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Title: A SCAFFOLD SEEDING METHOD

Abstract: The present invention provides methods for rapidly and efficiently seeding particles into a 3-dimensional scaffold, or onto a 2-dimentional scaffold. Cells, for example, may be efficiently transferred into, or onto, a scaffold in as little as 10 seconds using surface acoustic wave (SAW) actuation.

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A SCAFFOLD SEEDING METHOD FIELD OF THE INVENTION

The present invention relates to methods of seeding particles into, or onto the surface of, scaffolds. In particular, the invention relates to the seeding of particles, preferably cells, by surface acoustic wave (SAW) actuation. BACKGROUND OF THE INVENTION

The present invention is to be understood in light of what has previously been done in the field. However, the following discussion is not an acknowledgement or an admission that any of the material referred to was published, used or part of the common general knowledge in Australia as at the priority date of the application.

Tissue and organ transplantation is now an accepted and widely used therapy for the treatment of damaged or defective tissues and organs. Nevertheless, the transplant surgery process is extremely costly and can involve high risks due to possible complications. Long wait times are also common due to the perennial shortage of suitable donor tissues and organs. Tissue engineering, in which a patient's own cells can be grown, either within biodegradable and biocompatible three-dimensional scaffold matrices or on the surface of suitable substrate materials, and subsequently implanted in vivo to replicate the function of replacement tissues, or perhaps entire organs, is a promising alternative which could potentially alleviate inadequate donor tissue and organ supplies.

In order for in vitro cell cultures to occupy and grow within an extra-cellular matrix, the desired cells have first to be seeded into the scaffold while at the same time limiting any denaturing or

lysing that could potentially render the cells ineffective (Soletti L. et al., Biomaterials 2006; 27: 4863-4870). Achieving homogeneity in the cultured tissues is also clearly dependent on the uniformity of the cell distribution within the scaffolds and the efficiency of the seeding process. Any significant reduction in the total time required for cell seeding could also lead to substantial savings in cost. Further, an improvement in cell viability may occur since long seeding periods can result in adverse effects in which cells might expire or age beyond their useful state, which, in turn, affects the cells' adhesion, proliferation, and differentiation processes (Wendt D. et al., Biotechnol. Bioeng. 2003; 84: 205-214).

A further problem faced by those attempting to establish a viable population of cells within a scaffold is that when nutrients, growth and differentiation factors, and/or extracellular matrix molecules are provided in the culture medium, passive diffusion alone results in a decreasing concentration of the nutrients and/or extracellular matrix molecules from the surface to the innermost region of the scaffold. As it is important to maintain the viability and phenotype of the cells within the scaffold, it is highly desirable to develop a method to supply the inner cells with higher concentrations of the required factors. In relation to seeding into 3-dimensional polymeric scaffold materials, the typical hydrophobicity and small pore sizes (typically 10 - 150 Î¼m) of the material results in exceptionally slow ingress of, for example, a cell suspension into the scaffold in the absence of any external driving forces due to the large capillary resistance encountered. Under gravitational forces alone, perfusion of a cell suspension into the scaffold typically takes hours to days (Li and Chang, Biomaterials 2004; 25: 5473-5480: Li and Chang, J. Mater. Sci: Mater. Med. 2004; 15: 1089-1095). Nevertheless, this static seeding method has conventionally been used in tissue culturing due to its simplicity. The large capillary resistance also poses considerable difficulty in delivering cells deep within the scaffold structure in the absence of external driving forces. For example, studies have reported that new bone tissue forms easily at the surface of scaffolds but is hard to regenerate in the centre of the scaffolds due to the superficial penetration of the cell suspension with conventional seeding methods resulting in a large proportion of the cells being deposited merely on the surface of the scaffold.

As described supra, passive seeding of a cell suspension into a scaffold can take hours to days, which can result in cell stress due to the correspondingly poor diffusion of nutrients, growth factors and extracellular matrix molecules. It is highly desirable to be able to either include such beneficial factors with the cells at the time of seeding, or to later provide the cells which occupy the inner regions of the scaffold with a supply of such beneficial factors.

In relation to seeding onto the surface of an essentially 2-dimensional polymeric scaffold material the conventional method has been to apply, for

example, a cell suspension directly onto the surface and allow gravitational forces to cause the cells to gradually come into contact with the scaffold and adhere. A problem with this method is that the process of adhering the cells to the substrate by passive diffusion can typically take up to 24 hours. Moreover, when certain cell types are seeded by this method they adhere poorly to the scaffold material, which can lead to the loss of significant numbers of cells during subsequent culturing and/or handling and a non-uniform distribution.

Several improved methods are currently being explored for seeding cells within scaffolds and

achieving a greater homogeneous cell distribution within. These methods typically involve affixing a scaffold in an agitated vessel filled with the cell suspension. The cells then perfuse into the scaffold under a velocity gradient arising between the advected cells and the stationary scaffold. However, low seeding efficiencies have been reported in addition to long seeding times (hours to weeks) to finish a cell seeding process, and most of the cells aggregate on the surface of the scaffold which results in a non-uniform cell seeding (Vunjak- Novakovic G. et al., Biotechnol. Prog. 1998; 14: 193-202; Zhao F. and Ma T., Biotechnol. Bioeng. 2005; 91: 482-493; McFetridge PS. et al., J. Biomed. Mater. Res. 2004; 70: 224-234; Kitagawa T., et al., Biotechnol. Bioeng. 2005; 93: 947- 957; Burg KJL. et al., J. Biomed. Mater. Res. 2000; 51 : 642-649; Kim BS., et al., Biotechnol. Bioeng. 1998; 57: 46-54).

Soletti et al. (Biomaterials 2006; 27: 4863-4870) have proposed a technique for cell seeding in which cells within a suspension flowing through the annulus of a tubular scaffold perfuse through the scaffold under vacuum. The scaffold is concurrently rotated in order to achieve greater distribution uniformity. Although significantly reduced seeding times (several minutes) and improved distribution have been obtained, the use of vacuum and syringe pumps necessarily involves cumbersome airtight equipment and has an unknown effect on sensitive cells being infused with this equipment.

The present invention provides methods for rapidly and efficiently seeding particles into a 3-dimensional scaffold, or onto a 2-dimentional scaffold. Cells, for example, may be efficiently transferred into, or onto, a scaffold in as little as 10 seconds using SAW actuation. Moreover, cells transferred to the surface of a 2-

dimentional scaffold matrix adhere more strongly to the scaffold than cells allowed to passively adhere under normal gravitational force. SUMMARY OF THE INVENTION

This invention relates to a new and improved method of moving particles into, or onto the surface of, a scaffold matrix. Most preferably, it relates to a method of seeding cells into, or onto, a matrix suitable for tissue engineering applications.

It is therefore an object of the invention to provide a method of seeding particles into, or onto, a matrix which is improved over the prior art. According to one aspect of the invention, there is provided a method of driving a particle suspension into, or onto, a scaffold matrix by surface acoustic wave (SAW) actuation.

In a preferred embodiment of the invention, the method includes the steps of: (a) placing a scaffold matrix onto the surface of a SAW generating actuator;

(b) depositing a particle suspension onto the surface adjacent to, or in contact with, the matrix;

(c) generating a surface acoustic wave to thereby drive the suspension onto, or into, the matrix.

In a preferred embodiment of the invention, the matrix is either a 3- dimensional porous matrix or a planar, 2-dimensional matrix.

In a preferred embodiment of the invention, the particle is selected from the group including any

one or more of a mammalian cell, a non-mammalian cell, cell growth and/or differentiation factors, and extracellular matrix molecules.

In another preferred embodiment of the invention, the scaffold matrix is a biodegradable and/or biocompatible polymeric matrix.

In an alternate preferred embodiment of the invention, there is provided a method of driving a particle suspension onto, or into, a scaffold matrix by surface acoustic wave (SAW) generation, including the steps of:

(a) placing a scaffold matrix onto a piezoelectric substrate upon which has been fabricated an interdigital transducer (IDT);

(b) depositing a particle suspension onto the surface of the substrate between the IDT and the scaffold, and

(c) applying a radio frequency (RF) signal to the IDT to produce SAW radiation, which has the effect of driving the particle suspension onto, or into, the scaffold matrix.

Preferably, the matrix is a porous 3-dimensional matrix and the particle suspension is driven into the matrix by the surface acoustic wave radiation.

Preferably, the substrate is lithium niobate, $LiNbO_3$, though lead zirconate titanate (PZT) and closely related doped PZT materials and other piezoelectric materials with large electromechanical coupling coefficients, such as barium titanate, lead zirconium titanate, zinc oxide, aluminium nitride, lithium tantalate or quartz, would be suitable for this application.

According to another aspect of the invention, there is provided a method of driving a particle suspension onto a two-dimensional planar scaffold matrix by surface acoustic wave (SAW) generation.

In a preferred embodiment of the invention, the method includes the steps of:

(a) placing a two-dimensional scaffold matrix onto the surface of a SAW generating actuator; (b) depositing a particle suspension onto the surface of the matrix;

(c) generating a surface acoustic wave to thereby drive the suspension onto the matrix.

In another aspect of the invention, there is provided the use of SAW radiation to seed a porous three-dimensional scaffold matrix with a particle suspension.

In another preferred embodiment of the invention, the porous three- dimensional scaffold matrix is a biodegradable and/or biocompatible polymeric matrix.

In another aspect of the invention, there is provided a particle seeding apparatus for seeding a scaffold matrix, including a piezoelectric substrate upon which has been fabricated an interdigital transducer (IDT) which generates SAW radiation, upon excitation by an RF signal, to drive a

particle suspension onto, or into, said scaffold matrix.

The IDT may be of any suitable type, although preferred IDTs include alternating finger type transducers and single-phase uni-directional transducers (SPUDT).

Preferably, the piezoelectric substrate is $LiNbO_3$. In a preferred embodiment of the invention, the substrate has a titanium layer and an aluminium layer sputter-deposited thereon.

In another preferred embodiment of the invention, the IDT is an alternating finger IDT. More preferably, the IDT is an alternating finger IDT with 60 straight finger pairs and an aperture of 8 mm, and the strip and gap widths are 49 Î¼m, thus giving rise to a wavelength of Î» = 196 Î¼m for an operating frequency of 19.35 MHz.

In another preferred embodiment of the invention, the particle is a mammalian cell or a non-mammalian cell.

Recently, surface acoustic wave (SAW) technology has been developed for use in biotechnology, such as for biofluidic mixing, particle trapping, and as biosensors in microfluidic devices. A SAW is like an earthquake wave propagating along the surface of a piezoelectric substrate with an amplitude of a few tens of nanometres (Figure 1(a)). The wave is created by an interdigital transducer electrode (IDT) wherein the wavelength is defined by the width of each individual finger along the propagation direction of the wave and the gap between them. The amplitude of the electrical signal determines the amplitude of the wave. Though there are many forms of SAW, the present invention makes use of the Rayleigh wave, an axial-surface-normal polarized SAW (Morita T., et al., IEEE Trans. Ultrason. Ferroelec. Freq. Contr. 1999; 46:929-934). It has been shown that SAWs strongly interact with small amounts of liquid on the surface of a piezoelectric substrate by inducing bulk liquid recirculation through a process known as acoustic streaming (Figure 1(b)) (Wixforth A., Superlattice Microst. 2004; 33: 389-396). A SAW entering the droplet is diffracted at the Rayleigh angle ÎR into the fluid, where it generates a longitudinal pressure wave. For an infinite half space, this ÎR is given by the ratio of the sound velocities in the substrate and in the fluid, respectively. If the intensity of the acoustic radiation component into the fluid is sufficiently high, and the liquid consists of a free droplet whose contact line is not pinned, the SAW can induce the droplet to move in the

direction of the SAW (Wixforth A., et al., Anal. Bioanal. Chem. 2004; 379:982- 991 ; Guttenberg A., et al., P. Phys Rev E Stat Nonlin Soft Matter Phys 2004; 70: 056311). The technology is therefore a convenient mechanism for transporting a free droplet on a microfluidic device while requiring no mechanically moving components other than small deformations of the substrate itself.

According to the present invention, SAW actuation can be exploited as a method for quickly driving particles into, or onto, a scaffold, providing a means for rapid, uniformly distributed, and efficient cell seeding in various applications, including tissue engineering. DESCRIPTION OF THE DRAWINGS

Figure 1 : (a) Drawing illustrating the propagation of a surface acoustic wave (SAW) through a

lithium niobate substrate, (b) Drawing illustrating acoustic streaming generated within a small droplet by SAW.

Figure 2: (a) Drawing of the experimental setup for cell seeding using SAW actuation, (b) Drawing of the experimental setup for gravity-driven cell seeding, (c) Representation of six 1 - millimetre thick sections cut through the scaffold to evaluate the distribution of the cells within it.

Figure 3: Schematic of SAW method and processing for histological determination of depth of ingress of mouse bone cells. Figure 4: Graph of the effect of input RF power on the velocity of the droplet suspension prior to its contact with and entry into the scaffold, and the particle seeding efficiency within the scaffold.

Figure 5: (a) Cell seeding efficiency and distribution in each section of scaffold seeded by SAW-driven or static methods, (b) Viable cell seeding efficiency in each section, (c) Total cell seeding efficiency with and without (static) SAW actuation.

Figure 6: (a) SEM images of yeast cell morphology within scaffold seeded by SAW actuation, (b) Proliferation rates of yeast cells after SAW treatment.

Figure 7: Average Viability of mouse bone cells post-SAW treatment at different powers and frequencies.

Figure 8: Average Viability of mouse bone cells subjected to 20 Mhz/-1 dBm in SAW for different times.

Figure 9: (a) Average viability of mouse bone cells cells at different suspension densities subjected to SAW technology, (b) Difference in viability between treated and untreated samples at different cell densities.

Figure 10: Average cell proliferation of SAW-treated and untreated mouse bone cells as function of fluorescence via Alamar Blue uptake.

Figure 11: (a) Geometry of scaffold and droplet under bright illumination.

(b) Successive images acquired at 60 frames/s show a side view of SAW-driven perfusion process under fluoroscope in roughly 10 s. (c) A plan view of the static method in which the drop perfuses through the scaffold by pure diffusion alone, taking approximately 30 min.

Figure 12: Scaffold cross-sectional slices at various positions given in Fig. 2(b).

Figure 13: Particle distribution in each slice following seeding after ten seconds of SAW actuation, or 30 minutes of static diffusion. Figure 14a: Haematoxylin & Eosin staining of sections cut of PCL seeded with ~100,000 mouse bone cells.

Figure 14b: Red fluorescent images of sections cut of PCL seeded with ~100,000 mouse bone cells.

Figure 15: Methylene violet staining of yeast cells on the surface of seeded scaffolds following SAW actuation or static (passive) seeding.

DETAILED DESCRIPTION OF THE INVENTION

Previous attempts at seeding scaffold matrices, including both porous 3- dimensional and 2-dimensional planar matrices, have generally entailed methods which use no external driving forces, thus requiring long contact times between the particles to be seeded and the matrix and, in the case of cells, result in poor penetration into the matrix or poor adherence to the surface of the matrix, respectively. More recently developed methods for seeding 3-dimensional matrices rely on affixing a scaffold in an agitated vessel filled with the cell suspension, causing cells to perfuse under a velocity gradient, or which rely on vacuum pressure to cause cells to perfuse a matrix also have problems. The former method results in low seeding efficiency and long seeding times, whereas the latter method reduces seeding times but requires substantial airtight

equipment. In regard to the seeding of 2-dimentional matrices the method generally applied is that of passive diffusion, which requires sometimes several hours for the cells to settle onto the matrix. Moreover, the strength of adhesion to the matrix is often poor and the distribution of cells on the matrix is often uneven. The present invention provides a method for seeding scaffold matrices which is relatively fast and results in matrices with improved uniformity of seeding.

According to one aspect of the invention, there is provided a method of driving a particle suspension into a porous three-dimensional scaffold matrix by surface acoustic wave (SAW) generation, including the steps of: (a) placing a porous three-dimensional scaffold matrix onto the surface of a

SAW generating actuator;

(b) depositing a particle suspension onto the surface adjacent to, or in contact with, the matrix;

(c) generating a surface acoustic wave to thereby drive the suspension into the matrix.

According to another aspect of the invention, there is provided a method of driving a particle suspension onto a two-dimensional planar scaffold matrix by surface acoustic wave (SAW) generation, wherein the method includes the steps of: (a) placing a two-dimensional scaffold matrix onto the surface of a SAW generating actuator;

(b) depositing a particle suspension onto the surface of the matrix;

(c) generating a surface acoustic wave to thereby drive the suspension onto the matrix. It would be understood that, in the context of the invention, the particle may be selected from a variety of biological material. For example, the particle may be a prokaryotic or eukaryotic cell, a mammalian cell, a virus particle, or a nucleic acid such as a plasmid vector, a cell growth or differentiation factor, an extracellular matrix molecule, a polymer particle such as polycaprolactone, a metal particle such as gold or tantalum, or a ceramic particle such as hydroxyapatite or alumina. The cell type may be chosen for its suitability for tissue engineering applications. A cell such as a stem cell, in particular a human or animal stem cell, may be most

suitable for clinical applications.

In a preferred embodiment of the invention, the particle suspension is a mammalian cell suspension or a non-mammalian cell suspension.

The scaffold matrix may be made from any suitable material for the application. If the matrix is intended for tissue engineering applications, the material may be polymeric, such as polycaprolactone (PCL), bioresorbable polyurethane matrix enriched with laminin, Î³-poly glutamic acid (PGA)/chitosan composite matrices, poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (PHBV), hydrogel composed of collagen type I and matrigel, or synthesized or natural inorganic material matrices from materials such as hydroxyapatite or demineralized cancellous bone, coral, bioactive glass, and related calcium and phosphate-containing materials for bone and hard tissue engineering, and natural soft tissue based materials such as Alloderm Â®. Alloderm Â® is a human-derived collagen matrix, available from BioHorizons Inc., Birmingham, Alabama, USA. Preferably, the matrix is formed from polycaprolactone. In another preferred embodiment of the invention, the scaffold matrix is a biodegradable and/or biocompatible polymeric matrix.

