Technical Note

Seeding cells into needled felt scaffolds for tissue engineering applications

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Abstract: Tissue engineering methods are under development that will enable the repair or replacement of a variety of tissues, including articular cartilage and bone. To engineer functional tissue it is necessary that scaffolds initially be seeded with a large number of cells distributed evenly throughout the scaffold structure. It previously has been shown that, compared to static seeding conditions, seeding scaffolds under dynamic conditions facilitates high seeding densities and even distributions of cells (Li et al., Biotechnology Progress 2001;17:935–944). The efficiency of seeding HOSTE85 cells and bovine chondrocytes into needled felt scaffolds following agitation at different speeds was determined. Seeding efficiency was determined using the Hoechst 33258 assay, and cell viability was assessed using the Alamar Blue™ assay. The distribution of cells within the scaffolds was imaged using scanning electron microscopy. It was found that the optimum seeding conditions varied for HOSTE85 cells and bovine chondrocytes, with different agitation speeds leading to different seeding efficiencies, cell viabilities, and distributions of cells within scaffolds. The optimum agitation speeds for seeding a high number of viable cells into scaffolds so that they were arranged evenly were 300 rpm for HOSTE85 cells and 200 rpm for bovine chondrocytes. © 2003 Wiley Periodicals, Inc. J Biomed Mater Res 66A: 425–431, 2003

Key words: tissue engineering; articular cartilage; bone; scaffolds; seeding efficiency

INTRODUCTION

Musculoskeletal injuries affect one in seven Americans, causing chronic pain, severely reducing the person's quality of life, and costing the nation approximately $254 billion per year.[1] Damage to musculoskeletal tissues, such as articular cartilage and bone, may be the result of trauma, for example sports or road traffic accidents, or it may occur as a result of a disease, such as osteoarthritis.[2] It has been estimated that in excess of 40 million people in the United States suffer with arthritis each year.[3] Tissue engineering methods are being developed that will allow the repair or replacement of such diseased or damaged tissues.[4]

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Currently, many approaches for the engineering of organs and tissues are under development. One strategy is to take a biopsy of tissue, isolate the cells by enzymatic digestion, and then expand them in culture. The cells are then seeded into a suitable scaffold structure that will support the proliferating cells. The cell-seeded construct must then be cultured under appropriate conditions to allow extracellular matrix formation and tissue regeneration.[5]

There are many important parameters that must be optimized to enable the formation of functional tissues. It is important that the scaffold employed supports cell attachment, extracellular matrix and tissue formation, and that the constructs initially be seeded with a high number of cells that are distributed evenly throughout the entire scaffold.[6] For example, when tissue engineering articular cartilage, a high seeding density is necessary to prevent fibrous tissue formation.[7] It also is necessary for cells to be distributed evenly throughout the scaffold structure for normal tissue formation.[6]

It previously has been reported that higher seeding densities and more uniform distributions of cells in scaffolds can be achieved using dynamic rather than static seeding methods.[6,8] This technical note describes for two different cell types the relationship of cell seeding and cell viability with the rate at which cell-seeded scaffolds are agitated during the initial seeding process.

MATERIALS AND METHODS

Materials

Poly(ethylene terephthalate) (PET)-needled felt scaffolds (45 mg/cm^3) were manufactured and supplied by Smith and Nephew (York, UK). Lower legs from 30-month-old cows were obtained from a local abattoir. HOSTE85 cells were obtained from the European Collection of Cell Cultures (ECACC; Salisbury, UK). Dulbecco's modified Eagle's medium (DMEM) and gentamicin were from GIBCOBRL Life Technologies (Glasgow, Scotland). Pronase was from BDH (Lutterworth, UK). Alamar Blue™ was from Serotec (Kidlington, UK). Glutaraldehyde and osmium tetroxide were from Taab Laboratories (Berkshire, UK). All other reagents were purchased from Sigma (Poole, UK).

