mechanisms proposed to explain the influence of scaffold chemical composition on cell adhesion relate to degradation rate (Tsai *et al*., 2006), surface hydrophobicity (Evans and Steele, 1998) and the presence of adhesion peptides along the surface of the matrix (Jaschke *et al*., 2002). Among these parameters, the degradation rate of the PGA mesh used in our study would be most likely to account for its cell adhesion properties, since it dissolved in 21 days of incubation in acellular medium, and this polymer lacks functional groups for cell recognition (Tsai *et al*., 2006; Cui *et al*., 2003, Zhu *et al*., 2002). The chitosan used in our study was specifically selected for its high degree of deacetylation and low molecular weight, two characteristics expected to encourage cell adhesion (Hamilton *et al*., 2006; Mao *et al*., 2004). However, the cell adhesion characteristics of Chi–HA may not be directly related to direct cell–matrix interactions but to the differential adsorption of proteins present in the culture medium to the biomaterial (Chastain *et al*., 2006). The effects of HA on cell adhesion are mediated through CD44 receptors but may vary with the length of the molecule and its physical form, thereby explaining the conflicting results regarding HA and cell–matrix interactions (Chow *et al*., 1995; Boraldi *et al*., 2003).

The cellularity of CP constructs was 10 times greater than that of Chi–HA constructs seeded on an orbital shaker with or without incubation in a vacuum chamber. This finding may reflect a higher affinity of MSCs related to the hydrophobicity of CP (Evans and Steele, 1998). The average DNA/wet weight values of constructs correlated strongly ($R^2 = 0.995$) and inversely with the water retention of each scaffold tested in our study, regardless of the seeding technique. This suggests that proliferation of cells subsequent to their attachment may relate to the amount of fluid imbibed by the matrices. From our data, Chi–HA held the largest amount of fluid, but contained the least amount of cells at the end of the seeding period, while calcium phosphate exhibited the opposite behaviour, by retaining the least amount of fluid but containing more cells. Since the internal partition of polymer scaffolds is not exposed to fluid flow in the techniques used in this study, mass transfer of nutrients must occur via molecular diffusion (Raimondi *et al*., 2004). Thus, the mass transfer of nutrients would be challenged by the large amount of fluid retained in Chi–HA and may subsequently not be able to satisfy the metabolic needs of adherent cells, thereby affecting the downstream proliferation of MSCs compared to CP scaffolds.

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5. Conclusions

Initial attachment of MSCs onto 3D matrices is influenced by both seeding techniques and scaffolds. Direct loading techniques optimize the binding kinetic rate, but dynamic seeding in a spinner flask tended to improve the cellularity of polymer constructs, especially mesh. Regardless of the seeding method, bone marrow derived MSCs displayed a superior affinity for calcium phosphate scaffolds, which may be related to their hydrophobicity.

While this study provided insights which should enhance the quality of MSC–scaffold tissue engineering, several potential lines of study can be explored in the future. The effect of dynamic seeding techniques on the differentiation phenomenon warrants further investigation. The tendency of MSCs to condense and form flat sheets at the surface of the scaffold also needs to be addressed, as this behaviour potentially prevents the passage of cells to the inner interstices of porous scaffolds, thus affecting the uniformity of cell distribution.

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