In an alternate preferred embodiment of the invention, there is provided a method of driving a particle suspension into a porous three-dimensional scaffold matrix by surface acoustic wave (SAW) generation, including the steps of: (a) placing a porous three-dimensional scaffold matrix onto a piezoelectric substrate upon which has been fabricated an interdigital transducer (IDT);

(b) depositing a particle suspension onto the surface of the substrate between the IDT and the scaffold, and

(c) applying a radio frequency (RF) signal to the IDT to produce SAW radiation, which has the effect of driving the particle suspension into the scaffold matrix.

In another aspect of the invention, there is provided the use of SAW radiation to seed a scaffold matrix with a particle suspension.

In another aspect of the invention, there is provided a particle seeding apparatus for seeding a scaffold matrix, including a piezoelectric substrate upon which has been fabricated an interdigital transducer (IDT) which generates SAW radiation, upon excitation by an RF signal, to drive a particle suspension onto, or into, said three-dimensional scaffold matrix.

Preferably the substrate is lithium niobate ($LiNbO_3$), although other types of piezoelectric material such as barium titanate, lead zirconium titanate, zinc oxide, aluminium nitride, lithium tantalate, and quartz may also be used.

The piezoelectric substrate may be elongate in shape having opposing ends, and at least one IDT electrode may be located at one end thereof.

In a preferred embodiment of the invention, the substrate has a 4 nm titanium layer and a 150 nm aluminium layer sputter-deposited thereon.

The IDT may be of any suitable type, preferably an alternating finger IDT or a single-phase unidirectional transducer (SPUDT). In a preferred embodiment of the invention, the IDT is an alternating finger

IDT.

In another preferred embodiment of the invention, the IDT is an alternating finger IDT with 60 straight finger pairs and an aperture of 8 mm, and the strip and gap widths are 49 Î¼m, thus giving rise to a wavelength of Î» = 196 Î¼m for a SAW device operating at 19.35 MHz. In choosing other operating frequencies between 5 and 500 MHz, the wavelength and therefore the finger and gap widths are adjusted accordingly. Generally, the number of finger pairs increases in proportion to the operating frequency of the device. The configuration of the finger pairs may be altered to incorporate internal reflectors (SPUDT configuration, Campbell US Patent 6,462,698 and Campbell and Saw, 1987 IEEE TUFFC v34(3) pp357-) or focused with curved fingers to increase the efficiency of the device and concentrate the power transmission into the fluid.

The RF input power required to actuate a SAW to drive the particle suspension onto, or into, the scaffold matrix will vary depending on the particular characteristics of the particle and matrix. In the present invention, RF power in the order of 100 mW up to 860 mW has been sufficient to drive a particle suspension into a porous three-dimensional scaffold matrix. Preferably the RF power input is in the order of 100 mW to about 1 W, depending on the SAW frequency chosen for the device, which is preferably between 5 MHz and 500 MHz.

Method of seeding a 3-dimensional scaffold

In a preferred method of driving a particle suspension into a porous three- dimensional scaffold matrix by surface acoustic wave (SAW) generation, a porous

three-dimensional polycaprolactone (PCL) scaffold is placed onto an X- propagating lithium niobate ($LiNbO_3$) piezoelectric substrate, upon which has been fabricated an alternating finger interdigital transducer (IDT) coated with a 4- nm titanium layer and a 150-nm aluminium layer. A 20-Î¼l particle suspension is deposited onto the surface of the substrate between the IDT and the scaffold, and a radio frequency (RF) signal (input power of 400 mW, resonance frequency of 19.35 MHz)Js applied to the IDT for 10 seconds to produce SAW (Rayleigh wave) radiation, which has the effect of driving the particle suspension into the scaffold matrix. EXAMPLES

1. MATERIALS AND METHODS

1.1 Scaffolds

1.1.1 3-Dimensional

Three-dimensional scaffolds were prepared using a conventional solvent casting-particulate leaching method (Li H. and Chang J., J. Mater. ScL: Mater. Med.

2004; 15: 1089-1095) using polycaprolactone (PCL, MW = 65,000, MP = 65Â°C)

(Sigma Chemical Co., USA). Briefly, PCL powders were dissolved in chloroform with a concentration of 10% (w/v). Sodium chloride (NaCI) particles sieved as porogens (100-150 Î¼m) were then incorporated into the suspension (NaCI / polymer = 1 :9) (w/w), and the dispersion was cast into a 60-mm Teflon mould.

The samples were air-dried under a fume hood for 24 hours to allow the solvent to evaporate and were subsequently vacuum-dried at 60^0C for 48 hours to remove any remaining solvent. The samples were then immersed in deionised water to leach out porogens in the resulting polymer/ salt composites. The samples were finally vacuum-dried to obtain a set of sponge-like scaffolds with thicknesses of 2 mm. Before seeding, the scaffolds were carefully cut with a razor blade into 6 * 5 mm squares and were stored in a desiccator. The porosity of the scaffold was about 90% Â± 1.5%.

1.1.2 2-Dimensional Sheet Two-dimensional, or sheet-like, scaffolds such as the collagen matrix commercially available as Alloderm Â® may be obtained from BioHorizons Inc.,

Birmingham, Alabama, USA, or produced according to conventional methods using suitable matrix material such as PCL or hydroxyapatite.

1.2 Fluorescent microspheres

An aqueous suspension of 5-Î¼m green fluorescent polystyrene (PS) microspheres (Duke Scientific Corporation, USA) was used to facilitate observation of the seeding process and their spatial distribution in the scaffold.

The excitation maxima wavelength for these particles is 468 nm (blue) and the emission maxima wavelength is 508 nm (green). These PS microparticles were obtained in the form of a 1% (w/v) aqueous suspension, containing $1.4*10^8$ particles in 1 ml. Using deionised (Dl) water, the original suspension was diluted to obtain a concentration of $1.4x10^6$ particles/ml.

1.3 Yeast cells

Preliminary studies were performed on yeast cells to determine their ability to survive the SAW seeding process. Stock cultures of the yeast cells were maintained on standard agar consisting of 1% yeast extract, 0.5% neutralized bacteriological peptone, and 1% glucose solidified with 1.5% agar (w/v). All media were autoclaved immediately after preparation at 121^CC and 15 psi for 15 min. Yeast cells were grown aerobically to the required cell density at room temperature. For yeast cell seeding, a 40-Î¼l cell suspension with a concentration of 1*

10^8 cells/ml was slowly pipetted onto the surface of the SAW device between the IDT and the scaffold. The seeding end point was defined as when there was no suspension left on the surface of the SAW device, determined through visual inspection. 1.4 Human stem cells

Preliminary studies were performed on human stem cells to determine their ability to survive the SAW seeding process. Primary human bone cells were deposited onto the surface of a lithium niobate (LiNbOa) piezoelectric substrate. The cells were subjected to low (400 mW) and high

(860 mW) RF levels, or no treatment, for a period of 10 seconds and their viability determined after 2 days (Table 1). 1.5 Mouse primary bone cells

In order to further examine the effects of various SAW parameters on the viability of mammalian cells and their ability to enter a scaffold matrix, primary bone cells from mice were isolated using standard techniques.

1.5.1 Cell Isolation Briefly, long bones or calvaria were isolated from 6-8 week old C57bl/6 mice. Long bones were flushed from marrow using normal saline and the bones were crushed/minced using bone crunchers into smaller particles (calvaria were minced as described). They were then subjected to serial collagenase digestion to isolate bone and stromal cell populations. Cells were collected at the end of each digest, collagenase activity stopped by the addition of 15% FBS and cells were strained through a cell strainer, centrifuged, washed and resuspended in alpha-MEM supplemented with 10-15% FBS and plated (P1) in tissue culture flask for 4 days to allow recovery of cells. At this stage, cells were removed from the tissue culture flask using trypsin/ EDTA, collected, washed and centrifuged, resuspended in freeze medium (1x 10^6 cells /ml) and frozen under liquid nitrogen vapour until required.

7.5.2 Experimental cells

Frozen cells were thawed at 37Â°C and resuspended in alpha-MEM supplemented with 10% FBS, then plated and allowed to expand (P2). These cells were subsequently used for SAW experiments. These cells are able to form mineralised nodules in culture in the presence of Ascorbic acid and beta- glycerophosphate, indicative of their bone forming activity in vivo.

15.3 Mouse cell viability

1.5.3.1 Determination of optimal SAW power and frequency For bone cell seeding, 10Î¼l droplets with a concentration of 5x10^6 cells/ml were subjected to a SAW for 10 seconds at powers of -2 dBm, -1 dBm, 1 dBm or 2 dBm in combination with frequencies of 10 Mhz, 20 Mhz or 50 Mhz (in triplicate). Untreated controls were included for comparison.

A FACS Vantage DIVA (Becton Dickinson) was used to assess cell viability. Propidium iodide dye uptake is used to detect cells whose membranes are disrupted, thus providing an indication of total cell viability.

1.5.3.2 Determination of optimal time of SAW treatment

For bone cell seeding, 10Î¼l droplets with a concentration of 5x10^6 cells/ml were subjected to a SAW for 10, 30, 45 or 60 seconds, in triplicate at optimal power/frequency. Untreated controls were included for comparison. Viability was assessed as above. 1.5.3.3 Determination of optimal cell suspension density

Five different cell suspension seeding densities were tested for their effect on viability when subjected to SAW: 5 x 10^5 cells/ml, 1 x 10^6 cells/ml, 2 x 10^6 cells/ml, 4 x 10^6 cells/ml and 8 x

10^6 cells/ml, each in triplicate, at 20 MHzM dBm.

Untreated controls were included for comparison. Viability was assessed as above.

1.5.3.4 Assessment of cell proliferation post-SAW treatment

The ability of cells to proliferate after SAW treatment was assessed as a measure of whether SAW has an effect on this aspect of cell viability.

Cell suspensions at 5 x 10^3 cells/ml were subjected to SAW treatment for 10 seconds at 20 MHzM dBm, in triplicate, and compared to untreated controls. Cells were then seeded into a 48-well plate, followed by the addition of Alamar Blue (a reagent taken up by cells, leading to fluorescence which increases with increasing cell number). Proliferation was assessed by measurement of the total fluorescence in each well, using a plate reader, at various time points. 1.6 Seeding techniques

1.6.1 Particle seeding using SAW radiation

A method for direct pumping of a fluorescent particle suspension through the pores of 3D scaffolds using SAW radiation is described. A SAW is excited by the application of a radio frequency (RF) signal to an interdigital transducer (IDT) fabricated onto a piezoelectric substrate. The wavelength of the excited SAW, and therefore its resonance frequency, is defined by the geometry of the IDT. A 0.5-mm thick substrate, 127.68Â°YX cut, X-propagating lithium niobate ($LiNbO_3$ or LN, Roditi, London UK) single crystal was used as the SAW substrate in this experiment to generate a Rayleigh wave upon excitation of the IDT by an RF signal. A 4-nm titanium layer and a 150-nm aluminium layer were sputter- deposited upon this substrate. A standard alternating finger IDT with 60 straight finger pairs and an aperture of 8 mm was fabricated using photolithography; the

strip and gap widths were 49 Î¼m, thus giving rise to a wavelength of Î» = 196 Î¼m.

The IDT was driven with a sinusoidal signal at the resonance frequency f_0, defined as $f_0 = c/\hat{I}»$. The SAW velocity c = 3788 m/s, and hence f_0 Â« 19.35 MHz.

Such an IDT efficiently converts the applied RF signal into an acoustic wave, which in this case travels from the IDT in both directions along the substrate surface and perpendicular to the direction of the strip electrodes with low divergence.

Figure 2(a) shows a schematic of the experimental setup as seen from above. Situated on the left side is the input IDT. The wires in contact with the bus bars of the IDT are connected to the RF power source. A 20-Î¼l drop containing the fl uorescent PS suspension with a concentration of $1.4*10^6$ particles/ml is then deposited onto the surface of the SAW device between the IDT and the scaffold.

To observe the fluorescent PS particles, a high-speed video camera (Olympus i- speed, Tokyo, Japan) was used through a reflected fluorescent microscope system (Olympus BXFM, Tokyo,

Japan) to monitor the dynamics of the seeding process. Wideband blue light with an intensity maximum at 468 nm was used to excite the particle fluorescence.

1.6.2 Static seeding

In order to benchmark the efficiency of the SAW-driven cell seeding process, the results from Section 1.6.1 above were compared with a static seeding process in which the cell suspension is allowed to perfuse through the scaffold solely by pure diffusion. First, PCL scaffolds were placed onto a SAW substrate. A 20-Î¼l drop of fluorescent particulate suspension with a concentration of $1.4^{Ï}10^{6}$ particles/ml was pipetted onto the top surface of the scaffold. The same observation system was used to monitor the seeding process. The approximate time required for the scaffold to completely absorb the droplet suspension, i.e., no liquid apparent above the surface, was determined through visual inspection using the microscope system.

1.7 Image analysis After the seeding process, the scaffolds were cut perpendicularly to the long axis into six 1-mm thick slices (Figure 2(b)). The cross-section of each slice (a-f) was then observed under the reflected fluorescent microscope system. Slice a is the side where the cell suspension first enters in the two methods described

above. The fluorescent pixel intensities of each image were acquired using Photoshop CS (Adobe Systems Software Ltd, Ireland); each image's intensity was normalised against the intensity of the image of the initially-wetted surface of the scaffold. 1.8 Particle seeding efficiency

A digital frame-by-frame analysis of the high-speed video was employed to determine the area of the image under which the fluorescent signal was evident. The velocity was computed by dividing the distance that the drop suspension travelled prior to entry into the scaffold with the time taken. Preliminary investigations were aimed at determining the influence of the input RF power on the suspension's velocity prior to entry into the scaffold and the particle seeding efficiency of the scaffolds. The particle seeding efficiency was evaluated according to the following method: the SAW radiation was terminated when the entire suspension entered the scaffold completely, i.e., no liquid external to the scaffold remained on the SAW surface. None of the liquid was permitted to circumvent the scaffold or to atomize. Generally, the power required to atomize a droplet is higher than that used in this study. The SAW substrate was then rinsed using Dl water and the solution was collected. The number of fluorescent particles in this solution, N-i, was counted by using a hemocytometer (Hausser Scientific, Horsham, PA, Sigma, USA). There were $2.8x10^{4}$ fluorescent particles in the 20-Î¼l droplet according to the original fluorescent particle concentration ($1.4x10^{6}$ particles/ml). The "particle seeding efficiency of the scaffold is then given by

Particle seeding efficiency % = $100^{Ï}$ $(2.8x10^{4}$-Ni) / $2.8x10^{4}$ 1.9 Yeast cell seeding efficiency

The cell seeding efficiency, defined as the percentage of initially loaded cells to the scaffolds, was determined as follows. Samples were allowed to dry at room temperature overnight. The dehydrated seeded scaffolds were cut into six 1 mm thick slices and each slice was digested in 1 ml_ proteinase K solution for 18 h at 56^{0}C. Proteinase K (Sigma Chemical Co., USA) was

prepared at a concentration of 1 mg/mL in 50 mM Tris.[17] To terminate the reaction, phenylmethylsulfonyl fluoride (PMSF, Sigma Chemical Co., USA), an inhibitor of

proteinase K, was used. Aliquots of the digest for each section were analyzed using a hemocytometer to obtain the cell concentration at each section (C_a~C_f). The concentration of original seeding suspension was defined as C_0 (is $1 * 10^8$ cells/ml). The "cell seeding efficiency of each section" was therefore given by % cell seeding efficiency in each section = $100*(C_x/C_0)$ (2)

Where x represent section position (a, b, c, d, e, f). Total cell seeding efficiency can be obtained by summing the cell seeding efficiency in each section. In addition, the cell seeding efficiency in each section can also describe the cell distribution within the whole scaffold. The following section will describe how to quantify the cell distribution in each slice. 1.10 Yeast cell viability

Viability of living yeast cells after seeding was qualitatively confirmed using physiological staining with methylene violet 3 RAX and scanning electron microscopy (SEM). After seeding, a 0.5 mm thick cross-sectional slice was taken from the middle of the each slice immediately and stained with methylene violet 3 RAX solution according to standard procedures [Smart, K.A., Chambers, K.M., Lambert, I., Jenkins, C. and Smart, CA: J. Am. Soc. Brew. Chem, 57 (1), pp 18 23, 1999]. Images were segmented and violet cells were counted under a microscope to derive the fraction of nonviable cells. The latter parameter was then used in combination with the "cell seeding efficiency of each section" to estimate the "viable cell seeding efficiency in each section", i.e., the percentage of initially seeded cells that attached to the slice and were alive at the time of the assessment. For SEM observation, the dehydrated samples were mounted on stubs and coated in vacuum with gold before being observed by SEM (Model JEOL-6300F, Japan) at an accelerating voltage of 10 kV to observe the cells.

For quantitative assessment of yeast cells[1] viability after being treated by SAW, a yeast suspension with the identical in volume to the aliquot seeded into the scaffold was pipetted on the surface of SAW device and subjected to the SAW radiation at the input RF power applied to driven cells within scaffold. After being treated, aliquots of the yeast suspension were collected and diluted by adding culture media. The cell concentration of this suspension was counted subsequently using a haemocytometer and was defined as the cell concentration

at t = 0 day. Then, the yeast cells were cultured for an additional 14 days and the concentration of the yeast cells in the media was determined at 2, 5, 7 and 14 days. The yeast cells treated by SAW were considered to be viable if they could proliferate in prolonged culture conditions. 1.11 Mouse bone cell seeding using optimised SAW exposure

The optimised cell viability parameters determined under 1.5 above were used in a SAW-actuated cell seeding of 3-dimensional hydroxyapatite or polycaprolactone scaffolds. Either a 10Î¼l or 50Î¼l droplet of cells at 5×10^3 cells/ml was placed in contact with the scaffold matrix and subjected to SAW treatment for 10 seconds at 20 MHzM dBm. The cells were from a transgenic red fluorescent mouse, thus the cells glow red when subjected to appropriate light energy in vitro. Untreated controls (passive seeding) were included for comparison.