Culture of HOSTE85 cells

HOSTE85 cells were cultured in DMEM supplemented with 10% (v/v) fetal calf serum (FCS), 1% (v/v) nonessential amino acids (NEAA; ×100), 2 m*M* of L-glutamine, 100 units/mL of penicillin, 0.25 μg/mL of amphotericin B, 0.1 mg/mL of streptomycin, and 0.15 mg/mL of ascorbic acid (HOSTE85 media). When confluent, a cell suspension was obtained by enzymatic digestion with 0.25% trypsin/0.02% ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS; pH 7) and diluted to 4×10^6 cells per mL in HOSTE85 media.

Isolation of chondrocytes

Full-thickness cartilage was aseptically removed from the metacarpophalangeal joint of freshly slaughtered 30-month-old cows. Chondrocytes were isolated, as previously described by Archer et al.[9] Briefly, cartilage pieces were washed in PBS and finely diced. Chondrocytes were obtained by enzymatic digestion with pronase (700 units/mL of activity) for 1 h and collagenase (300 units/mL of activity) for 3 h at 37°C and 5% CO_2. Chondrocytes were diluted to 4×10^6 cells per mL in DMEM supplemented with 10% (v/v) FCS, 1% (v/v) NEAA (×100), 2 m*M* of L-glutamine, and 50 μg/mL of gentamicin (chondrocyte media).

Needled felt scaffolds

Sheets of the PET needled felt material were cut into discs 8 mm in diameter and 3 mm in thickness. Scaffolds were autoclaved at 120°C for 20 min and presoaked in either chondrocyte or HOSTE85 media overnight at 37°C and 5% CO_2 prior to seeding.

Seeding cells into scaffolds

Scaffolds were placed in triplicate for biochemical analysis and in duplicate for image analysis in 24-well plates. One mL of media containing 4×10^6 cells was added to each well and pipetted through the scaffold matrix. The plates were immediately placed on one/quarter of the orbital shaker (IKA®, Schüttler MTS4, Germany) with a balance-plate placed diagonally opposite and agitated at 0, 100, 200, 300, or 400 rpm (for chondrocytes) or 100, 200, 300, or 400 rpm (for HOSTE85 cells) overnight in an incubator at 5% CO_2 and 37°C. Control scaffolds were agitated in the same way but with media alone. The plates were placed in the same position on the shaker for every agitation speed.

Determination of cell viability

The viability of cells within the scaffolds was determined with the Alamar Blue™ assay, using a method adapted from that reported by Fields and colleagues.[10] The scaffolds were removed from culture, washed three times in PBS, and incubated with 10% Alamar Blue™ in Hank's balanced salt solution (HBSS) without phenol red (pH 7) for 90 min. Aliquots (200 μL) of Alamar Blue™/HBSS were placed in a 96-well plate in triplicate, and the fluorescence was measured at an excitation wavelength of 530 nm and an emission wavelength of 595 nm using a fluorescence plate reader (F2 Microplate Fluorescence, Absorbance and Luminescence System, Labtech, UK).

Determination of DNA content

For the determination of the number of cells within scaffolds, the scaffolds were washed three times in PBS, frozen, and lyophilized. Pellets of 8×10^6 chondrocytes and HOSTE85 cells also were frozen and lyophilized. The scaffolds and cell pellets were digested overnight in 1 mL of papain (1.06 mg/mL) at 60°C, as described by Kim and colleagues.[11]

Serial dilutions of the digested cell pellets were prepared as standards (0, 1.25×10^5, 2.5×10^5, 5×10^5, 1×10^6, 2×10^6, 4×10^6, and 8×10^6 cells/mL). The amount of DNA within the controls, sample triplicates, and standards was determined using Hoechst 33258 dye.[11] Fluorescence was measured at an excitation wavelength of 355 nm and an emission wavelength of 460 nm on a fluorescence plate

reader. The amount of DNA was related to cell numbers using a standard curve of cell number versus average fluorescence (nm).

Scanning electron microscopy

Scaffolds were prepared for scanning electron microscopy, as described in a method by Robinson and Gray.[12] Briefly, scaffolds were removed from culture, washed three times in PBS, and fixed overnight in 3% glutaraldehyde in PBS at 4°C. The scaffolds were washed three times in PBS and fixed further in 1% osmium tetroxide for 2 h. The scaffolds then were washed three times in distilled water, dehydrated with increasing concentrations of ethanol (25, 50, 70, 90, 95, 100%, v/v), and dried with hexamethyldisilazane (HMDS). The scaffolds were placed on carbon-coated stubs and sputter-coated with gold. They then were imaged using a scanning electron microscope (Philips IL505).