Post-treated matrices were embedded in frozen embedding medium, followed by sections (slices of matrix) being cut onto slides, according to the scheme provided in Figure 3. Sections were stained using haematoxylin/eosin, which stains for cellular material (contrasting staining for cells vs. tissue/matrix). The degree of egress of cells into the matrix was determined by viewing the H&E- stained sections or fluorescence emitted by cells in the sections. 1.12 Statistical analysis

All values are presented as mean Â± standard deviation. Differences among experimental groups were assessed using a two-tailed Student's f-test and considered statistically significant if $p < 0.05$. 2. RESULTS 2.1 Effect of input RF power on the particle seeding efficiency

Figure 4 describes the relationship between the velocity at which the particle suspension enters the scaffold and the particle seeding efficiency within the scaffold. Below approximately 325 mW, the particle seeding efficiency is low (<50%) as there is insufficient fluid velocity to drive the suspension into the scaffold. The majority of particles were found to aggregate on the SAW substrate. Above this input power, however, there was a marked increase in the particle seeding efficiency, approximately 50% to 85% despite only a modest increase in

the fluid droplet velocity to 1 mm/s. Above approximately 400 mW, however, the particle seeding appeared to plateau despite a sharp increase in the droplet velocity as the input RF power was increased. The highest seeding efficiency was about 92% at an RF power of 570 mW. Above this power, the intense SAW radiation resulted in the atomization of the droplet containing the suspension even before it reached the scaffold. This thus imposes an upper limit on the input RF power. Since there is no significant increase in the seeding efficiency beyond 400 mW, the subsequent experiments were conducted using this input power level.

The plateau in the cell seeding efficiency above 400 mW, on the other hand, is associated with the elastic-plastic transition for the scaffold, which demonstrates the typical behaviour of porous polymer materials undergoing deformation. For a typical polycaprolactone porous scaffold structure, the yield stress (transition from elastic to plastic behaviour) occurs at about 3 MPa, while its stiffness or Young's modulus is about 42 MPa. 2.2 Effect of input RF power on cell survival

2.2.1 Yeast cells

Recent studies have shown that a cell's viability will not be damaged when it is treated by ultrasonic standing wave fields (Radel S. et al., Ultrasonics 2000; 38: 633-637; Cousins SM. et al., Ultrasound Med Biol 2000; 26: 881-888). Studies on freely suspended yeast acoustically treated in ultrasonic standing wave devices for longer periods (up to 2 h) suggest that the viability of the yeast cells was not significantly affected (Radel et al., supra). Similarly, no disruption of red blood cells was noted when using a tubular acoustic resonator optimised for diagnostic purposes and subsequently used as an alternative method for concentrating red blood cells. The present inventors have confirmed that yeast are resistant to SAW-induced acoustic damage. Figure 5(a) shows the cell seeding efficiency in each slice cut from the whole scaffold. By summing the cell seeding efficiency in each slice, total cell seeding efficiency in whole scaffold can be obtained (Figure 5(b), grey bars). Cell seeding efficiency in each section of scaffold seeded by SAW actuation was relative average (about 20%) and the total cell seeding efficiency for these scaffolds

was 87%Â±5%. A non-uniform cell seeding was observed in scaffolds seeded by the static method and 68% cells

were seeded on slice a while almost no cells were delivered in slices d-f although the total cell seeding efficiencies for these scaffolds was 83%Â±5%, which was not statistically different to that seeded by SAW actuation.

Cell viability within the scaffolds was also quantified to differentiate between the contribution of living and dead cells to the seeding efficiency. Image analysis of each stained cross-section revealed that more cells remained viable in the scaffold seeded by SAW actuation than by the static method (Figure 5(b)). The percentage of viable cells in the whole scaffold was obtained by summing the data in each section, with the result that 80%Â±8% of cells within the scaffold seeded by SAW actuation were viable, whereas survival was significantly lower in scaffolds seeded statically (55%Â±10%, $p = 0.025$). Using the viability data in conjunction with the previously determined seeding efficiencies, we calculated that the percentage of the initially loaded cells that stayed and remained viable in the whole scaffold (i. e., "viable cell seeding efficiency") was significantly higher by SAW actuation than by the static method (1.5-times higher, $P < 0.0005$) (Figure 5(c), black bars).

The viability of yeast cells after SAW-driven seeding has been verified by methylene staining and the percentage of viable cells has been calculated (Figure 5(b)). In Figure 6(a), SEM morphology of yeast cells within the scaffolds further confirmed that the shape of the yeast cells loaded in the pores was preserved after SAW treatment. In addition, yeast cells treated by SAW maintained their proliferation ability during the prolonged culture in the following 14 days (Figure 6(b)), which further proved that the SAW radiation at the applied RF power has not damaged the yeast cells.

2.2.2 Human bone cells

The present inventors have also shown that human stem cell viability is not significantly reduced by SAW actuation itself at either low or high radio frequency compared with the (untreated) control group (Table 1).

Table 1

2.2.3 Mouse primary bone cells

For all power and frequencies tested, the cell viability ranged between 72- 88%, with an optimum survival rate at a power/frequency combination of -1 dBm/20MHz (Figure 7).

Studies on the effect of SAW exposure time on viability showed that for exposure times between 10 seconds and 60 seconds the viability ranged between about 92%, for 10 seconds exposure, and about 2% for a 60 second exposure (Figure 8).

The studies on the effect of the cell concentration in the seeding suspension on viability showed that survival at concentrations of 5×10^5 cells/ml was in the order of 45%, whereas survival was increased to over 70% when the concentration was at least 4×10^6 cells/ml (Figure 9a). When the

difference in viability between treated and corresponding untreated cell suspensions was considered, the optimal cell survival density tested was 8 x 10^6 cells/ml (Figure 9b).

No difference was seen between the proliferation of SAW-treated and untreated cells, indicating that SAW treatment had no effect on the potential for the cells to proliferate (Figure 10).

Therefore, there is sufficient evidence to suggest that SAW radiation at comparatively low power levels does not affect cell viability. 2.3 Dynamics of seeding process

High-speed video images of the particle seeding process at 400 mW were captured at 60 frames per second. The images in Figure 11 (a) show the geometry of the droplet and scaffold under bright illumination field at t = 0 prior to seeding. The left image shows the positions of the droplet and scaffold in the

SAW-driven method and the right image shows that the droplet was pipetted onto

the surface of the scaffold, maintaining a round shape, at least initially. Images in Figure 11(b), in which the seeding process is observed dynamically under fluoroscopic illumination, show that the SAW-driven particle seeding process is rapid. The green fluorescence indicates that approximately 85% of the particles in the initial suspension were distributed within the scaffold in just 10 seconds. The droplet was observed to approach the scaffold and subsequently to be driven into the scaffold by SAW actuation. The fluorescence almost completely disappears in the last image at t = 10 s, indicating that the majority of the fluorescent PS particles have completely entered the scaffold structure. In contrast, the static seeding process, in which the particle suspension is allowed to perfuse into the scaffold by pure diffusion, requires approximately 30 minutes (Figure 11(c)). Due to the small scaffold pores, of about 200 Î¼m, the capillary stress is extremely large. Gravity is insufficient to drive the suspension through the scaffold and the suspension permeates the scaffold by pure diffusion alone.

2.4 Spatial distribution of seeded particles 2.4.1 Fluorescent microspheres

The particle seeding efficiencies only provide information about the number of particles that enter the scaffold. By slicing the scaffold into several 1- mm sections, as shown in Figure 2(b), it is possible to determine the spatial distribution of fluorescent particles within the scaffold and hence evaluate the uniformity of the particle seeding process. Cross-sectional images obtained from fluorescence microscopy of the successive slices of the scaffold are shown in Figure 12. Images in the left column show the distribution of fluorescent particles in slices a to f when the particle seeding process is driven by SAW actuation. The corresponding particle distribution in each slice, obtained via pixel intensity analysis of the fluorescent microscopic images in Figure 12, is shown in Figure 13. This is compared to the particle distribution in each slice using the static seeding method, shown in the right column of Figure 12. In addition to rapid and efficient seeding, using SAW actuation to drive the particle suspension into the scaffold gives both greater particle penetration into the scaffold and a more uniform particle distribution. The particles are present in relative abundance in the last slice, i.e. slice f, of the scaffold compared to the corresponding slice with the

static seeding method (Figure 12). In the static method, most of the particles remained in the surface layer due to the inability of gravitational forces to overcome the large capillary pressure drop over the entire scaffold length. The penetration depth in this case was roughly 20% of the entire scaffold length; the particle's fluorescent intensity can be seen to drop drastically in slices c to f. 2.4.2 Mouse bone cells

Frozen cut sections of scaffolds which had been seeded with mouse bone cells under optimised SAW actuation conditions (~5 x 10^6 cells/ml, SAW actuation for 10 seconds at 20 MHzM dBm), or allowed to seed passively for 30 minutes, showed that a number of cells were driven into the PCL matrix by SAW, whereas no cells entered the scaffold by passive diffusion within the time-frame observed (Figures 14a and 14b). Cells within the SAW-driven PCL scaffold are clearly visible with both H&E staining (Figure 14a) and under fluorescence excitation of red fluorescent mouse bone cells (Figure 14b). 2.4.3 Yeast cells

Methylene violet staining of the surface of a scaffold, which had been seeded with yeast cells by SAW actuation or passive loading, showed that SAW- driven cells were present and distributed uniformly over the surface of the scaffold (Figure 15a), whereas cells seeded by the static method were found to be in clusters (indicated by arrows in 15b) distributed non-uniformly on the surface of the scaffold (Figure 15b-d). 3. CONCLUSIONS

The present invention provides a promising low-cost and miniaturizable alternative for driving a particle suspension onto, or into, a scaffold matrix through SAW actuation. Cells seeded onto the surface of a scaffold matrix by SAW actuation appear to adhere more strongly to the matrix during later applications. The SAW method has applications in, for example, the seeding of cells onto an Alloderm Â® collagen matrix for use in the formation of a wound patch or skin substitute, or the creation of a cellular patch which could be placed on the surface of a defective organ. The process is rapid; a particle suspension can be driven into, or onto, a scaffold in about 10 seconds, much quicker than if the seeding occurred by pure diffusion alone or by other techniques proposed to date. In addition to rapid seeding, a higher seeding efficiency was also achieved in which

higher particle concentrations were observed to perfuse deeper into the scaffold using SAW. Moreover, the particle can be distributed in the scaffold more uniformly than when delivered by the static method. The proposed SAW-driven method therefore holds promise for improving cell seeding for various applications in tissue and orthopaedic engineering.

It will be appreciated that the present invention described herein is not to be limited to specific examples or features disclosed.

For example, persons skilled in the art would appreciate that the SAW device itself may be tailored to seed scaffolds in different ways. In the case of seeding a porous 3-dimensional scaffold matrix, SAW actuation may be employed such that a proportion of the particles in a particle suspension are driven into the matrix and a proportion of the particles are driven onto the surface of the matrix. Further, a particle suspension may be driven into, and/or onto, a scaffold from the side of, or from below, the scaffold. The present invention provides a particle seeding method which is simple to perform, achieves good particle penetration into a 3-dimensional scaffold, or good distribution and adherence to a 2-dimensional scaffold, in a very short period of time, and

thus could be therapeutically applied at the point of patient contact. Moreover, the SAW apparatus may be scaled down such that seeding of a scaffold matrix could be achieved in a hand-held device.

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CLAIMS:

1. A method of driving a particle suspension into, onto, or both onto and into, a scaffold matrix by surface acoustic wave (SAW) actuation.

2. The method of claim 1 , including the steps of: a) placing a scaffold matrix onto the surface of a SAW generating actuator; b) depositing a particle suspension onto the surface adjacent to, or in contact with, the matrix; c) generating a surface acoustic wave to thereby drive the suspension onto, or into, the matrix.

3. The method of claim 1 , wherein the matrix is a 3-dimentional matrix or a planar 2-dimentional matrix.

4. The method of claim 1 , wherein the method includes the steps of:

(a) placing a two-dimensional scaffold matrix onto the surface of a SAW generating actuator;

(b) depositing a particle suspension onto the surface of the matrix;

(c) generating a surface acoustic wave to thereby drive the suspension onto the matrix.

5. The method of claim 1 , wherein the matrix is a porous 3-dimensional matrix and the particle suspension is driven into the matrix by the surface acoustic wave.

6. The method of claim 1 , wherein the particle is selected from the group including any one or more of a mammalian cell, a non-mammalian cell, cell growth and/or differentiation factors, or extracellular matrix molecules.

7. The method of claim 1 , wherein the scaffold matrix is a biodegradable and/or biocompatible polymeric matrix.

8. The method of claim 2 further comprising the steps of:

a) placing a scaffold matrix onto a piezoelectric substrate upon which has been fabricated an interdigital transducer (IDT); b) depositing a particle suspension onto the surface of the substrate

between the IDT and the scaffold, and c) applying a radio frequency (RF) signal to the IDT to produce SAW radiation, which has the effect of driving the particle suspension onto, or into, the scaffold matrix.

9. The method of any one of claims 1 to 8, wherein the substrate is selected from the group including, lithium niobate (LiNbOa), barium titanate, lead zirconium titanate, zinc oxide, aluminium nitride, lithium tantalate, and quartz.

10. Use of SAW radiation to seed a three-dimensional scaffold matrix or a planar 2-dimentional matrix with a particle suspension.

11. The use of claim 10, wherein the particle is selected from the group including any one or more of a mammalian cell, a non-mammalian cell, cell growth and/or differentiation factors, and extracellular matrix molecules.

12. The use of claim 10 or 11, wherein the scaffold matrix is a biodegradable and/or biocompatible polymeric matrix.

13. A particle seeding apparatus for seeding a scaffold matrix, including a piezoelectric substrate upon which has been fabricated an interdigital transducer (IDT) which generates SAW radiation, upon excitation by an RF signal, to drive a particle suspension onto, or into, a scaffold matrix.

14. The apparatus according to claim 13, wherein the piezoelectric substrate is selected from the group including, lithium niobate ($LiNbO_3$), barium titanate, lead zirconium titanate, zinc oxide, aluminium nitride, lithium tantalate, and quartz.

15. The apparatus according to claim 13 or 14, wherein the substrate has a titanium layer and an aluminium layer sputter-deposited thereon.

16. The apparatus according to any one of claims 13 to 15, wherein the IDT is an alternating finger IDT.

17. The apparatus according to claim 16, substantially as hereinbefore described with reference to the examples.

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(54) Title: A SCAFFOLD SEEDING METHOD

(57) Abstract: The present invention provides methods for rapidly and efficiently seeding particles into a 3-dimensional scaffold, or onto a 2-dimentional scaffold. Cells, for example, may be efficiently transferred into, or onto, a scaffold in as little as 10 seconds using surface acoustic wave (SAW) actuation.

A SCAFFOLD SEEDING METHOD

FIELD OF THE INVENTION

The present invention relates to methods of seeding particles into, or onto the surface of, scaffolds. In particular, the invention relates to the seeding of particles, preferably cells, by surface acoustic wave (SAW) actuation.

BACKGROUND OF THE INVENTION

The present invention is to be understood in light of what has previously been done in the field. However, the following discussion is not an acknowledgement or an admission that any of the material referred to was published, used or part of the common general knowledge in Australia as at the priority date of the application.

Tissue and organ transplantation is now an accepted and widely used therapy for the treatment of damaged or defective tissues and organs. Nevertheless, the transplant surgery process is extremely costly and can involve high risks due to possible complications. Long wait times are also common due to the perennial shortage of suitable donor tissues and organs. Tissue engineering, in which a patient's own cells can be grown, either within biodegradable and biocompatible three-dimensional scaffold matrices or on the surface of suitable substrate materials, and subsequently implanted *in vivo* to replicate the function of replacement tissues, or perhaps entire organs, is a promising alternative which could potentially alleviate inadequate donor tissue and organ supplies.

In order for *in vitro* cell cultures to occupy and grow within an extra-cellular matrix, the desired cells have first to be seeded into the scaffold while at the same time limiting any denaturing or lysing that could potentially render the cells ineffective (Soletti L. et al., Biomaterials 2006; 27: 4863-4870). Achieving homogeneity in the cultured tissues is also clearly dependent on the uniformity of the cell distribution within the scaffolds and the efficiency of the seeding process. Any significant reduction in the total time required for cell seeding could also lead to substantial savings in cost. Further, an improvement in cell viability may occur since long seeding periods can result in adverse effects in which cells might expire or age beyond their useful state, which, in turn, affects the cells' adhesion, proliferation, and differentiation processes (Wendt D. et al., Biotechnol. Bioeng. 2003; 84: 205-214).

A further problem faced by those attempting to establish a viable population of cells within a scaffold is that when nutrients, growth and differentiation factors, and/or extracellular matrix molecules are provided in the culture medium, passive diffusion alone results in a decreasing concentration of the nutrients and/or extracellular matrix molecules from the surface to the innermost region of the scaffold. As it is important to maintain the viability and phenotype of the cells within the scaffold, it is highly desirable to develop a method to supply the inner cells with higher concentrations of the required factors.

In relation to seeding into 3-dimensional polymeric scaffold materials, the typical hydrophobicity and small pore sizes (typically 10 – 150 μm) of the material results in exceptionally slow ingress of, for example, a cell suspension into the scaffold in the absence of any external driving forces due to the large capillary resistance encountered. Under gravitational forces alone, perfusion of a cell suspension into the scaffold typically takes hours to days (Li and Chang, Biomaterials 2004; 25: 5473-5480: Li and Chang, J. Mater. Sci: Mater. Med. 2004;15: 1089-1095). Nevertheless, this static seeding method has conventionally been used in tissue culturing due to its simplicity. The large capillary resistance also poses considerable difficulty in delivering cells deep within the scaffold structure in the absence of external driving forces. For example, studies have reported that new bone tissue forms easily at the surface of scaffolds but is hard to regenerate in the centre of the scaffolds due to the superficial penetration of the cell suspension with conventional seeding methods resulting in a large proportion of the cells being deposited merely on the surface of the scaffold.