Statistics

The statistical significance of the results was assessed using GraphPad InStat, v. 3.00 (GraphPad Software Inc, San Diego). One-way analysis of variance (ANOVA) and the Tukey-Kramer multiple comparisons post-test were performed.

RESULTS AND DISCUSSION

HOS cells

Seeding efficiency and cell viability

The effect of agitation speed on the number of HOSTE85 cells seeded into needled felt scaffolds is shown in Figure 1(a). Increasing the rate at which scaffolds were agitated led to an increase in the number of cells within the scaffolds. The relative total viability of HOSTE85 cells in needled felt scaffolds seeded at each of the speeds is shown in Figure 1(b). No significant difference in relative viability was detected for any of the agitation speeds. The relative viability per cell can be determined by normalizing the viability reading with respect to the number of cells present in the scaffolds and is shown in Figure 1(c). A decrease in relative viability per cell with increasing agitation speed was observed. For all control scaffolds, no DNA or cell viability was detected.

Spatial arrangement

Representative scanning electron micrographs in Figure 2 show the arrangement of HOSTE85 cells within needled felt scaffolds agitated at 100 and 300 rpm. Following agitation at 100 rpm, few HOSTE85 cells were visible within the scaffolds [Fig. 2(a,b)]. Agitation at 300 rpm caused a greater number of HOSTE85 cells to be retained within the scaffolds, and cells were distributed evenly throughout the entire scaffold structure, spreading between fibers, as shown in Figure 2(c,d). Agitation at 400 rpm led to a further

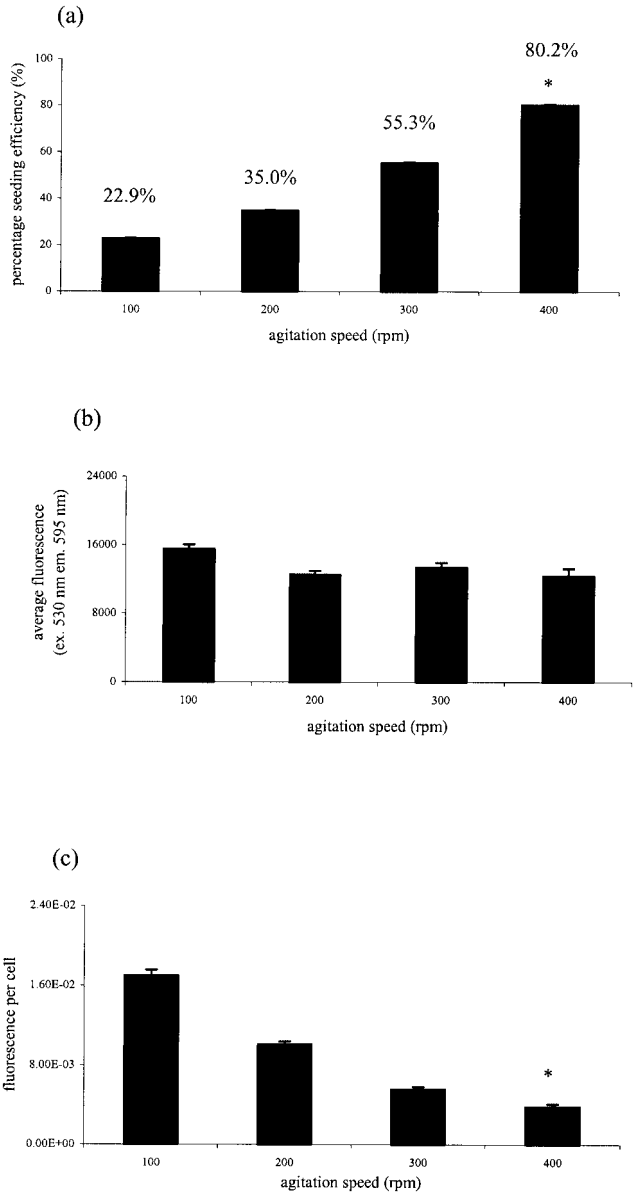

Figure 1. Analysis of needled felt scaffolds seeded with HOSTE85 cells at 100, 200, 300, and 400 rpm. (a) The effect of shaking intensity on the efficiency with which cells were seeded into scaffolds. Seeding efficiency is expressed as the number of cells within scaffold/initial number of cells (4×10^6) $\times$ 100%. (b) The effect of shaking intensity on the total viability of cells within scaffolds. (c) The effect of shaking intensity on viability per cell. The viability per cell is calculated by total viability/number of cells within the scaffold. Data are the mean $\pm$ SEM ($n = 3$ for each speed). *Indicates $p < 0.001$.