As described supra, passive seeding of a cell suspension into a scaffold can take hours to days, which can result in cell stress due to the correspondingly poor diffusion of nutrients, growth factors and extracellular matrix molecules. It is highly desirable to be able to either include such beneficial factors with the cells at the time of seeding, or to later provide the cells which occupy the inner regions of the scaffold with a supply of such beneficial factors.

In relation to seeding onto the surface of an essentially 2-dimensional polymeric scaffold material the conventional method has been to apply, for

example, a cell suspension directly onto the surface and allow gravitational forces to cause the cells to gradually come into contact with the scaffold and adhere. A problem with this method is that the process of adhering the cells to the substrate by passive diffusion can typically take up to 24 hours. Moreover, when certain cell types are seeded by this method they adhere poorly to the scaffold material, which can lead to the loss of significant numbers of cells during subsequent culturing and/or handling and a non-uniform distribution.

Several improved methods are currently being explored for seeding cells within scaffolds and achieving a greater homogeneous cell distribution within. These methods typically involve affixing a scaffold in an agitated vessel filled with the cell suspension. The cells then perfuse into the scaffold under a velocity gradient arising between the advected cells and the stationary scaffold. However, low seeding efficiencies have been reported in addition to long seeding times (hours to weeks) to finish a cell seeding process, and most of the cells aggregate on the surface of the scaffold which results in a non-uniform cell seeding (Vunjak-Novakovic G. et al., Biotechnol. Prog. 1998; 14: 193-202; Zhao F. and Ma T., Biotechnol. Bioeng. 2005; 91: 482-493; McFetridge PS. et al., J. Biomed. Mater. Res. 2004; 70: 224-234; Kitagawa T., et al., Biotechnol. Bioeng. 2005; 93: 947-957; Burg KJL. et al., J. Biomed. Mater. Res. 2000; 51: 642-649; Kim BS., et al., Biotechnol. Bioeng. 1998; 57: 46-54).

Soletti *et al.* (Biomaterials 2006; 27: 4863-4870) have proposed a technique for cell seeding in which cells within a suspension flowing through the annulus of a tubular scaffold perfuse through the scaffold under vacuum. The scaffold is concurrently rotated in order to achieve greater distribution uniformity. Although significantly reduced seeding times (several minutes) and improved distribution have been obtained, the use of vacuum and syringe pumps necessarily involves cumbersome airtight equipment and has an unknown effect on sensitive cells being infused with this equipment.

The present invention provides methods for rapidly and efficiently seeding particles into a 3-dimensional scaffold, or onto a 2-dimentional scaffold. Cells, for example, may be efficiently transferred into, or onto, a scaffold in as little as 10 seconds using SAW actuation. Moreover, cells transferred to the surface of a 2-

dimentional scaffold matrix adhere more strongly to the scaffold than cells allowed to passively adhere under normal gravitational force.

SUMMARY OF THE INVENTION

This invention relates to a new and improved method of moving particles into, or onto the surface of, a scaffold matrix. Most preferably, it relates to a method of seeding cells into, or onto, a matrix suitable for tissue engineering applications.

It is therefore an object of the invention to provide a method of seeding particles into, or onto, a matrix which is improved over the prior art.

According to one aspect of the invention, there is provided a method of driving a particle suspension into, or onto, a scaffold matrix by surface acoustic wave (SAW) actuation.

In a preferred embodiment of the invention, the method includes the steps of:

(a) placing a scaffold matrix onto the surface of a SAW generating actuator;

(b) depositing a particle suspension onto the surface adjacent to, or in contact with, the matrix;

(c) generating a surface acoustic wave to thereby drive the suspension onto, or into, the matrix.

In a preferred embodiment of the invention, the matrix is either a 3-dimensional porous matrix or a planar, 2-dimensional matrix.

In a preferred embodiment of the invention, the particle is selected from the group including any one or more of a mammalian cell, a non-mammalian cell, cell growth and/or differentiation factors, and extracellular matrix molecules.

In another preferred embodiment of the invention, the scaffold matrix is a biodegradable and/or biocompatible polymeric matrix.

In an alternate preferred embodiment of the invention, there is provided a method of driving a particle suspension onto, or into, a scaffold matrix by surface acoustic wave (SAW) generation, including the steps of:

(a) placing a scaffold matrix onto a piezoelectric substrate upon which has been fabricated an interdigital transducer (IDT);

(b) depositing a particle suspension onto the surface of the substrate between the IDT and the scaffold, and

(c) applying a radio frequency (RF) signal to the IDT to produce SAW radiation, which has the effect of driving the particle suspension onto, or into, the scaffold matrix.

Preferably, the matrix is a porous 3-dimensional matrix and the particle suspension is driven into the matrix by the surface acoustic wave radiation.

Preferably, the substrate is lithium niobate, $LiNbO_3$, though lead zirconate titanate (PZT) and closely related doped PZT materials and other piezoelectric materials with large electromechanical coupling coefficients, such as barium titanate, lead zirconium titanate, zinc oxide, aluminium nitride, lithium tantalate or quartz, would be suitable for this application.

According to another aspect of the invention, there is provided a method of driving a particle suspension onto a two-dimensional planar scaffold matrix by surface acoustic wave (SAW) generation.

In a preferred embodiment of the invention, the method includes the steps of:

(a) placing a two-dimensional scaffold matrix onto the surface of a SAW generating actuator;

(b) depositing a particle suspension onto the surface of the matrix;

(c) generating a surface acoustic wave to thereby drive the suspension onto the matrix.

In another aspect of the invention, there is provided the use of SAW radiation to seed a porous three-dimensional scaffold matrix with a particle suspension.

In another preferred embodiment of the invention, the porous three-dimensional scaffold matrix is a biodegradable and/or biocompatible polymeric matrix.

In another aspect of the invention, there is provided a particle seeding apparatus for seeding a scaffold matrix, including a piezoelectric substrate upon which has been fabricated an interdigital transducer (IDT) which generates SAW radiation, upon excitation by an RF signal, to drive a particle suspension onto, or into, said scaffold matrix.

The IDT may be of any suitable type, although preferred IDTs include alternating finger type transducers and single-phase uni-directional transducers (SPUDT).

Preferably, the piezoelectric substrate is $LiNbO_3$.

In a preferred embodiment of the invention, the substrate has a titanium layer and an aluminium layer sputter-deposited thereon.

In another preferred embodiment of the invention, the IDT is an alternating finger IDT. More preferably, the IDT is an alternating finger IDT with 60 straight finger pairs and an aperture of 8 mm, and the strip and gap widths are 49 μm, thus giving rise to a wavelength of λ = 196 μm for an operating frequency of 19.35 MHz.

In another preferred embodiment of the invention, the particle is a mammalian cell or a non-mammalian cell.

Recently, surface acoustic wave (SAW) technology has been developed for use in biotechnology, such as for biofluidic mixing, particle trapping, and as biosensors in microfluidic devices. A SAW is like an earthquake wave propagating along the surface of a piezoelectric substrate with an amplitude of a few tens of nanometres (Figure 1(a)). The wave is created by an interdigital transducer electrode (IDT) wherein the wavelength is defined by the width of each individual finger along the propagation direction of the wave and the gap between them. The amplitude of the electrical signal determines the amplitude of the wave. Though there are many forms of SAW, the present invention makes use of the Rayleigh wave, an axial-surface-normal polarized SAW (Morita T., et al., IEEE Trans. Ultrason. Ferroelec. Freq. Contr. 1999; 46:929-934). It has been shown that SAWs strongly interact with small amounts of liquid on the surface of a piezoelectric substrate by inducing bulk liquid recirculation through a process known as acoustic streaming (Figure 1(b)) (Wixforth A., Superlattice Microst. 2004; 33: 389-396). A SAW entering the droplet is diffracted at the Rayleigh angle θR into the fluid, where it generates a longitudinal pressure wave. For an infinite half space, this θR is given by the ratio of the sound velocities in the substrate and in the fluid, respectively. If the intensity of the acoustic radiation component into the fluid is sufficiently high, and the liquid consists of a free droplet whose contact line is not pinned, the SAW can induce the droplet to move in the

direction of the SAW (Wixforth A., et al., Anal. Bioanal. Chem. 2004; 379:982-991; Guttenberg A., et al., P. Phys Rev E Stat Nonlin Soft Matter Phys 2004; 70: 056311). The technology is therefore a convenient mechanism for transporting a free droplet on a microfluidic device while requiring no mechanically moving components other than small deformations of the substrate itself.

According to the present invention, SAW actuation can be exploited as a method for quickly driving particles into, or onto, a scaffold, providing a means for rapid, uniformly distributed, and efficient cell seeding in various applications, including tissue engineering.

DESCRIPTION OF THE DRAWINGS

Figure 1: (a) Drawing illustrating the propagation of a surface acoustic wave (SAW) through a lithium niobate substrate. (b) Drawing illustrating acoustic streaming generated within a small droplet by SAW.

Figure 2: (a) Drawing of the experimental setup for cell seeding using SAW actuation. (b) Drawing of the experimental setup for gravity-driven cell seeding. (c) Representation of six 1-millimetre thick sections cut through the scaffold to evaluate the distribution of the cells within it.

Figure 3: Schematic of SAW method and processing for histological determination of depth of ingress of mouse bone cells.

Figure 4: Graph of the effect of input RF power on the velocity of the droplet suspension prior to its contact with and entry into the scaffold, and the particle seeding efficiency within the scaffold.

Figure 5: (a) Cell seeding efficiency and distribution in each section of scaffold seeded by SAW-driven or static methods. (b) Viable cell seeding efficiency in each section. (c) Total cell seeding efficiency with and without (static) SAW actuation.

Figure 6: (a) SEM images of yeast cell morphology within scaffold seeded by SAW actuation. (b) Proliferation rates of yeast cells after SAW treatment.

Figure 7: Average Viability of mouse bone cells post-SAW treatment at different powers and frequencies.

Figure 8: Average Viability of mouse bone cells subjected to 20 Mhz/-1 dBm in SAW for different times.

Figure 9: (a) Average viability of mouse bone cells cells at different suspension densities subjected to SAW technology. (b) Difference in viability between treated and untreated samples at different cell densities.

Figure 10: Average cell proliferation of SAW-treated and untreated mouse bone cells as function of fluorescence via Alamar Blue uptake.

Figure 11: (a) Geometry of scaffold and droplet under bright illumination. (b) Successive images acquired at 60 frames/s show a side view of SAW-driven perfusion process under fluoroscope in roughly 10 s. (c) A plan view of the static method in which the drop perfuses through the scaffold by pure diffusion alone, taking approximately 30 min.

Figure 12: Scaffold cross-sectional slices at various positions given in Fig. 2(b).

Figure 13: Particle distribution in each slice following seeding after ten seconds of SAW actuation, or 30 minutes of static diffusion.

Figure 14a: Haematoxylin & Eosin staining of sections cut of PCL seeded with ~100,000 mouse bone cells.

Figure 14b: Red fluorescent images of sections cut of PCL seeded with ~100,000 mouse bone cells.

Figure 15: Methylene violet staining of yeast cells on the surface of seeded scaffolds following SAW actuation or static (passive) seeding.

DETAILED DESCRIPTION OF THE INVENTION

Previous attempts at seeding scaffold matrices, including both porous 3-dimensional and 2-dimensional planar matrices, have generally entailed methods which use no external driving forces, thus requiring long contact times between the particles to be seeded and the matrix and, in the case of cells, result in poor penetration into the matrix or poor adherence to the surface of the matrix, respectively. More recently developed methods for seeding 3-dimensional matrices rely on affixing a scaffold in an agitated vessel filled with the cell suspension, causing cells to perfuse under a velocity gradient, or which rely on vacuum pressure to cause cells to perfuse a matrix also have problems. The former method results in low seeding efficiency and long seeding times, whereas the latter method reduces seeding times but requires substantial airtight

equipment. In regard to the seeding of 2-dimentional matrices the method generally applied is that of passive diffusion, which requires sometimes several hours for the cells to settle onto the matrix. Moreover, the strength of adhesion to the matrix is often poor and the distribution of cells on the matrix is often uneven. The present invention provides a method for seeding scaffold matrices which is relatively fast and results in matrices with improved uniformity of seeding.

According to one aspect of the invention, there is provided a method of driving a particle suspension into a porous three-dimensional scaffold matrix by surface acoustic wave (SAW) generation, including the steps of:

(a) placing a porous three-dimensional scaffold matrix onto the surface of a SAW generating actuator;

(b) depositing a particle suspension onto the surface adjacent to, or in contact with, the matrix;

(c) generating a surface acoustic wave to thereby drive the suspension into the matrix.

According to another aspect of the invention, there is provided a method of driving a particle suspension onto a two-dimensional planar scaffold matrix by surface acoustic wave (SAW) generation, wherein the method includes the steps of:

(a) placing a two-dimensional scaffold matrix onto the surface of a SAW generating actuator;

(b) depositing a particle suspension onto the surface of the matrix;

(c) generating a surface acoustic wave to thereby drive the suspension onto the matrix.

It would be understood that, in the context of the invention, the particle may be selected from a variety of biological material. For example, the particle may be a prokaryotic or eukaryotic cell, a mammalian cell, a virus particle, or a nucleic acid such as a plasmid vector, a cell growth or differentiation factor, an extracellular matrix molecule, a polymer particle such as polycaprolactone, a metal particle such as gold or tantalum, or a ceramic particle such as hydroxyapatite or alumina. The cell type may be chosen for its suitability for tissue engineering applications. A cell such as a stem cell, in particular a human or animal stem cell, may be most suitable for clinical applications.

In a preferred embodiment of the invention, the particle suspension is a mammalian cell suspension or a non-mammalian cell suspension.

The scaffold matrix may be made from any suitable material for the application. If the matrix is intended for tissue engineering applications, the material may be polymeric, such as polycaprolactone (PCL), bioresorbable polyurethane matrix enriched with laminin, γ-poly glutamic acid (PGA)/chitosan composite matrices, poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (PHBV), hydrogel composed of collagen type I and matrigel, or synthesized or natural inorganic material matrices from materials such as hydroxyapatite or demineralized cancellous bone, coral, bioactive glass, and related calcium and phosphate-containing materials for bone and hard tissue engineering, and natural soft tissue based materials such as Alloderm ®. Alloderm ® is a human-derived collagen matrix, available from BioHorizons Inc., Birmingham, Alabama, USA. Preferably, the matrix is formed from polycaprolactone.

In another preferred embodiment of the invention, the scaffold matrix is a biodegradable and/or biocompatible polymeric matrix.

In an alternate preferred embodiment of the invention, there is provided a method of driving a particle suspension into a porous three-dimensional scaffold matrix by surface acoustic wave (SAW) generation, including the steps of:

(a) placing a porous three-dimensional scaffold matrix onto a piezoelectric substrate upon which has been fabricated an interdigital transducer (IDT);

(b) depositing a particle suspension onto the surface of the substrate between the IDT and the scaffold, and

(c) applying a radio frequency (RF) signal to the IDT to produce SAW radiation, which has the effect of driving the particle suspension into the scaffold matrix.

In another aspect of the invention, there is provided the use of SAW radiation to seed a scaffold matrix with a particle suspension.

In another aspect of the invention, there is provided a particle seeding apparatus for seeding a scaffold matrix, including a piezoelectric substrate upon which has been fabricated an interdigital transducer (IDT) which generates SAW radiation, upon excitation by an RF signal, to drive a particle suspension onto, or into, said three-dimensional scaffold matrix.

Preferably the substrate is lithium niobate ($LiNbO_3$), although other types of piezoelectric material such as barium titanate, lead zirconium titanate, zinc oxide, aluminium nitride, lithium tantalate, and quartz may also be used.

The piezoelectric substrate may be elongate in shape having opposing ends, and at least one IDT electrode may be located at one end thereof.

In a preferred embodiment of the invention, the substrate has a 4 nm titanium layer and a 150 nm aluminium layer sputter-deposited thereon.

The IDT may be of any suitable type, preferably an alternating finger IDT or a single-phase unidirectional transducer (SPUDT).

In a preferred embodiment of the invention, the IDT is an alternating finger IDT.

In another preferred embodiment of the invention, the IDT is an alternating finger IDT with 60 straight finger pairs and an aperture of 8 mm, and the strip and gap widths are 49 μm, thus giving rise to a wavelength of λ = 196 μm for a SAW device operating at 19.35 MHz. In choosing other operating frequencies between 5 and 500 MHz, the wavelength and therefore the finger and gap widths are adjusted accordingly. Generally, the number of finger pairs increases in proportion to the operating frequency of the device. The configuration of the finger pairs may be altered to incorporate internal reflectors (SPUDT configuration, Campbell US Patent 6,462,698 and Campbell and Saw, 1987 IEEE TUFFC v34(3) pp357-) or focused with curved fingers to increase the efficiency of the device and concentrate the power transmission into the fluid.

The RF input power required to actuate a SAW to drive the particle suspension onto, or into, the scaffold matrix will vary depending on the particular characteristics of the particle and matrix. In the present invention, RF power in the order of 100 mW up to 860 mW has been sufficient to drive a particle suspension into a porous three-dimensional scaffold matrix. Preferably the RF power input is in the order of 100 mW to about 1 W, depending on the SAW frequency chosen for the device, which is preferably between 5 MHz and 500 MHz.

Method of seeding a 3-dimensional scaffold

In a preferred method of driving a particle suspension into a porous three-dimensional scaffold matrix by surface acoustic wave (SAW) generation, a porous

three-dimensional polycaprolactone (PCL) scaffold is placed onto an X-propagating lithium niobate ($LiNbO_3$) piezoelectric substrate, upon which has been fabricated an alternating finger interdigital transducer (IDT) coated with a 4-nm titanium layer and a 150-nm aluminium layer. A 20-µl particle suspension is deposited onto the surface of the substrate between the IDT and the scaffold, and a radio frequency (RF) signal (input power of 400 mW, resonance frequency of 19.35 MHz) is applied to the IDT for 10 seconds to produce SAW (Rayleigh wave) radiation, which has the effect of driving the particle suspension into the scaffold matrix.