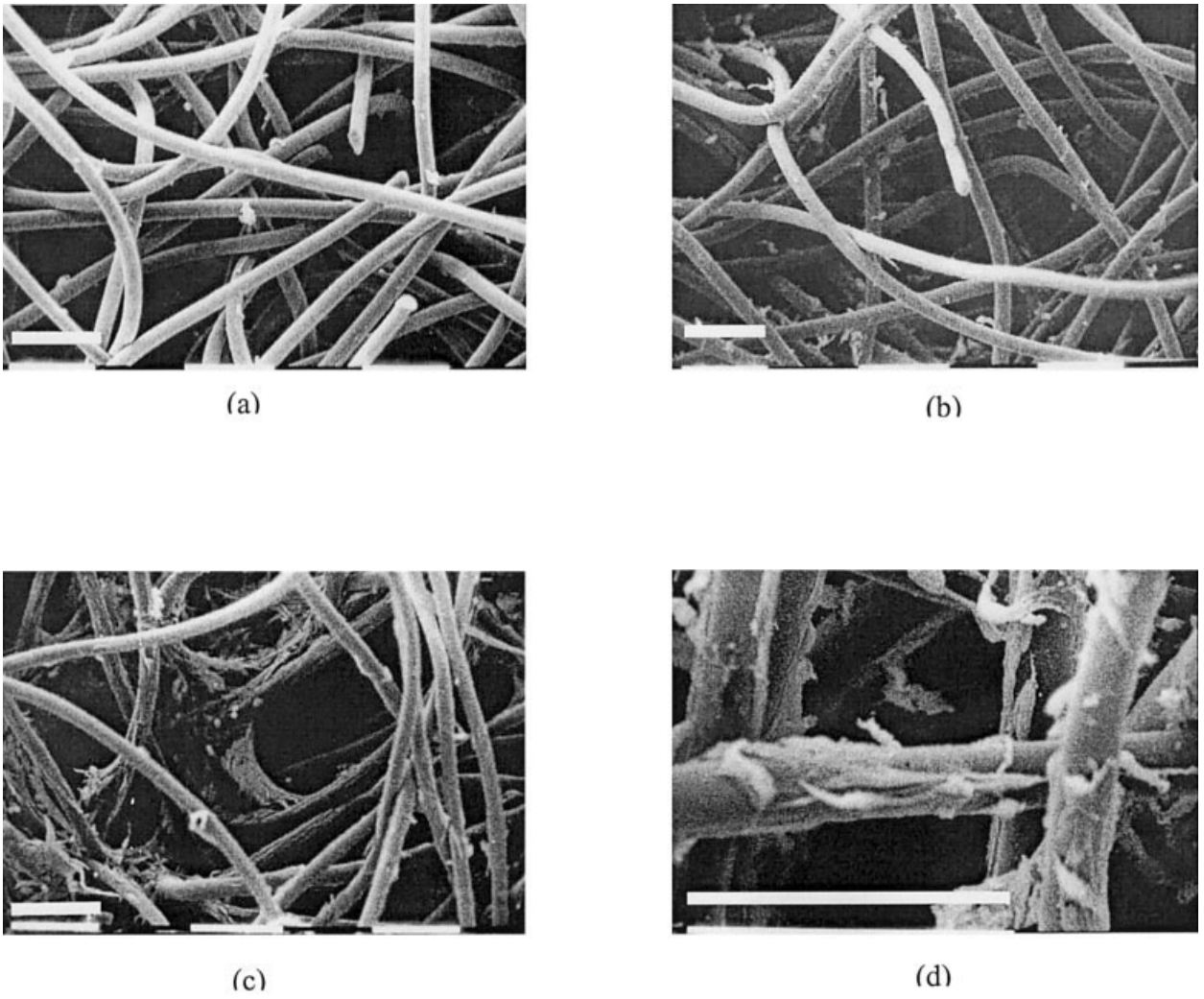

Figure 2. Arrangement of HOSTE85 cells within needled felt scaffolds shaken at 100 and 300 rpm. The distribution of HOSTE85 cells within scaffolds agitated at (a) & (b) 100 rpm and (c) & (d) 300 rpm. The images are representative examples (for each speed $n = 2$). Micron bar represents 100 μm.

increase in the number of HOSTE85 cells present, but the cells were concentrated in the center of the scaffold and normal cell morphology was compromised (images not shown).

Increasing the rate of agitation during seeding increases the number of HOSTE85 cells retained within the scaffolds, but it also results in a decrease in the viability of the cells. Agitating the scaffolds at 400 rpm also led to an uneven distribution of cells within the scaffolds and normal cell morphology was compromised.

Chondrocytes

Seeding efficiency and cell viability

The seeding efficiency with which chondrocytes were seeded into needled felt scaffolds following shaking at 0, 100, 200, 300, and 400 rpm is shown in Figure 3(a). Increasing the speed of agitation from 0 to 200 rpm led to an increased number of cells within the scaffold, but, surprisingly, increasing the speed further resulted in a reduction in the number of cells retained in the scaffolds. The relative overall viability of chondrocytes seeded into needled felt scaffolds is shown in Figure 3(b).

The scaffolds seeded statically showed statistically lower relative viability than the scaffolds agitated at the other speeds. The relative viability per cell for chondrocytes in each scaffold was calculated by dividing the average viability readings by the number of chondrocytes in each needled felt scaffold [see Fig. 3(c)]. No significant difference among the relative viabilities of individual chondrocytes in needled felt scaffolds agitated at different speeds was detected. For all control scaffolds, no DNA or cell viability was detected.

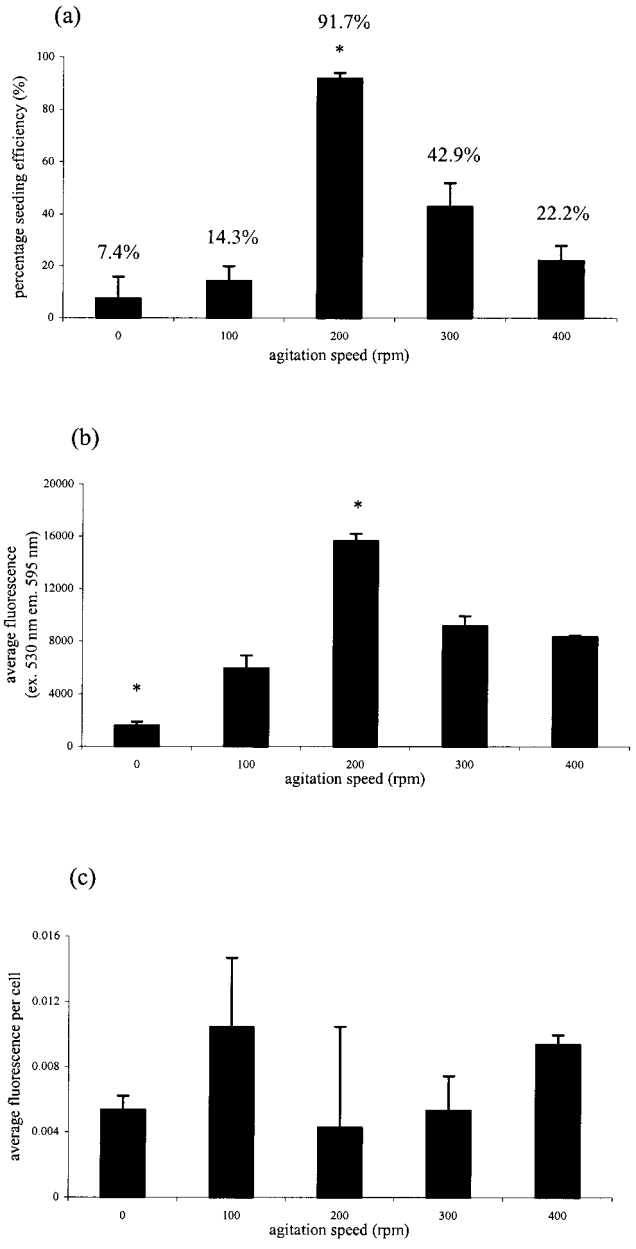

Figure 3. Analysis of needled felt scaffolds seeded with bovine chondrocytes at 0, 100, 200, 300, and 400 rpm. (a) The effect of shaking intensity on the efficiency with which cells were seeded into scaffolds. Seeding efficiency is expressed as the number of cells within scaffold/initial number of cells (4×10^6) $\times$ 100%. (b) The effect of shaking intensity on the total viability of cells within scaffolds. (c) The effect of shaking intensity on viability per cell. Viability per cell is calculated by total viability/number of cells present within the scaffold. Data are the mean $\pm$ SEM ($n = 3$ for each speed). *Indicates $p < 0.001$.

Spatial arrangement

Figure 4 shows representative scanning electron micrographs of chondrocytes in needled felt scaffolds agitated at 0, 200, and 400 rpm. Culturing the scaffolds overnight at 0 rpm caused only a few chondrocytes to be retained within the needled felt scaffolds [Fig. 4(a,b)]. Agitating the scaffolds at 100 rpm resulted in an increase in the number of chondrocytes both at the surface and in the center of the scaffolds (images not shown). Further increasing the speed of agitation to 200 rpm caused a great increase in the number of chondrocytes, and they were distributed evenly throughout the scaffolds [Fig. 4(c,d)]. Agitating the scaffolds at 400 rpm [Fig. 4(e,f)] lead to a less even distribution of cells throughout the scaffolds, with the chondrocytes concentrated in the centers of the scaffolds.

Agitating needled felt scaffolds containing bovine chondrocytes at 200 rpm produced optimum seeding. At this agitation speed, a high density of chondrocytes was seeded into scaffolds and evenly distributed throughout them without their viability being compromised.

CONCLUSIONS

Tissue engineering methods are under development that will allow the formation of functional tissues and organs. The density with which cells initially are seeded into scaffolds and their arrangement within the scaffolds are important parameters in tissue engineering. These factors affect cell proliferation, differentiation, and migration, and ultimately they affect the quality of the engineered tissue.[6]

The agitation speed required for obtaining optimum seeding for HOSTE85 cells in needled felt scaffolds, as determined from this study, is 300 rpm. At this speed a large number of cells are retained within the scaffolds, they are distributed evenly throughout, and their morphology is not compromised. The optimum seeding efficiency for bovine chondrocytes in needled felt scaffolds in this study was achieved by agitating the scaffolds at 200 rpm. Chondrocytes were distributed evenly throughout the scaffolds and the viability of each chondrocyte was not compromised.

The findings of this study support previous studies that have shown improved seeding of cells into scaffolds using dynamic rather than static seeding methods.[6,8] The optimum seeding conditions for the two cell types were different, as shown by the efficiency with which cells were seeded into the scaffolds and the arrangement of cells within the scaffolds. It is unknown why the cells respond differently to agitation; however, it is postulated that it may be due to differences in cell size or density. A study of this nature therefore is vital prior to commencing tissue engineering work in order to determine the optimum parameters for seeding scaffolds.