EXAMPLES

1. MATERIALS AND METHODS

1.1 Scaffolds

1.1.1 3-Dimensional

Three-dimensional scaffolds were prepared using a conventional solvent casting-particulate leaching method (Li H. and Chang J., J. Mater. Sci.: Mater. Med. 2004; 15: 1089-1095) using polycaprolactone (PCL, MW = 65,000, MP = 65°C) (Sigma Chemical Co., USA). Briefly, PCL powders were dissolved in chloroform with a concentration of 10% (w/v). Sodium chloride (NaCl) particles sieved as porogens (100-150 µm) were then incorporated into the suspension (NaCl / polymer = 1:9) (w/w), and the dispersion was cast into a 60-mm Teflon mould. The samples were air-dried under a fume hood for 24 hours to allow the solvent to evaporate and were subsequently vacuum-dried at 60°C for 48 hours to remove any remaining solvent. The samples were then immersed in deionised water to leach out porogens in the resulting polymer/salt composites. The samples were finally vacuum-dried to obtain a set of sponge-like scaffolds with thicknesses of 2 mm. Before seeding, the scaffolds were carefully cut with a razor blade into 6 × 5 mm squares and were stored in a desiccator. The porosity of the scaffold was about 90% ± 1.5%.

1.1.2 2-Dimensional Sheet

Two-dimensional, or sheet-like, scaffolds such as the collagen matrix commercially available as Alloderm ® may be obtained from BioHorizons Inc.,

Birmingham, Alabama, USA, or produced according to conventional methods using suitable matrix material such as PCL or hydroxyapatite.

1.2 Fluorescent microspheres

An aqueous suspension of 5-µm green fluorescent polystyrene (PS) microspheres (Duke Scientific Corporation, USA) was used to facilitate observation of the seeding process and their spatial distribution in the scaffold. The excitation maxima wavelength for these particles is 468 nm (blue) and the emission maxima wavelength is 508 nm (green). These PS microparticles were obtained in the form of a 1% (w/v) aqueous suspension, containing 1.4×10^8 particles in 1 ml. Using deionised (DI) water, the original suspension was diluted to obtain a concentration of 1.4×10^6 particles/ml.

1.3 Yeast cells

Preliminary studies were performed on yeast cells to determine their ability to survive the SAW seeding process. Stock cultures of the yeast cells were maintained on standard agar consisting of 1% yeast extract, 0.5% neutralized bacteriological peptone, and 1% glucose solidified with 1.5% agar (w/v). All media were autoclaved immediately after preparation at 121°C and 15 psi for 15 min. Yeast cells were grown aerobically to the required cell density at room temperature.

For yeast cell seeding, a 40-µl cell suspension with a concentration of 1×10^8 cells/ml was slowly pipetted onto the surface of the SAW device between the IDT and the scaffold. The seeding end point was defined as when there was no suspension left on the surface of the SAW device, determined through visual inspection.

1.4 Human stem cells

Preliminary studies were performed on human stem cells to determine their ability to survive the SAW seeding process. Primary human bone cells were deposited onto the surface of a lithium niobate ($LiNbO_3$) piezoelectric substrate. The cells were subjected to low (400 mW) and high (860 mW) RF levels, or no treatment, for a period of 10 seconds and their viability determined after 2 days (Table 1).

1.5 Mouse primary bone cells

In order to further examine the effects of various SAW parameters on the viability of mammalian cells and their ability to enter a scaffold matrix, primary bone cells from mice were isolated using standard techniques.

1.5.1 Cell Isolation

Briefly, long bones or calvaria were isolated from 6-8 week old C57bl/6 mice. Long bones were flushed from marrow using normal saline and the bones were crushed/minced using bone crunchers into smaller particles (calvaria were minced as described). They were then subjected to serial collagenase digestion to isolate bone and stromal cell populations. Cells were collected at the end of each digest, collagenase activity stopped by the addition of 15% FBS and cells were strained through a cell strainer, centrifuged, washed and resuspended in alpha-MEM supplemented with 10-15% FBS and plated (P1) in tissue culture flask for 4 days to allow recovery of cells. At this stage, cells were removed from the tissue culture flask using trypsin/EDTA, collected, washed and centrifuged, resuspended in freeze medium (1x 10^6 cells /ml) and frozen under liquid nitrogen vapour until required.

1.5.2 Experimental cells

Frozen cells were thawed at 37°C and resuspended in alpha-MEM supplemented with 10% FBS, then plated and allowed to expand (P2). These cells were subsequently used for SAW experiments. These cells are able to form mineralised nodules in culture in the presence of Ascorbic acid and beta-glycerophosphate, indicative of their bone forming activity in vivo.

1.5.3 Mouse cell viability

1.5.3.1 Determination of optimal SAW power and frequency

For bone cell seeding, 10µl droplets with a concentration of 5x10^6 cells/ml were subjected to a SAW for 10 seconds at powers of -2 dBm, -1 dBm, 1 dBm or 2 dBm in combination with frequencies of 10 Mhz, 20 Mhz or 50 Mhz (in triplicate). Untreated controls were included for comparison.

A FACS Vantage DIVA (Becton Dickinson) was used to assess cell viability. Propidium iodide dye uptake is used to detect cells whose membranes are disrupted, thus providing an indication of total cell viability.

1.5.3.2 Determination of optimal time of SAW treatment

For bone cell seeding, 10µl droplets with a concentration of $5x10^6$ cells/ml were subjected to a SAW for 10, 30, 45 or 60 seconds, in triplicate at optimal power/frequency. Untreated controls were included for comparison. Viability was assessed as above.

1.5.3.3 Determination of optimal cell suspension density

Five different cell suspension seeding densities were tested for their effect on viability when subjected to SAW: 5×10^5 cells/ml, 1×10^6 cells/ml, 2×10^6 cells/ml, 4×10^6 cells/ml and 8×10^6 cells/ml, each in triplicate, at 20 MHz/-1 dBm. Untreated controls were included for comparison. Viability was assessed as above.

1.5.3.4 Assessment of cell proliferation post-SAW treatment

The ability of cells to proliferate after SAW treatment was assessed as a measure of whether SAW has an effect on this aspect of cell viability.

Cell suspensions at 5×10^3 cells/ml were subjected to SAW treatment for 10 seconds at 20 MHz/-1 dBm, in triplicate, and compared to untreated controls. Cells were then seeded into a 48-well plate, followed by the addition of Alamar Blue (a reagent taken up by cells, leading to fluorescence which increases with increasing cell number). Proliferation was assessed by measurement of the total fluorescence in each well, using a plate reader, at various time points.

1.6 Seeding techniques

1.6.1 Particle seeding using SAW radiation

A method for direct pumping of a fluorescent particle suspension through the pores of 3D scaffolds using SAW radiation is described. A SAW is excited by the application of a radio frequency (RF) signal to an interdigital transducer (IDT) fabricated onto a piezoelectric substrate. The wavelength of the excited SAW, and therefore its resonance frequency, is defined by the geometry of the IDT. A 0.5-mm thick substrate, 127.68°YX cut, X-propagating lithium niobate ($LiNbO_3$ or LN, Roditi, London UK) single crystal was used as the SAW substrate in this experiment to generate a Rayleigh wave upon excitation of the IDT by an RF signal. A 4-nm titanium layer and a 150-nm aluminium layer were sputter-deposited upon this substrate. A standard alternating finger IDT with 60 straight finger pairs and an aperture of 8 mm was fabricated using photolithography; the

strip and gap widths were 49 μm, thus giving rise to a wavelength of λ = 196 μm. The IDT was driven with a sinusoidal signal at the resonance frequency f_0, defined as $f_0 = c/\lambda$. The SAW velocity c = 3788 m/s, and hence $f_0 \approx 19.35$ MHz. Such an IDT efficiently converts the applied RF signal into an acoustic wave, which in this case travels from the IDT in both directions along the substrate surface and perpendicular to the direction of the strip electrodes with low divergence.

Figure 2(a) shows a schematic of the experimental setup as seen from above. Situated on the left side is the input IDT. The wires in contact with the bus bars of the IDT are connected to the RF power source. A 20-μl drop containing the fluorescent PS suspension with a concentration of 1.4×10^6 particles/ml is then deposited onto the surface of the SAW device between the IDT and the scaffold. To observe the fluorescent PS particles, a high-speed video camera (Olympus i-speed, Tokyo, Japan) was used through a reflected fluorescent microscope system (Olympus BXFM, Tokyo, Japan) to monitor the dynamics of the seeding process. Wideband blue light with an intensity maximum at 468 nm was used to excite the particle fluorescence.

1.6.2 Static seeding

In order to benchmark the efficiency of the SAW-driven cell seeding process, the results from Section 1.6.1 above were compared with a static seeding process in which the cell suspension is allowed to perfuse through the scaffold solely by pure diffusion. First, PCL scaffolds were placed onto a SAW substrate. A 20-μl drop of fluorescent particulate suspension with a concentration of 1.4×10^6 particles/ml was pipetted onto the top surface of the scaffold. The same observation system was used to monitor the seeding process. The approximate time required for the scaffold to completely absorb the droplet suspension, i.e., no liquid apparent above the surface, was determined through visual inspection using the microscope system.

1.7 Image analysis

After the seeding process, the scaffolds were cut perpendicularly to the long axis into six 1-mm thick slices (Figure 2(b)). The cross-section of each slice (*a-f*) was then observed under the reflected fluorescent microscope system. Slice *a* is the side where the cell suspension first enters in the two methods described

above. The fluorescent pixel intensities of each image were acquired using Photoshop CS (Adobe Systems Software Ltd, Ireland); each image's intensity was normalised against the intensity of the image of the initially-wetted surface of the scaffold.

1.8 Particle seeding efficiency

A digital frame-by-frame analysis of the high-speed video was employed to determine the area of the image under which the fluorescent signal was evident. The velocity was computed by dividing the distance that the drop suspension travelled prior to entry into the scaffold with the time taken. Preliminary investigations were aimed at determining the influence of the input RF power on the suspension's velocity prior to entry into the scaffold and the particle seeding efficiency of the scaffolds. The particle seeding efficiency was evaluated according to the following method: the SAW radiation was terminated when the entire suspension entered the scaffold completely, i.e., no liquid external to the scaffold remained on the SAW surface. None of the liquid was permitted to circumvent the scaffold or to atomize. Generally, the power required to atomize a droplet is higher than that used in this study. The SAW substrate was then rinsed using DI water and the solution was collected. The number of fluorescent particles in this solution, N_1, was counted by using a hemocytometer (Hausser Scientific, Horsham, PA, Sigma, USA). There were 2.8×10^4 fluorescent particles in the 20-µl droplet according to the original fluorescent particle concentration (1.4×10^6 particles/ml). The "*particle seeding efficiency*" of the scaffold is then given by

$$\textit{Particle seeding efficiency } \% = 100\times (2.8\times10^4 - N_1) / 2.8\times10^4$$

1.9 Yeast cell seeding efficiency

The cell seeding efficiency, defined as the percentage of initially loaded cells to the scaffolds, was determined as follows. Samples were allowed to dry at room temperature overnight. The dehydrated seeded scaffolds were cut into six 1 mm thick slices and each slice was digested in 1 mL proteinase K solution for 18 h at 56°C. Proteinase K (Sigma Chemical Co., USA) was prepared at a concentration of 1 mg/mL in 50 mM Tris.[17] To terminate the reaction, phenylmethylsulfonyl fluoride (PMSF, Sigma Chemical Co., USA), an inhibitor of

proteinase K, was used. Aliquots of the digest for each section were analyzed using a hemocytometer to obtain the cell concentration at each section (C_a~C_f). The concentration of original seeding suspension was defined as C_0 (is 1×10^8 cells/ml). The "cell seeding efficiency of each section" was therefore given by

$$\%\ cell\ seeding\ efficiency\ in\ each\ section = 100*(C_x/C_0) \quad (2)$$

Where x represent section position (a, b, c, d, e, f). Total cell seeding efficiency can be obtained by summing the cell seeding efficiency in each section. In addition, the cell seeding efficiency in each section can also describe the cell distribution within the whole scaffold. The following section will describe how to quantify the cell distribution in each slice.

1.10 Yeast cell viability

Viability of living yeast cells after seeding was qualitatively confirmed using physiological staining with methylene violet 3 RAX and scanning electron microscopy (SEM). After seeding, a 0.5 mm thick cross-sectional slice was taken from the middle of the each slice immediately and stained with methylene violet 3 RAX solution according to standard procedures [Smart, K.A., Chambers, K.M., Lambert, I., Jenkins, C. and Smart, CA: J. Am. Soc. Brew. Chem, 57 (1), pp 18 23, 1999]. Images were segmented and violet cells were counted under a microscope to derive the fraction of nonviable cells. The latter parameter was then used in combination with the "cell seeding efficiency of each section" to estimate the "viable cell seeding efficiency in each section", i.e., the percentage of initially seeded cells that attached to the slice and were alive at the time of the assessment. For SEM observation, the dehydrated samples were mounted on stubs and coated in vacuum with gold before being observed by SEM (Model JEOL-6300F, Japan) at an accelerating voltage of 10 kV to observe the cells.

For quantitative assessment of yeast cells' viability after being treated by SAW, a yeast suspension with the identical in volume to the aliquot seeded into the scaffold was pipetted on the surface of SAW device and subjected to the SAW radiation at the input RF power applied to driven cells within scaffold. After being treated, aliquots of the yeast suspension were collected and diluted by adding culture media. The cell concentration of this suspension was counted subsequently using a haemocytometer and was defined as the cell concentration

at t = 0 day. Then, the yeast cells were cultured for an additional 14 days and the concentration of the yeast cells in the media was determined at 2, 5, 7 and 14 days. The yeast cells treated by SAW were considered to be viable if they could proliferate in prolonged culture conditions.

1.11 Mouse bone cell seeding using optimised SAW exposure

The optimised cell viability parameters determined under 1.5 above were used in a SAW-actuated cell seeding of 3-dimensional hydroxyapatite or polycaprolactone scaffolds. Either a 10µl or 50µl droplet of cells at 5 x 10^3 cells/ml was placed in contact with the scaffold matrix and subjected to SAW treatment for 10 seconds at 20 MHz/-1 dBm. The cells were from a transgenic red fluorescent mouse, thus the cells glow red when subjected to appropriate light energy in vitro. Untreated controls (passive seeding) were included for comparison.

Post-treated matrices were embedded in frozen embedding medium, followed by sections (slices of matrix) being cut onto slides, according to the scheme provided in Figure 3. Sections were stained using haematoxylin/eosin, which stains for cellular material (contrasting staining for cells vs. tissue/matrix). The degree of egress of cells into the matrix was determined by viewing the H&E-stained sections or fluorescence emitted by cells in the sections.

1.12 Statistical analysis

All values are presented as mean ± standard deviation. Differences among experimental groups were assessed using a two-tailed Student's *t*-test and considered statistically significant if $p < 0.05$.

2. RESULTS

2.1 Effect of input RF power on the particle seeding efficiency

Figure 4 describes the relationship between the velocity at which the particle suspension enters the scaffold and the particle seeding efficiency within the scaffold. Below approximately 325 mW, the particle seeding efficiency is low (<50%) as there is insufficient fluid velocity to drive the suspension into the scaffold. The majority of particles were found to aggregate on the SAW substrate. Above this input power, however, there was a marked increase in the particle seeding efficiency, approximately 50% to 85% despite only a modest increase in

the fluid droplet velocity to 1 mm/s. Above approximately 400 mW, however, the particle seeding appeared to plateau despite a sharp increase in the droplet velocity as the input RF power was increased. The highest seeding efficiency was about 92% at an RF power of 570 mW. Above this power, the intense SAW radiation resulted in the atomization of the droplet containing the suspension even before it reached the scaffold. This thus imposes an upper limit on the input RF power. Since there is no significant increase in the seeding efficiency beyond 400 mW, the subsequent experiments were conducted using this input power level.

The plateau in the cell seeding efficiency above 400 mW, on the other hand, is associated with the elastic-plastic transition for the scaffold, which demonstrates the typical behaviour of porous polymer materials undergoing deformation. For a typical polycaprolactone porous scaffold structure, the yield stress (transition from elastic to plastic behaviour) occurs at about 3 MPa, while its stiffness or *Young's modulus* is about 42 MPa.

2.2 Effect of input RF power on cell survival

2.2.1 Yeast cells

Recent studies have shown that a cell's viability will not be damaged when it is treated by ultrasonic standing wave fields (Radel S. et al., Ultrasonics 2000; 38: 633-637; Cousins SM. et al., Ultrasound Med Biol 2000; 26: 881-888). Studies on freely suspended yeast acoustically treated in ultrasonic standing wave devices for longer periods (up to 2 h) suggest that the viability of the yeast cells was not significantly affected (Radel et al., supra). Similarly, no disruption of red blood cells was noted when using a tubular acoustic resonator optimised for diagnostic purposes and subsequently used as an alternative method for concentrating red blood cells. The present inventors have confirmed that yeast are resistant to SAW-induced acoustic damage. Figure 5(a) shows the cell seeding efficiency in each slice cut from the whole scaffold. By summing the cell seeding efficiency in each slice, total cell seeding efficiency in whole scaffold can be obtained (Figure 5(b), grey bars). Cell seeding efficiency in each section of scaffold seeded by SAW actuation was relative average (about 20%) and the total cell seeding efficiency for these scaffolds was 87%±5%. A non-uniform cell seeding was observed in scaffolds seeded by the static method and 68% cells

were seeded on slice *a* while almost no cells were delivered in slices *d-f* although the total cell seeding efficiencies for these scaffolds was 83%±5%, which was not statistically different to that seeded by SAW actuation.