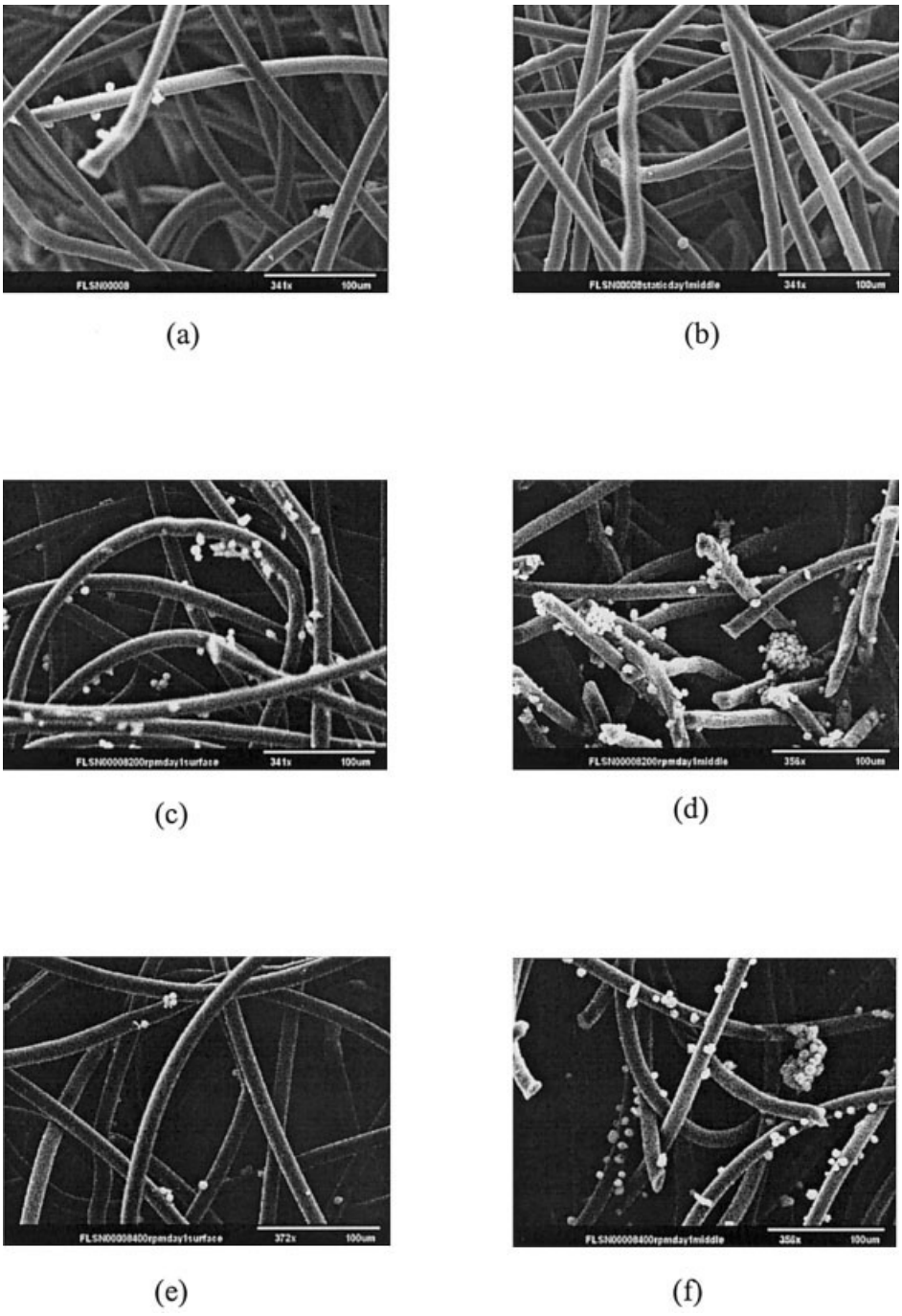

Figure 4. Arrangement of chondrocytes within needled felt scaffolds shaken at 0, 200, and 400 rpm. The distribution of cells at the surface of scaffolds agitated at (a) 0 rpm, (c) 200 rpm, and (e) 400 rpm can be seen. The distribution of cells within the scaffolds agitated at (b) 0 rpm, (d) 200 rpm, and (f) 400 rpm also are shown. The images are representative examples (for each speed $n = 2$).

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