Cell viability within the scaffolds was also quantified to differentiate between the contribution of living and dead cells to the seeding efficiency. Image analysis of each stained cross-section revealed that more cells remained viable in the scaffold seeded by SAW actuation than by the static method (Figure 5(b)). The percentage of viable cells in the whole scaffold was obtained by summing the data in each section, with the result that 80%±8% of cells within the scaffold seeded by SAW actuation were viable, whereas survival was significantly lower in scaffolds seeded statically (55%±10%, p = 0.025). Using the viability data in conjunction with the previously determined seeding efficiencies, we calculated that the percentage of the initially loaded cells that stayed and remained viable in the whole scaffold (i. e., "viable cell seeding efficiency") was significantly higher by SAW actuation than by the static method (1.5-times higher, P <0.0005) (Figure 5(c), black bars).

The viability of yeast cells after SAW-driven seeding has been verified by methylene staining and the percentage of viable cells has been calculated (Figure 5(b)). In Figure 6(a), SEM morphology of yeast cells within the scaffolds further confirmed that the shape of the yeast cells loaded in the pores was preserved after SAW treatment. In addition, yeast cells treated by SAW maintained their proliferation ability during the prolonged culture in the following 14 days (Figure 6(b)), which further proved that the SAW radiation at the applied RF power has not damaged the yeast cells.

2.2.2 Human bone cells

The present inventors have also shown that human stem cell viability is not significantly reduced by SAW actuation itself at either low or high radio frequency compared with the (untreated) control group (Table 1).

Table 1

RF levels of SAW Treatment of human stem cells	Average % viability (n=3) (±SD)
Untreated	84.3 ± 2.6
Low (400 mW)	84.6 ± 1.7
High (860 mW)	80.9 ± 2.1

2.2.3 Mouse primary bone cells

For all power and frequencies tested, the cell viability ranged between 72-88%, with an optimum survival rate at a power/frequency combination of -1 dBm/20MHz (Figure 7).

Studies on the effect of SAW exposure time on viability showed that for exposure times between 10 seconds and 60 seconds the viability ranged between about 92%, for 10 seconds exposure, and about 2% for a 60 second exposure (Figure 8).

The studies on the effect of the cell concentration in the seeding suspension on viability showed that survival at concentrations of 5×10^5 cells/ml was in the order of 45%, whereas survival was increased to over 70% when the concentration was at least 4×10^6 cells/ml (Figure 9a). When the difference in viability between treated and corresponding untreated cell suspensions was considered, the optimal cell survival density tested was 8×10^6 cells/ml (Figure 9b).

No difference was seen between the proliferation of SAW-treated and untreated cells, indicating that SAW treatment had no effect on the potential for the cells to proliferate (Figure 10).

Therefore, there is sufficient evidence to suggest that SAW radiation at comparatively low power levels does not affect cell viability.

2.3 Dynamics of seeding process

High-speed video images of the particle seeding process at 400 mW were captured at 60 frames per second. The images in Figure 11(a) show the geometry of the droplet and scaffold under bright illumination field at $t = 0$ prior to seeding. The left image shows the positions of the droplet and scaffold in the SAW-driven method and the right image shows that the droplet was pipetted onto

the surface of the scaffold, maintaining a round shape, at least initially. Images in Figure 11(b), in which the seeding process is observed dynamically under fluoroscopic illumination, show that the SAW-driven particle seeding process is rapid. The green fluorescence indicates that approximately 85% of the particles in the initial suspension were distributed within the scaffold in just 10 seconds. The droplet was observed to approach the scaffold and subsequently to be driven into the scaffold by SAW actuation. The fluorescence almost completely disappears in the last image at t = 10 s, indicating that the majority of the fluorescent PS particles have completely entered the scaffold structure.

In contrast, the static seeding process, in which the particle suspension is allowed to perfuse into the scaffold by pure diffusion, requires approximately 30 minutes (Figure 11(c)). Due to the small scaffold pores, of about 200 μm, the capillary stress is extremely large. Gravity is insufficient to drive the suspension through the scaffold and the suspension permeates the scaffold by pure diffusion alone.

2.4 Spatial distribution of seeded particles

2.4.1 Fluorescent microspheres

The particle seeding efficiencies only provide information about the number of particles that enter the scaffold. By slicing the scaffold into several 1-mm sections, as shown in Figure 2(b), it is possible to determine the spatial distribution of fluorescent particles within the scaffold and hence evaluate the uniformity of the particle seeding process. Cross-sectional images obtained from fluorescence microscopy of the successive slices of the scaffold are shown in Figure 12. Images in the left column show the distribution of fluorescent particles in slices a to f when the particle seeding process is driven by SAW actuation. The corresponding particle distribution in each slice, obtained via pixel intensity analysis of the fluorescent microscopic images in Figure 12, is shown in Figure 13. This is compared to the particle distribution in each slice using the static seeding method, shown in the right column of Figure 12. In addition to rapid and efficient seeding, using SAW actuation to drive the particle suspension into the scaffold gives both greater particle penetration into the scaffold and a more uniform particle distribution. The particles are present in relative abundance in the last slice, i.e. slice f, of the scaffold compared to the corresponding slice with the

static seeding method (Figure 12). In the static method, most of the particles remained in the surface layer due to the inability of gravitational forces to overcome the large capillary pressure drop over the entire scaffold length. The penetration depth in this case was roughly 20% of the entire scaffold length; the particle's fluorescent intensity can be seen to drop drastically in slices c to f.

2.4.2 Mouse bone cells

Frozen cut sections of scaffolds which had been seeded with mouse bone cells under optimised SAW actuation conditions (~5 x 10^6 cells/ml, SAW actuation for 10 seconds at 20 MHz/-1 dBm), or allowed to seed passively for 30 minutes, showed that a number of cells were driven into the PCL matrix by SAW, whereas no cells entered the scaffold by passive diffusion within the time-frame observed (Figures 14a and 14b). Cells within the SAW-driven PCL scaffold are clearly visible with both H&E staining (Figure 14a) and under fluorescence excitation of red fluorescent mouse bone cells (Figure 14b).

2.4.3 Yeast cells

Methylene violet staining of the surface of a scaffold, which had been seeded with yeast cells by SAW actuation or passive loading, showed that SAW-driven cells were present and distributed uniformly over the surface of the scaffold (Figure 15a), whereas cells seeded by the static method were found to be in clusters (indicated by arrows in 15b) distributed non-uniformly on the surface of the scaffold (Figure 15b-d).

3. CONCLUSIONS

The present invention provides a promising low-cost and miniaturizable alternative for driving a particle suspension onto, or into, a scaffold matrix through SAW actuation. Cells seeded onto the surface of a scaffold matrix by SAW actuation appear to adhere more strongly to the matrix during later applications. The SAW method has applications in, for example, the seeding of cells onto an Alloderm ® collagen matrix for use in the formation of a wound patch or skin substitute, or the creation of a cellular patch which could be placed on the surface of a defective organ. The process is rapid; a particle suspension can be driven into, or onto, a scaffold in about 10 seconds, much quicker than if the seeding occurred by pure diffusion alone or by other techniques proposed to date. In addition to rapid seeding, a higher seeding efficiency was also achieved in which

higher particle concentrations were observed to perfuse deeper into the scaffold using SAW. Moreover, the particle can be distributed in the scaffold more uniformly than when delivered by the static method. The proposed SAW-driven method therefore holds promise for improving cell seeding for various applications in tissue and orthopaedic engineering.

It will be appreciated that the present invention described herein is not to be limited to specific examples or features disclosed.

For example, persons skilled in the art would appreciate that the SAW device itself may be tailored to seed scaffolds in different ways. In the case of seeding a porous 3-dimensional scaffold matrix, SAW actuation may be employed such that a proportion of the particles in a particle suspension are driven into the matrix and a proportion of the particles are driven onto the surface of the matrix. Further, a particle suspension may be driven into, and/or onto, a scaffold from the side of, or from below, the scaffold.

The present invention provides a particle seeding method which is simple to perform, achieves good particle penetration into a 3-dimensional scaffold, or good distribution and adherence to a 2-dimensional scaffold, in a very short period of time, and thus could be therapeutically applied at the point of patient contact. Moreover, the SAW apparatus may be scaled down such that seeding of a scaffold matrix could be achieved in a hand-held device.

CLAIMS:

1. A method of driving a particle suspension into, onto, or both onto and into, a scaffold matrix by surface acoustic wave (SAW) actuation.

2. The method of claim 1, including the steps of:

a) placing a scaffold matrix onto the surface of a SAW generating actuator;

b) depositing a particle suspension onto the surface adjacent to, or in contact with, the matrix;

c) generating a surface acoustic wave to thereby drive the suspension onto, or into, the matrix.

3. The method of claim 1, wherein the matrix is a 3-dimentional matrix or a planar 2-dimentional matrix.

4. The method of claim 1, wherein the method includes the steps of:

(a) placing a two-dimensional scaffold matrix onto the surface of a SAW generating actuator;

(b) depositing a particle suspension onto the surface of the matrix;

(c) generating a surface acoustic wave to thereby drive the suspension onto the matrix.

5. The method of claim 1, wherein the matrix is a porous 3-dimensional matrix and the particle suspension is driven into the matrix by the surface acoustic wave.

6. The method of claim 1, wherein the particle is selected from the group including any one or more of a mammalian cell, a non-mammalian cell, cell growth and/or differentiation factors, or extracellular matrix molecules.

7. The method of claim 1, wherein the scaffold matrix is a biodegradable and/or biocompatible polymeric matrix.

8. The method of claim 2 further comprising the steps of:

a) placing a scaffold matrix onto a piezoelectric substrate upon which has been fabricated an interdigital transducer (IDT);

b) depositing a particle suspension onto the surface of the substrate between the IDT and the scaffold, and

c) applying a radio frequency (RF) signal to the IDT to produce SAW radiation, which has the effect of driving the particle suspension onto, or into, the scaffold matrix.

9. The method of any one of claims 1 to 8, wherein the substrate is selected from the group including, lithium niobate ($LiNbO_3$), barium titanate, lead zirconium titanate, zinc oxide, aluminium nitride, lithium tantalate, and quartz.

10. Use of SAW radiation to seed a three-dimensional scaffold matrix or a planar 2-dimentional matrix with a particle suspension.

11. The use of claim 10, wherein the particle is selected from the group including any one or more of a mammalian cell, a non-mammalian cell, cell growth and/or differentiation factors, and extracellular matrix molecules.

12. The use of claim 10 or 11, wherein the scaffold matrix is a biodegradable and/or biocompatible polymeric matrix.

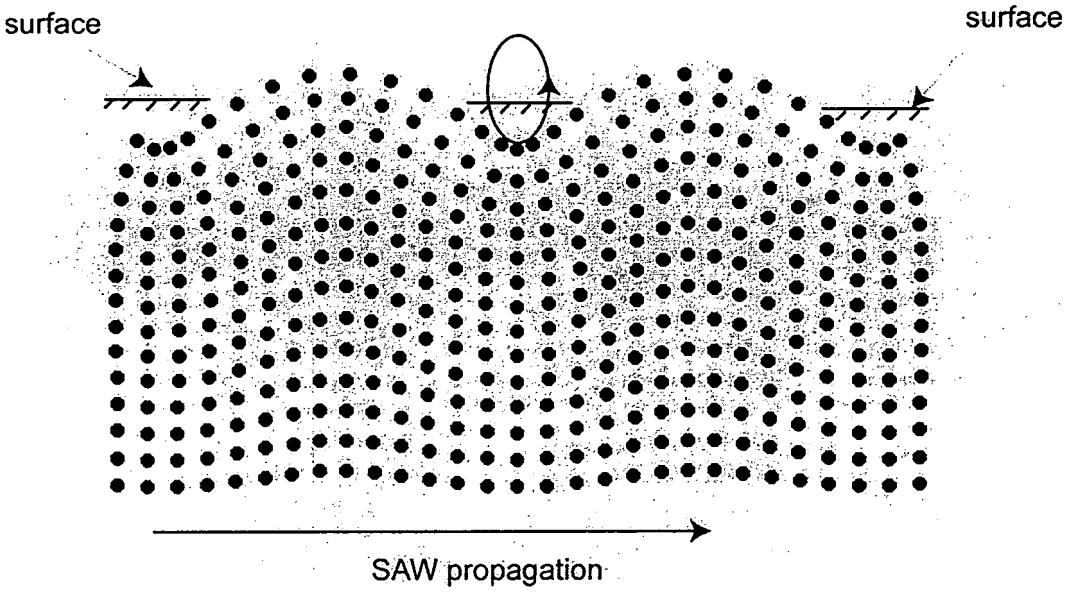
13. A particle seeding apparatus for seeding a scaffold matrix, including a piezoelectric substrate upon which has been fabricated an interdigital transducer (IDT) which generates SAW radiation, upon excitation by an RF signal, to drive a particle suspension onto, or into, a scaffold matrix.

14. The apparatus according to claim 13, wherein the piezoelectric substrate is selected from the group including, lithium niobate ($LiNbO_3$), barium titanate, lead zirconium titanate, zinc oxide, aluminium nitride, lithium tantalate, and quartz.

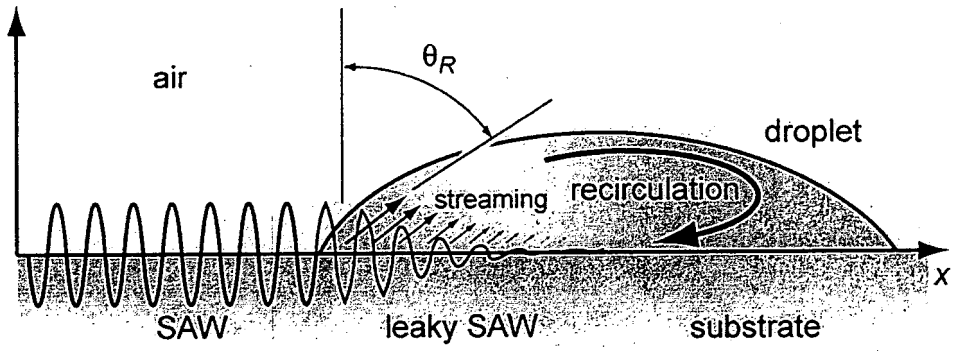
15. The apparatus according to claim 13 or 14, wherein the substrate has a titanium layer and an aluminium layer sputter-deposited thereon.

16. The apparatus according to any one of claims 13 to 15, wherein the IDT is an alternating finger IDT.

17. The apparatus according to claim 16, substantially as hereinbefore described with reference to the examples.

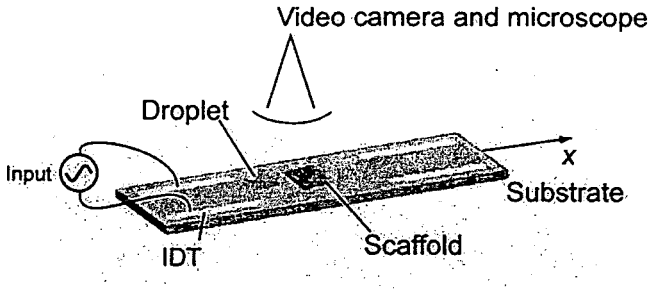

surface
surface
SAW propagation

(a)

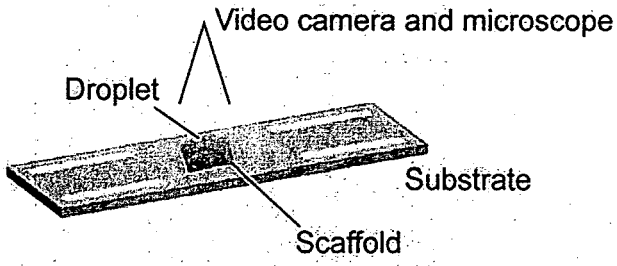

air
θR
droplet
streaming
recirculation
x
SAW
leaky SAW
substrate

(b)

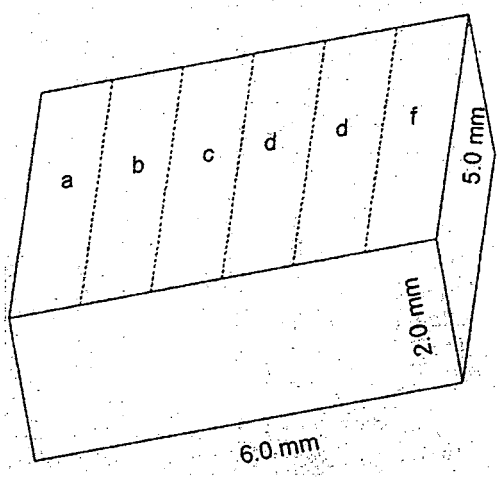
FIGURE 1


Video camera and microscope
Droplet
Input
x
Substrate
IDT
Scaffold

(a)


Video camera and microscope
Droplet
Substrate
Scaffold

(b)


a
b
c
d
d
f
5.0 mm
2.0 mm
6.0 mm

(c)

FIGURE 2

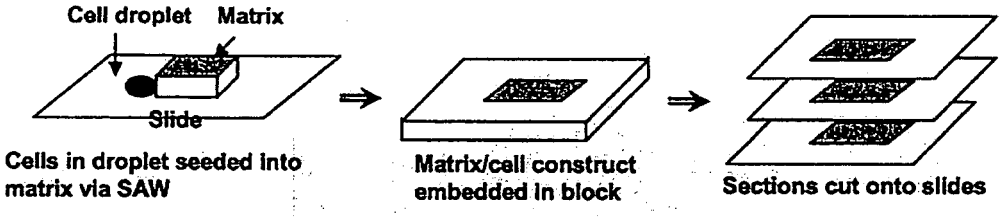


FIGURE 3

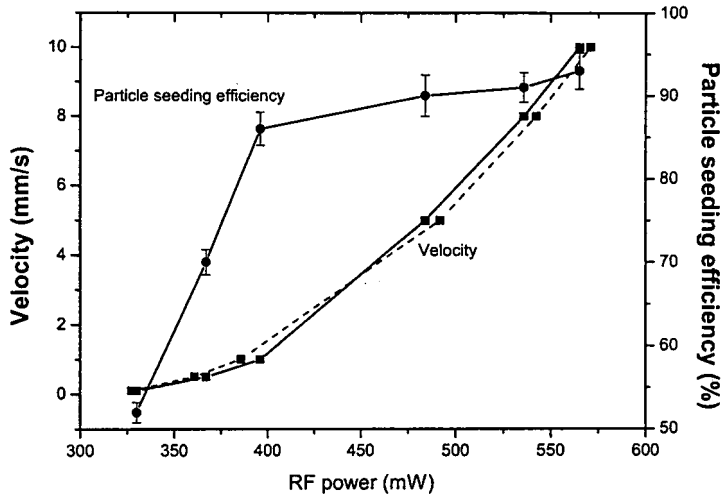


FIGURE 4

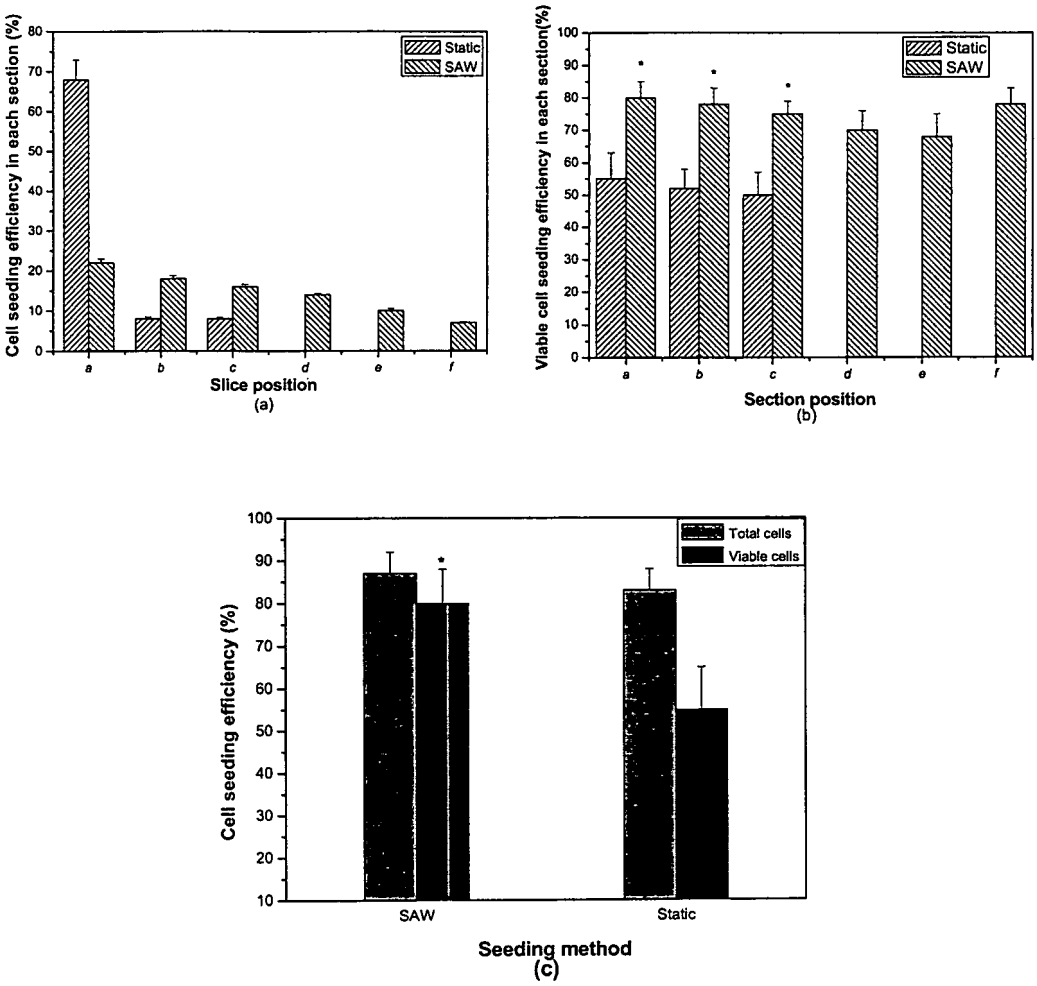
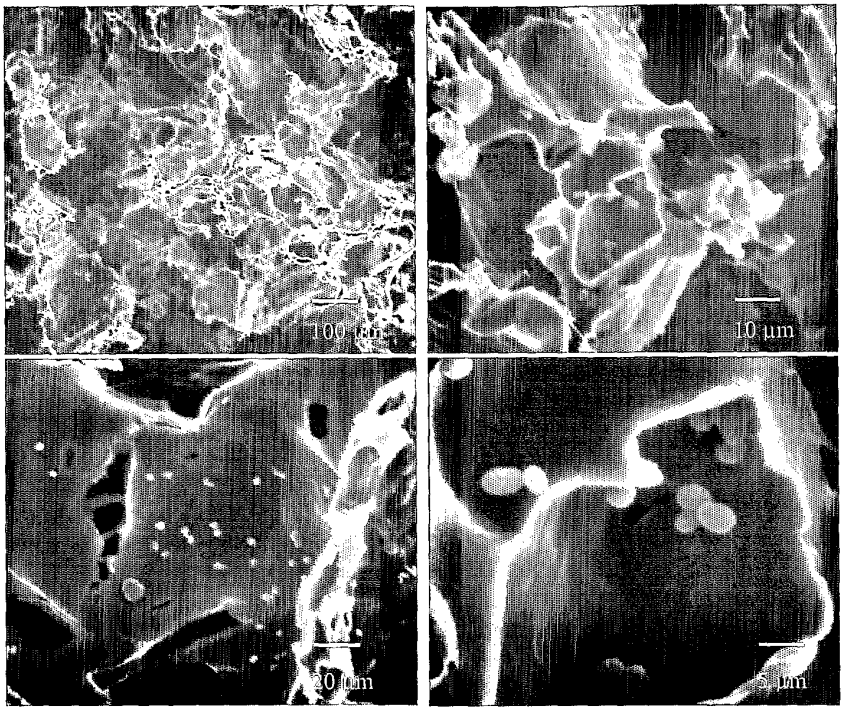
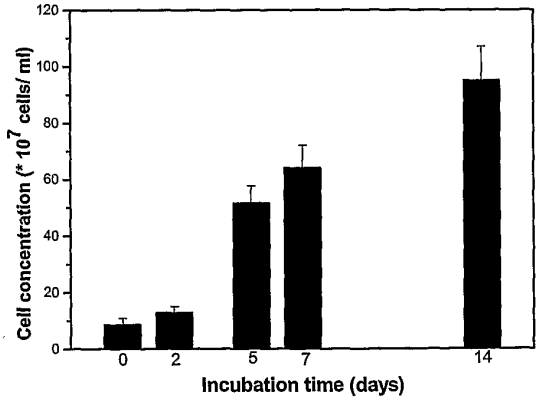

Cell seeding efficiency in each section (%)
Static
SAW
Slice position
(a)
Viable cell seeding efficiency in each section(%)
Static
SAW
Section position
(b)
Cell seeding efficiency (%)
Total cells
Viable cells
SAW
Static
Seeding method
(c)

FIGURE 5


100 µm
10 µm
30 µm
5 µm

(a)


Cell concentration (* 10^7 cells/ ml)
120
100
80
60
40
20
0
0
2
5
7
14
Incubation time (days)

(b)

FIGURE 6

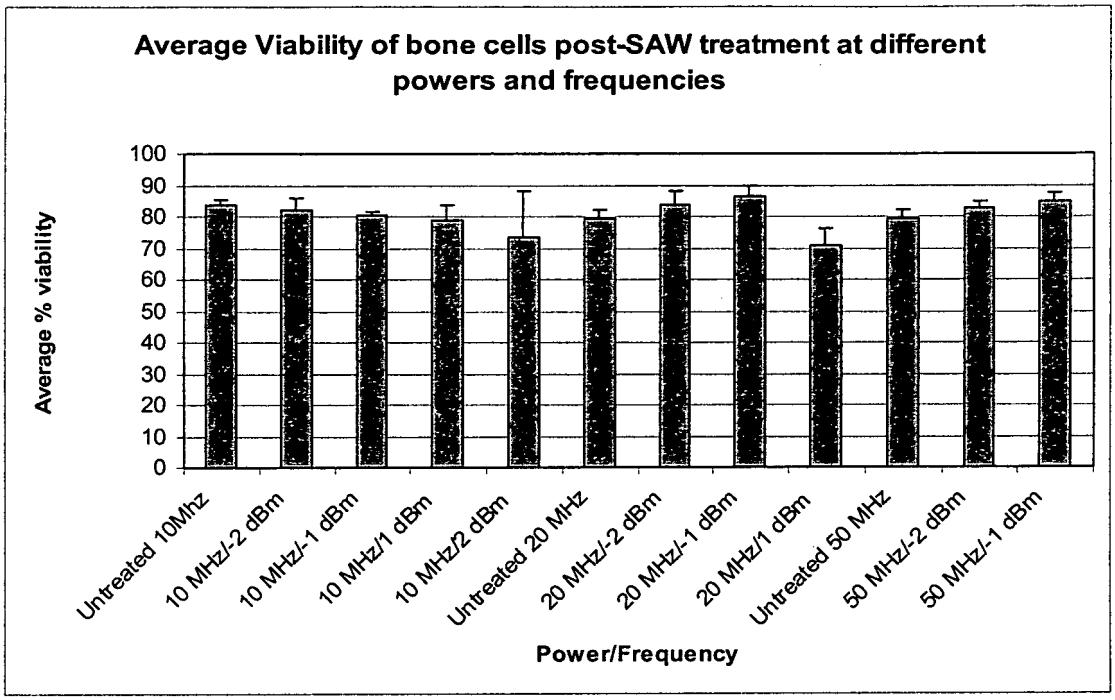

Average Viability of bone cells post-SAW treatment at different powers and frequencies
Average % viability
100
90
80
70
60
50
40
30
20
10
0
Untreated 10Mhz
10 MHz/-2 dBm
10 MHz/-1 dBm
10 MHz/1 dBm
10 MHz/2 dBm
Untreated 20 MHz
20 MHz/-2 dBm
20 MHz/-1 dBm
20 MHz/1 dBm
Untreated 50 MHz
50 MHz/-2 dBm
50 MHz/-1 dBm
Power/Frequency

FIGURE 7

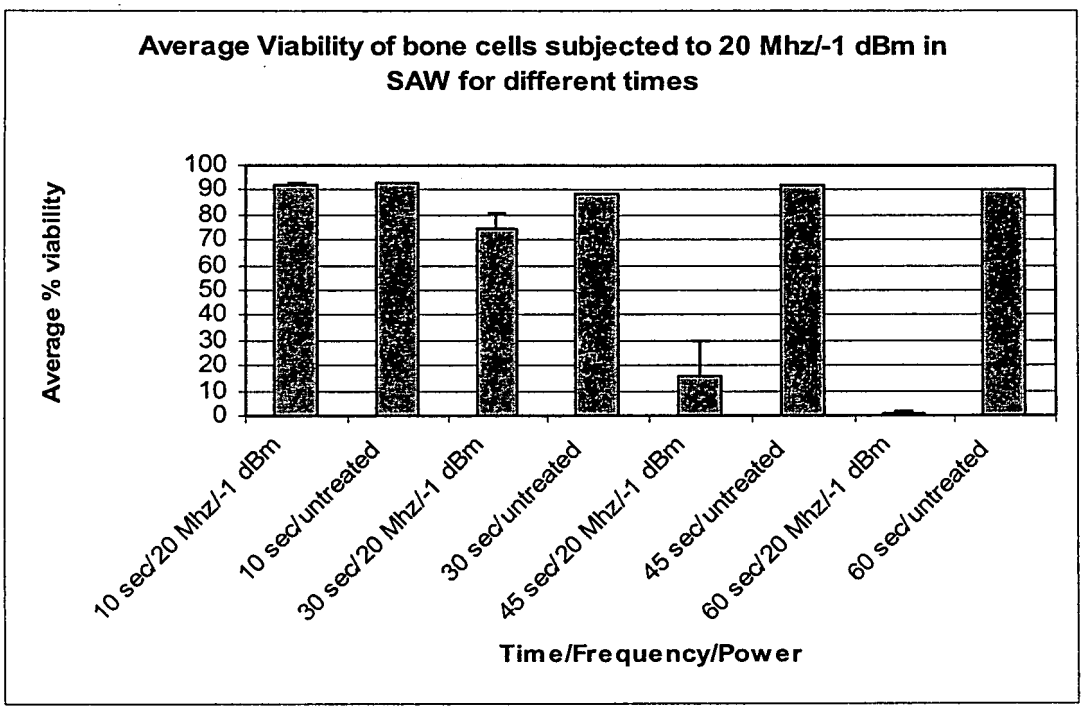
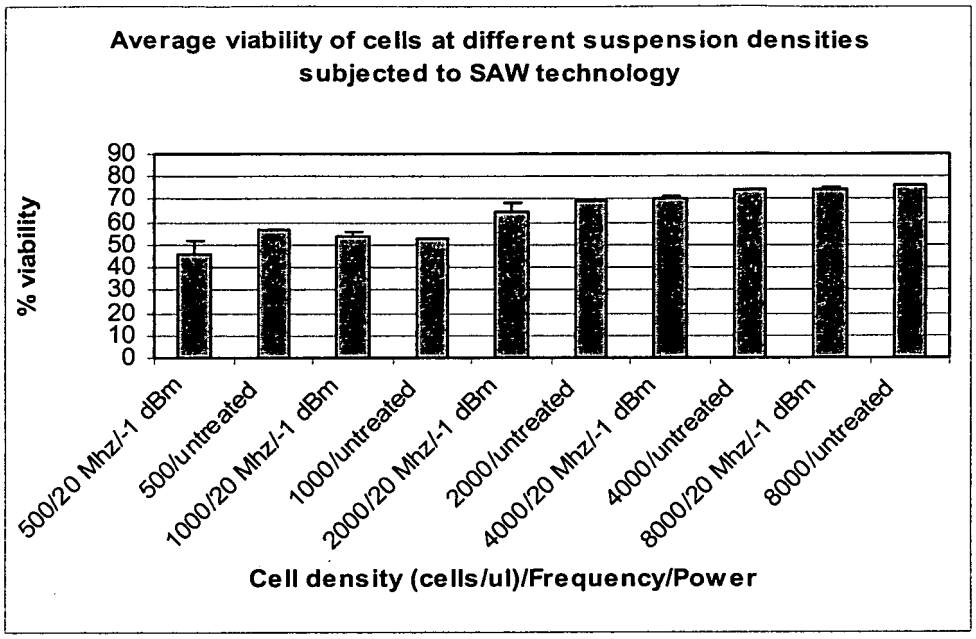
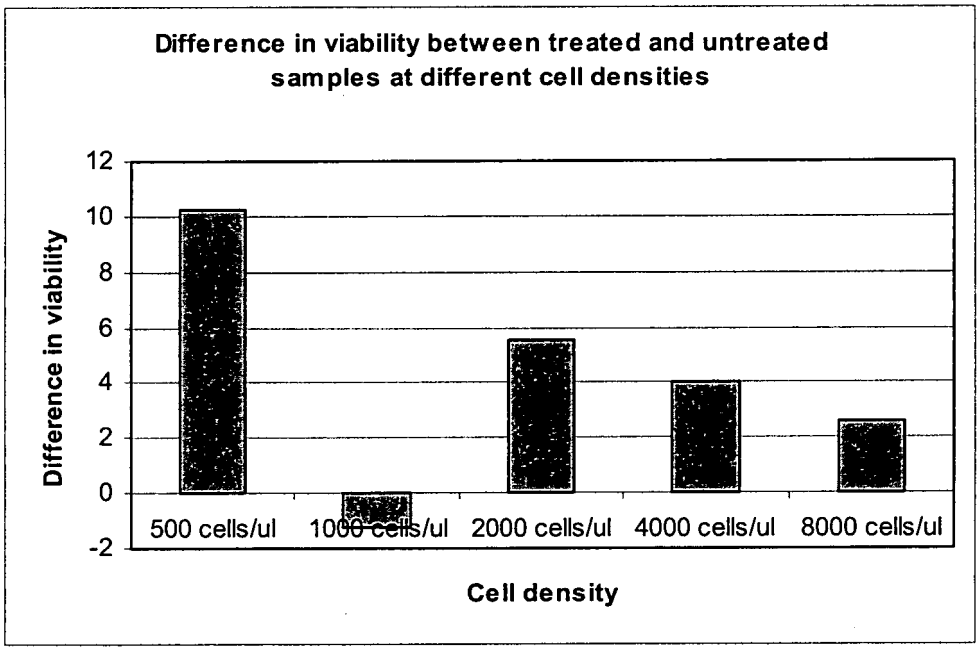

Average Viability of bone cells subjected to 20 Mhz/-1 dBm in SAW for different times
Average % viability
100
90
80
70
60
50
40
30
20
10
0
10 sec/20 Mhz/-1 dBm
10 sec/untreated
30 sec/20 Mhz/-1 dBm
30 sec/untreated
45 sec/20 Mhz/-1 dBm
45 sec/untreated
60 sec/20 Mhz/-1 dBm
60 sec/untreated
Time/Frequency/Power

FIGURE 8


Average viability of cells at different suspension densities subjected to SAW technology
% viability
90
80
70
60
50
40
30
20
10
0
500/20 Mhz/-1 dBm
500/untreated
1000/20 Mhz/-1 dBm
1000/untreated
2000/20 Mhz/-1 dBm
2000/untreated
4000/20 Mhz/-1 dBm
4000/untreated
8000/20 Mhz/-1 dBm
8000/untreated
Cell density (cells/ul)/Frequency/Power

(a)


Difference in viability between treated and untreated samples at different cell densities
Difference in viability
12
10
8
6
4
2
0
-2
500 cells/ul
1000 cells/ul
2000 cells/ul
4000 cells/ul
8000 cells/ul
Cell density

(b)

FIGURE 9

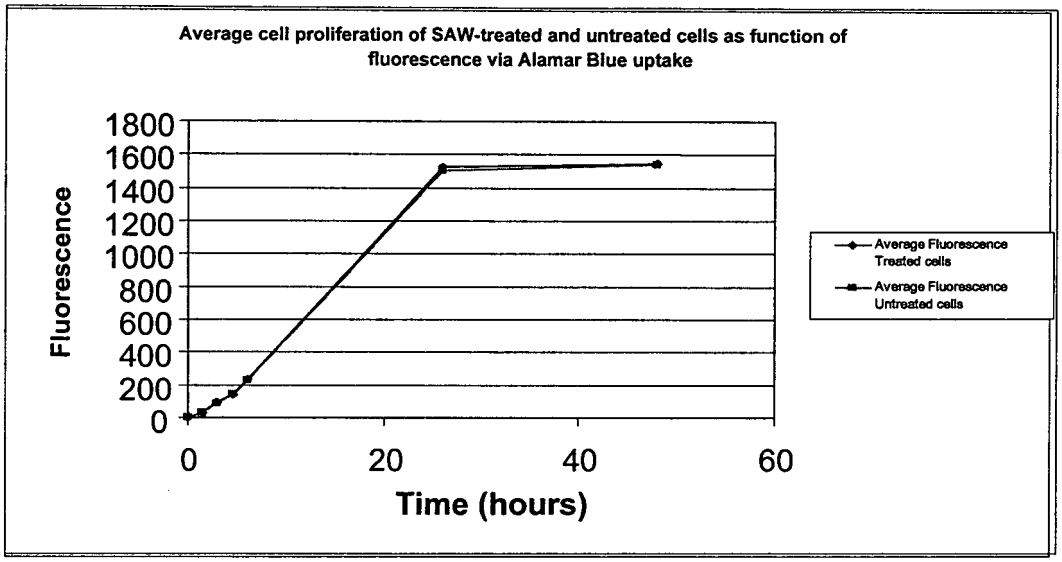

Average cell proliferation of SAW-treated and untreated cells as function of fluorescence via Alamar Blue uptake
Fluorescence
1800
1600
1400
1200
1000
800
600
400
200
0
0
20
40
60
Time (hours)
Average Fluorescence Treated cells
Average Fluorescence Untreated cells

FIGURE 10

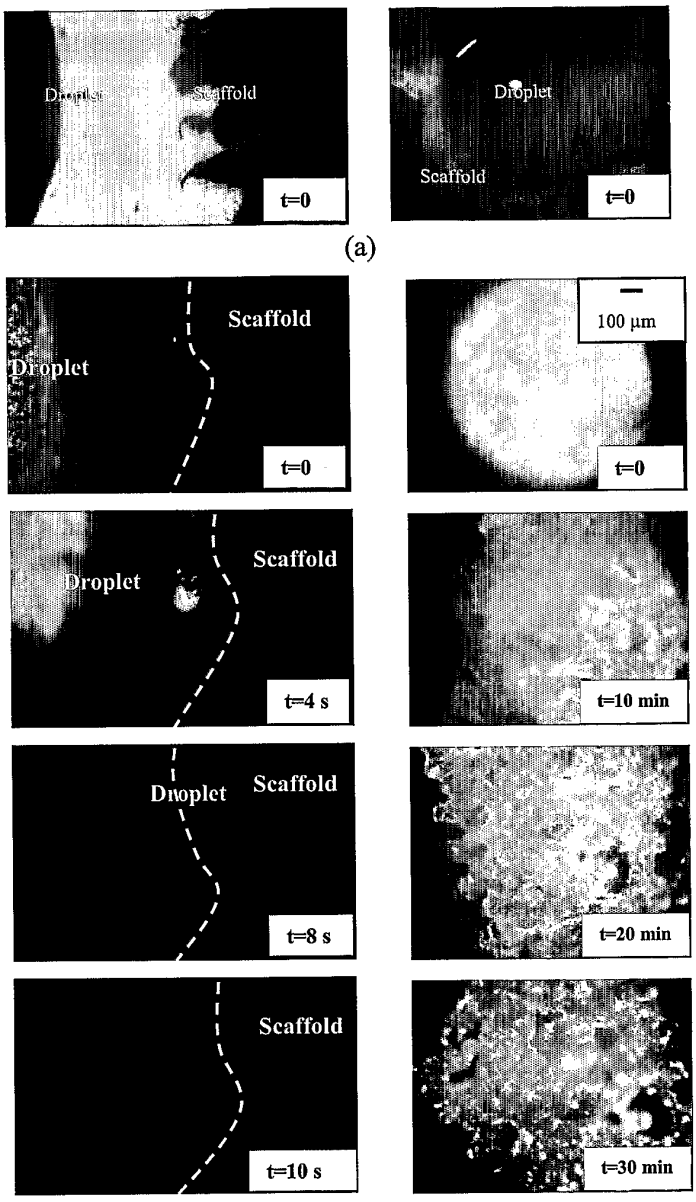

Droplet
Scaffold
t=0
Droplet
Scaffold
t=0
(a)
Scaffold
Droplet
t=0
100 µm
t=0
Droplet
Scaffold
t=4 s
t=10 min
Droplet
Scaffold
t=8 s
t=20 min
Scaffold
t=10 s
t=30 min
(b)
(c)

FIGURE 11

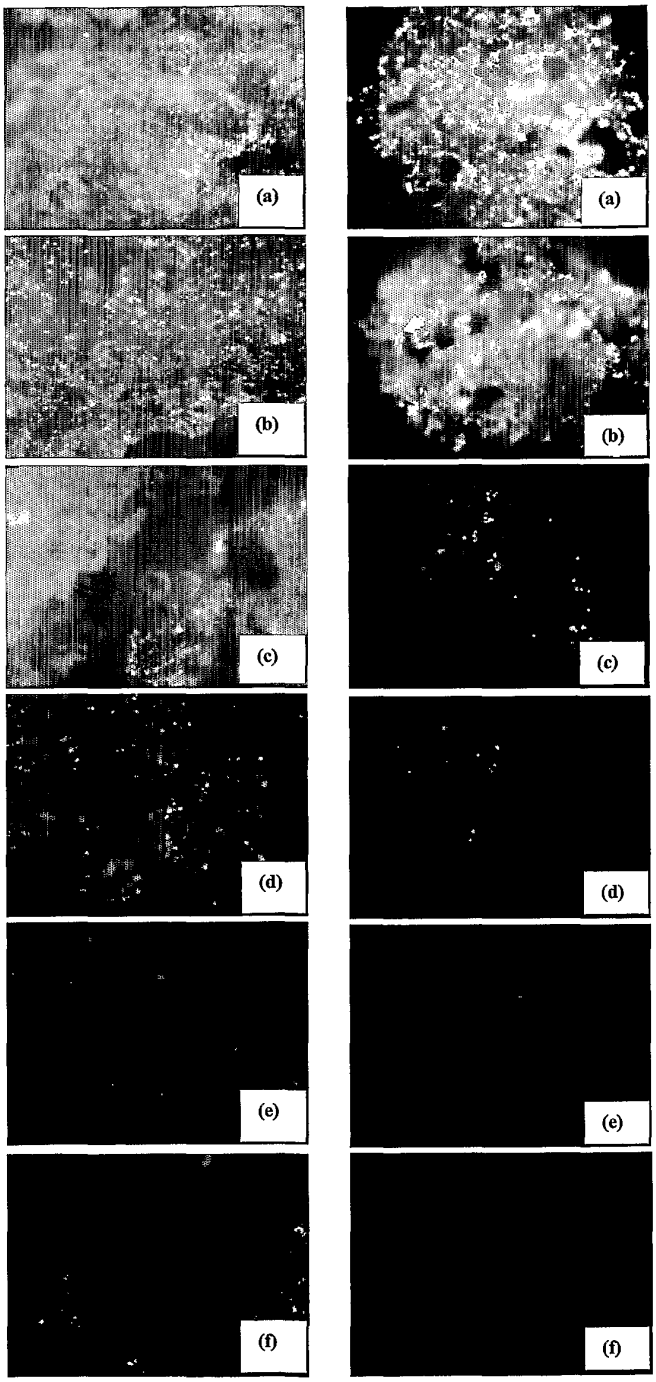


FIGURE 12

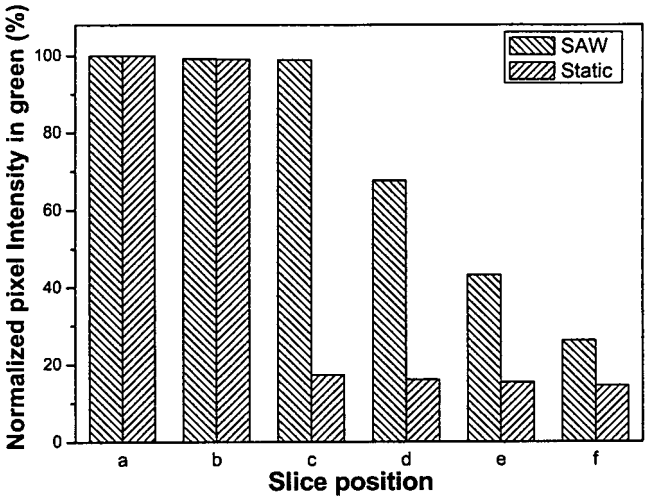
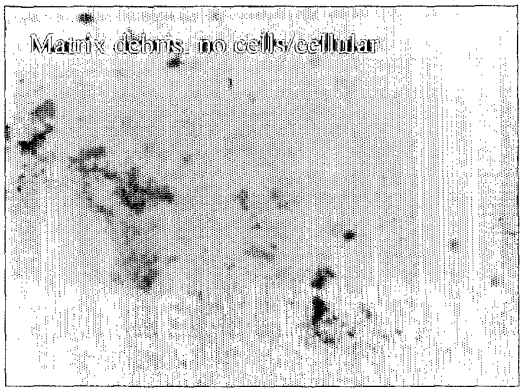
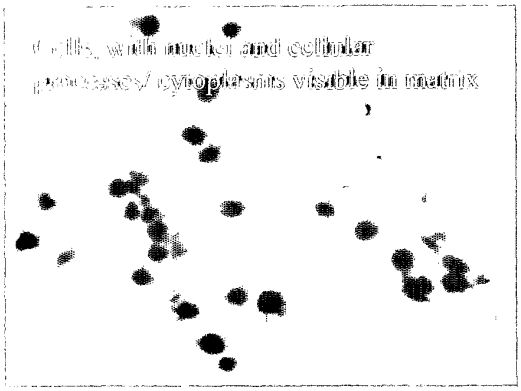

Normalized pixel Intensity in green (%)
100
80
60
40
20
0
SAW
Static
a
b
c
d
e
f
Slice position

FIGURE 13

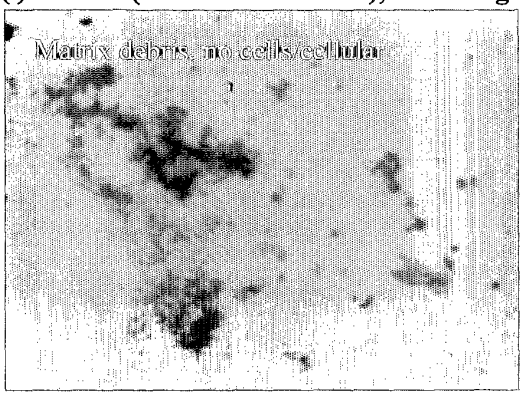
Haematoxylin&Eosin staining of sections cut of PCL seeded with ~100,000 cells



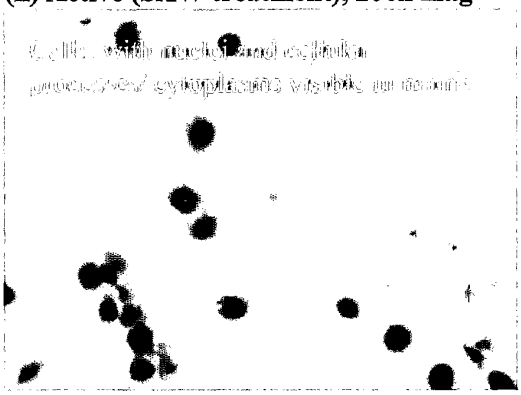
(i) Passive (no SAW treatment), 200x mag



(ii) Active (SAW treatment), 200x mag



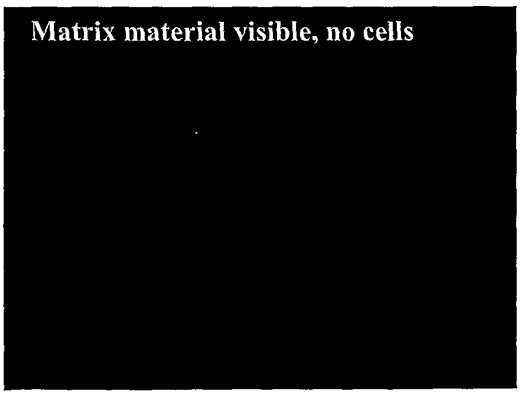
(iii) Passive (no SAW treatment), 400x mag



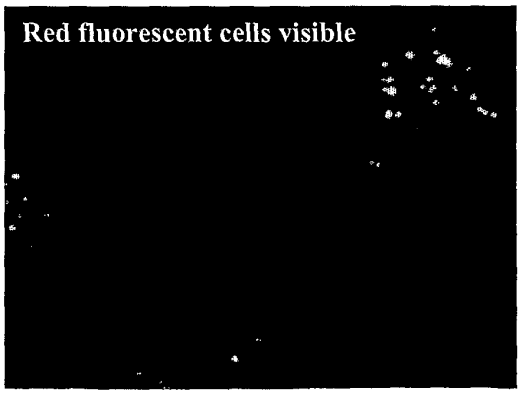
(iv) Active (SAW treatment), 400x mag

(a)

Red fluorescent images of sections cut of PCL seeded with ~100,000 cells



(i) Passive (no SAW treatment), 40x mag



(ii) Active (SAW treatment), 40x mag

(b)

FIGURE 14

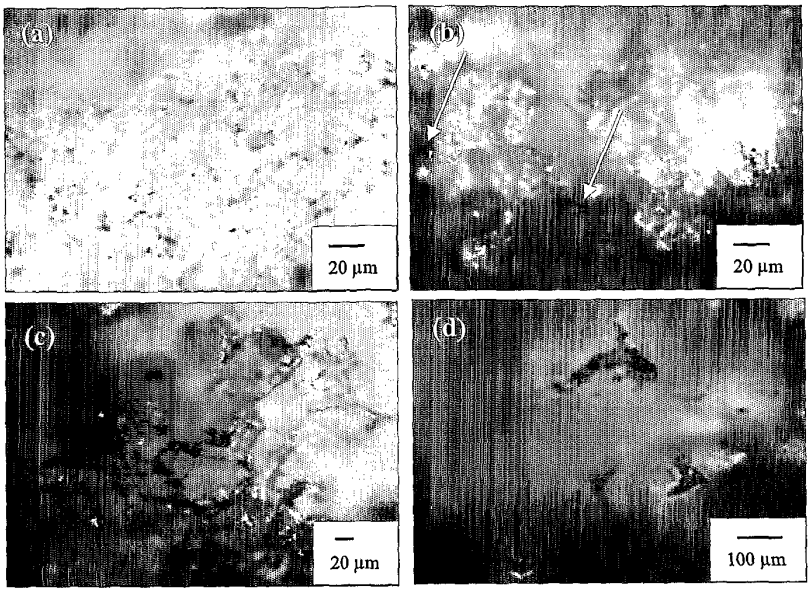


FIGURE 15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2008/000457

A. CLASSIFICATION OF SUBJECT MATTER (Int. Cl.)

A61L 27/38 (2006.01) *A61L 27/50* (2006.01) *A61L 31/12* (2006.01)
A61L 27/02 (2006.01) *A61L 27/54* (2006.01) *H02N 2/00* (2006.01)
A61L 27/40 (2006.01) *A61L 27/56* (2006.01)
A61L 27/44 (2006.01) *A61L 27/58* (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Databases: WPIDS, JAPIO, CAPlus, Medline; Google;
Keywords: particl?/cell?/molecul?/factor/growth/differentiat?/extracellular(w)matrix, scaffold/matrix, SAW/surface(w)acoustic(w)wave, 2D/3D/dimension?, seed?/deposit?/driv?, porous/biodegradab?/biocompatib?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
L	Biomaterials 28 (2007) 4098-4104, "*A Scaffold Cell Seeding Method driven by Surface Acoustic Waves*", Haiyan Li, James R. Friend, Leslie Y. Yeo; (Whole document (and references therein))	1-17
L	Conference Proceedings: 16th Australasian Fluid Mechanics Conference, 2-7 December 2007, "*Driving Cell Seeding Using Surface Acoustic Wave Fluid Actuation*", Haiyan Li, James R. Friend, Leslie Y. Yeo; (Whole document (and references therein))	1-17
P, X A	WO 2007/100731 A2 (NANOVIBRONIX, INC.) 07 September 2007 See especially [0032]-[0072]; & US 2007/0232962 A1 (ZUMERIS ET AL.) 04 October 2007 (see especially [0058])	13 1-12, 14-17

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search
15 April 2008

Date of mailing of the international search report 1 MAY 2008

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2008/000457

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,A	Medline Online Abstract Accession No. 2007410771 Tissue Engineering, (2007 Jul) Vol. 13, No. 7, pp. 1561-72 (ISSN: 1076-3279) Abstract	1-12
A	Derwent Online Abstract Accession No. 2002-230278/29 & GB 2360789 A (MASON C) 03 October 2001 Abstract	1-12
A	Derwent Online Abstract Accession No. 2005-779478/79 & WO 2005/108550 A1 (UNIV HOSPITAL BASEL) 17 November 2005 Abstract	1-12
A	Derwent Online Abstract Accession No. 2005-078642/09 & US2004254668 A1 (JANG B Z) 16 December 2004 Abstract	1-12

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/AU2008/000457

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member			
WO	2007100731	US	2007213645	US	2007232962
GB	2360789				
WO	2005108550	EP	1745121		
US	2004254668				

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX