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(54) Title: SUPER-SPARGER MICROCARRIER BEADS AND PRECISION EXTRUSION DEPOSITED POLY-EPSILON-CAPROLACTONE STRUCTURES FOR BIOLOGICAL APPLICATIONS

(57) Abstract: A microcarrier bead having a porous three-dimensional core having at least 99% interconnected pores and an outer protective layer for growing cells in a bioreactor using higher sparging rates than cells would ordinary withstand. A method of making an artificial scaffold wherein a scaffolding material is extruded into a coolant and thereby creating a porous material.

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SUPER-SPARGER MICROCARRIER BEADS AND PRECISION EXTRUSION DEPOSITED POLY-EPSILON-CAPROLACTONE STRUCTURES FOR BIOLOGICAL APPLICATIONS

SPECIFICATION

BACKGROUND OF THE INVENTION

1. FIELD OF INVENTION

This invention relates to microcarrier beads for use in bioreactors and methods for growing cells and specifically for growing anchorage dependent cells. Further, this invention relates to methods of creating scaffolds for artificial tissues and scaffolds and tissues made by these methods, specifically, the invention relates to methods of making scaffolds containing biomaterial.

2. DESCRIPTION OF RELATED ART

Anchorage dependent cells are difficult to culture in high density because they cannot grow in suspension. Mammalian cells require the presence of oxygen, but they are highly sensitive to the shear and bubbles created by the fluid motion and sparging required for creating a high DO count.

Ability to grow mammalian cells is important for laboratory and industrial levels to produce materials for cellular behavior studies and manufacture of biomaterials such as, for example, proteins, hormones, vaccines, antibiotics, insulin, etc. Cells can be produced in bioreactors using microcarries beads. Ability to produce cells in large quantities in a controlled environment is important. Bioreactors and microcarrier beads have been described in U.S. Patent No. 5,073491 to Familletti, U.S. Patent No. 5,175,093 to Seifert, U.S. Patent No. 5,654,197 to Jem, et al., U.S. Patent No. 5,153,133 to Schwarz, et al., U.S. Patent No. 4,906,577 to Armstrong, et al., U.S. Patent No. 4,824,946 to Schwengers, et al., U.S. Patent No. 4,189,534 to Levine, et al., and U.S. Patent No. 6,150,581 to Jiang, et al. CULTISPHER beads are constructed of porous gelatin, with random pore orientation and unpredictable interconnectivity. However, these microcarrier beads do not provide the needed porosity and protection from agitated fluid environment to produce higher yields of cells. CYTODEX is another type of microcarrier beads, which also does not provide sufficient cell survival at a more agitated fluid environment. Noon-porous beads grow anchorage dependent cells upon their surface.

Despite the foregoing developments, there is a need in the art for improved microcarrier beads capable of supporting cell growth in agitated conditions and providing larger areas for cell growth as well as improved methods of cell growth in bioreactors.

Three-dimensional (3D) scaffolds play important roles in scaffold guided tissue engineering because they provide critical functions as extracellular matrices onto which cells can attach, grow, and form new tissues. Design and fabrication of tissue scaffolds are important issues in regenerative medicine, particularly for load bearing scaffolds in bone and cartilage tissue engineering application (see U.S. Pat. No. 5,900,207 to Danforth et al., U.S. Pat. No. 6,712,850 to Vyakarnam et al., U.S. Pat. No. 6,730,252 to Teoh et al., and U.S. Pat. No.6,645,412 to Priedman Jr.).

To design a 3D scaffold, one needs to address multiple biological, mechanical and geometrical design constraints and take into account scaffold external and internal geometry, porosity, pore size and interconnectivity in order to provide the needed structural integrity, strength, transport property, and an ideal micro-environment for cell and tissue ingrowth and healing (Hollister *et al.*, 2002; Hutmacher, 2000; Sun and Lal, 2002). Advances in computer-aided tissue engineering and the use of biomimetic design approach made possible introducing biological and biophysical requirement into the scaffold design (Sun *et al.*, 2003). However, thus designed scaffolds often have intricate architectures that can only be fabricated through advanced manufacturing techniques. Most available scaffold fabrication methods, such as solvent casting, fiber bonding, phase separation, gas induced foaming, and salt leaching, are either limited to produce scaffolds with simple geometry, or to depend on in-direct casting method for scaffold fabrication (Taboas *et al.*, 2003; Yang *et al.*, 2002), so they are impractical for being used to manufacture scaffolds with complex structural architectures. To overcome this hurdle, solid freeform fabrication techniques, such as 3D printing, multi-phase jet solidification, and fused deposition modeling (FDM) have been widely adopted for scaffold fabrication (Koch *et al.*, 1998; Wu *et al.*, 1996; Zein *et al.*, 2002). Among the reported techniques, FDM-based extruding deposition seems to be one of the most promising processes because of its versatility of using different scaffolding materials, possibility of manufacturing scaffolds in a cell-friendly environment, and feasibility of controlled drop-on-demand high precision deposition (Vozzi *et al.*, 2002; Xiong *et al.*, 2001). On the other hand, the ability of quantification of the scaffold fabrication microstructure relationships, such as the effect of the process on the morphologies and the functional properties of the scaffolds, is as important as the scaffold fabrication itself because the biological and mechanical functions of the scaffold are in part dominated by the fabricated local micro-architecture of the scaffold. Micro-computed tomographic (micro-CT) imaging technology enables the characterization of the salient features of the scaffolds for tissue engineering applications. Recent reports have shown that micro-CT techniques are capable of

characterizing micro-architectural and mechanical properties of tissue scaffolds (Lin *et al.*, 2003), evaluating porous biomaterials (Muller *et al.*, 1996), quantifying the bone tissue morphologies and internal stress-strain behavior (Van *et al.*, 1999) and conducting nondestructive evaluation for tissue properties (Muller and Ruegsegger, 1997). The objective of this paper is to present our recent study on using a precision extruding deposition (PED) process to fabricate poly-ε-caprolactone (PCL) tissue scaffolds with designed micro-architecture, and to present the study on using micro-CT technique for evaluation and characterization of the morphologies and microstructures of the PED fabricated scaffolds. In contrast to the conventional FDM process that requires the use of precursor filaments, the presented PED process directly extrudes scaffolding materials in its granulated or pellet form without the filament preparation and freeform deposits according to the designed microscale features.

Currently, commercial applications of tissue engineering scaffolds are limited. Examples include films and gels such used in wound healing applications and rigid, porous filler materials such as those used to fill bone defects. Macro and microcarrier beads are more common. Some are rigid (such as CYTODEX carrier beads) and some are porous (such as CULTISPHER carrier beads), but there are no existing microcarrier beads with ~100% porous interconnectivity with pores of the size scales producible with PED.

An apparatus comprising a multi-nozzle biopolymer deposition system capable of extruding biopolymer solutions and living cells for freeform construction of three-dimensional tissue scaffolds is described in a PCT application Serial No. PCT/US2004/015316 filed on May 14, 2004 and U.S. Patent Application Serial No. 10/540,968 incorporated herein in their entireties. The apparatus and the method do not describe depositing polycaprolactone (PCL).

The most common process of creating biocompatible structures is mold fabrication. While mold fabrication can create structures of desirable exterior shape, there is limited ability in controlling internal architecture. Secondary processes (such as salt fusion and phase manipulation) may be used to add porosity, but such processes often create random pores with unknown connectivity.

Despite the foregoing developments, there is a need in the art for improved methods and apparatuses capable of producing porous structures with controllable pore sizes and interconnectivity and ability to incorporate biomaterial in a scaffold without affecting the biomaterial's viability.

All references cited herein are incorporated herein by reference in their entireties.

BRIEF SUMMARY OF THE INVENTION

The present invention provides a microcarrier bead comprising a porous three-dimensional core having at least 99% interconnected pores and an outer protective layer.

In certain embodiments, the microcarrier bead further comprises a filler material in communication with the polymeric porous three-dimensional core.

In certain embodiments, the porous three-dimensional core is made from a scaffold material comprising a biodegradable polyester, fibrin, collagen, hydroxyapatite, and mixtures thereof.

In certain embodiments, the scaffold material is at least one of poly-caprolactone, polylactic acid, polyglycolic acid, and polylactide co-glucolide. In certain embodiments, the filler material is at least one of fibrin, collagen, and dextran. In certain embodiments, the outer material is hydrogel. In certain embodiments, the scaffold material comprises a biodegradable polyester and the outer material comprises alginate.

In certain embodiments, the porous three-dimensional core is made by depositing poly-caprolactone using in a layered pattern such that at least 99% of pores is interconnected, and substantially all pores lead to the external surface of the microcarrier bead.

In certain embodiments, the microcarrier bead further comprises cells.

Further provided is an assembly of microcarrier beads.

Further provided is a method for making an artificial scaffold, the method comprising:

providing a scaffolding material;

providing a coolant having a temperature of at most 20°C, provided that the scaffolding material has a temperature of at least 25°C; and

extruding the scaffolding material into the coolant and thereby making the artificial scaffold.

In certain embodiments of the process, extruding the scaffolding material into the coolant is performed in a layered pattern such that the artificial scaffold has a porous three-dimensional core.

In certain embodiments of the process, at least 99% of pores in the porous three-dimensional core are interconnected and substantially all pores lead to the external surface of the porous three-dimensional core.

In certain embodiments of the process, the artificial scaffold comprises an artificial tissue.

In certain embodiments of the process, the scaffolding material comprises a biodegradable polyester, fibrin, collagen, hydroxyapatite, and mixtures thereof.

In certain embodiments of the process, the coolant is at least one of a liquid, a foam, and a gel. In certain embodiments of the process, the coolant comprises hydrogel. In certain embodiments of the process, at least one of the scaffolding material or the coolant comprises a biomaterial.

Also provided is a microcarrier bead made by the method of the invention, the method comprising:

providing a scaffolding material;

providing a coolant having a temperature of at most 20°C, provided that the scaffolding material has a temperature of at least 25°C; and

extruding the scaffolding material into the coolant and thereby making the artificial scaffold .

Also provided is an artificial tissue made by the method comprising:

providing a scaffolding material;

providing a coolant having a temperature of at most 20°C, provided that the scaffolding material has a temperature of at least 25°C; and

extruding the scaffolding material into the coolant and thereby making the artificial scaffold.

Further provided is an improvement method in a process for manufacturing complex parts and devices comprising (a) utilizing a computer aided design program to design a part or device to be created; (b) converting the computer aided design program designed part or device into a heterogeneous material and multi-part assembly model which can be used for multi-nozzle printing; and (c) printing the designed part or device using different, specialized nozzles, the improvement comprising extruding a scaffolding material into a coolant having a temperature of at most 20°C.

The microcarriers of the invention have a significantly greater surface area as they have an internal porosity. The protective alginate layer of the microcarriers of the invention also allows for a more agitated fluid environment than survivable by cells on CYTODEX beads. CULTISPHER beads are constructed of porous gelatin, with random pore orientation and unpredictable interconnectivity. In the Super-Sparger microcarrier beads, pore dimensions and nearly complete interconnectivity are known and measurable and allow growing cells in a controlled environment. Also, the rigidity of the precision extrusion deposited polymer and the

protective nature of the alginate film allow the microcarrier beads to withstand a more agitated environment than the CULTISPHER beads. In the present invention, the method of growing cells will produce more cells at higher oxygen rates and feed of nutrients.

Improved microcarrier beads provide the needed porosity and protection from agitated fluid environment to produce higher yields of anchorage dependent cells in a bioreactor. Precision extrusion deposited porous microcarrier beads within the reactor create large amounts of surface area for cell attachment. These pores are uniform with >99% interconnectivity and constructed of poly-ε-caprolactone or other polymer. Bubble and fluid kinetic resistance is provided through a thin hydrogel layer at the perimeter of the porous bead. This layer allows diffusion of particles smaller than 1300 daltons while providing mechanical resistance to fluid motion and preventing invasion of bubbles into the bead.

Inventors have discovered that biomaterial sensitive to elevated temperatures (i.e., above 37°C) can be deposited together with the scaffolding material (e.g., PCL) without compromising its viability if the scaffolding material is cooled off during the deposition step. Without the additional cooling step, the scaffolding material is deposited on a surface at a room temperature as described in the article by Wang, et al. Since the scaffolding material is being extruded at its glass transition temperature (about 90-60°C), it will take time to cool of to 37°C. During the cooling process, the scaffold materials endurance a phase transition from a liquid or gel state to a solid state. However, this time-dependent phase transition often causes difficulty in control of scaffold geometrical and structural configuration. Advantageously, the additional cooling step involving using a coolant addresses these shortcomings in several ways and also provides a method of making more viable biomaterial containing scaffolds.

Further, the inventors have discovered that extruding into a coolant to expedite phase transition allows for creation of pores less than 100 microns in size. A consistent mesh-like deposition pattern allows for ~100% porous interconnectivity. Any material may be used with a melting point within the range of the heating element, allowing for use of biocompatible materials such as poly-epsilon-caprolactone (PCL). PCL is a biocompatible material with a lengthy history of use in clinical products, and PED has demonstrated a capability of building anatomic or synthetic shaped structures. PCL structures may also be put through secondary processes, such as filling with a second material, as part of a multi-component bioactive scaffold.

In certain embodiments, the scaffolding material can be deposited in a layer of a coolant, which is kept at a temperature below 20°C, wherein the coolant is, for example, a liquid, a foam

or any other surface capable of being chilled. An example of a liquid is water and an example of a suitable foam is a surfactant. The coolant is preferably mixed with biomaterial such as, for example, cells, proteins, pharmacologically active agents, a component of a substrate-ligand pair, etc.

The coolant is provided as a layer of a constant depth or multiple layers which can be added as needed during the deposition of the scaffolding material. As the scaffolding material is being dropped into the coolant, it forms a controlled droplet or regular shapes rather than irregular shapes due to the phase transition without using a coolant, thus one can form a controllable structural configuration. This is another advantage of using a coolant.

In certain embodiments, the scaffolding material can be deposited simultaneously with the coolant and with other biomaterials or bioactive compounds through multi-nozzles, wherein the coolant is applied alone or preferably mixed with biomaterial such as, for example, cells. During such deposition, the scaffolding material is being chilled along the outer parts of its extruded mass. A nozzle for depositing the scaffolding material is positioned adjacent to two or more nozzles for depositing a coolant and/or with other biomaterials. In another variant, the nozzle for depositing the scaffolding material is nested inside a larger nozzle for depositing the coolant.

The above described ways of introducing a coolant can be combined. More than one type of the scaffolding material can be deposited. The scaffolding material can be deposited with other non-scaffolding material such as, for example, a filler material (e.g., hydrogel) which can also act as a coolant in some embodiments.

Non-limiting examples of the scaffold material are poly-caprolactone (PCL), polylactic acid (PLA), polyglycolic acid (PGA), polylactide co-glucolide poly(lactic acid) (PLGA), fibrin, collagen, hydroxyapatite, mixtures thereof and other biologically useful materials. The scaffold materials are preferably biocompatible.

In preferred embodiments, the hydrogel is a member selected from the group consisting of alginate, collagen, chitosan, fibrin, hyaluronic acid, agar, polyethylene glycol and its copolymers, and acrylamide-based and acrylic acid-based polymers, and the scaffold material is a member selected from the group consisting of polycaproactone, tricalcium phosphate, hydroxyapatite, polyglycolic acid, polylactic acid, and their co-polymers, polyhydroxybutyrate, and polypropylene fumarate. The scaffold material can have a wide range of biodegradability, depending on the desired properties and purpose of the scaffold.

The scaffold material can also be combined with various additives to better suit the type

of cell or tissue that is being used. For example, hydroxyapatite could be used when working with osteoblasts to create bone implant scaffolds. The scaffold could also be coated with proteins and receptors that facilitate cellular adhesion or migration onto the scaffold surface. Growth factors and other biologically active agents could also be included within the scaffold material.

Applications of the invention include construction of PCL tissue engineering scaffolds incorporating biomaterial during scaffold construction for research studies and clinical applications including implants for bone, cartilage, fibrous tissue, muscle, plastic surgery applications, and tissue transplants, and construction of carrier beads for culture of anchorage dependent cells.

To provide a volumetric cell attachment substrate within the structural pores, a filler material (e.g., hydrogel) must be added to the pore volume of the structural component. This can be accomplished by adding low viscosity liquid sodium alginate or fibrinogen solution to the scaffold. The liquid may be gelated through addition of a crosslinking agent, calcium chloride solution for alginate and thrombin for fibrinogen. The resulting alginate or fibrin hydrogel provides cell attachment volume while maintaining potential for nutrient transfer. Other applications of this filler material include factor release and diffusion networks within the scaffolds.

Areas of improvement described in this invention also include using new materials such as PCL/Hydroxyapatite mixtures, hydrogel filler materials such as collagen, and functionalization of hydrogel filler materials as well as improvement in deposition characteristics such as a nozzle and a strut size.

The advantages of using PCL over other material scaffolds are numerous. PCL is biocompatible and biodegradable on the long-term, but it is more rigid than other polymers frequently used in tissue engineering. This aspect of the polymer allows it to withstand forces exerted by cell growth in culture and by physiologic function in vivo after implantation. Cell types vary in their desired porosity and pore size. Due to the flexible PED manufacturing method, porous parameters are customizable, so a variety of cell types may be accommodated. Micro-CT characterization has illustrated >99.9% interconnectivity of pores in PED manufactured PCL scaffolds, a valuable parameter for nutrient transfer. Lastly, the resultant scaffolds may be supplemented with filler materials such as fibrin or collagen, enhancing cell attachment characteristics.

The advantages of a filler material hydrogel such as fibrin or alginate are numerous. The

addition of the hydrogel filler material allows for much greater cell attachment, cell seeding efficiency of 100%, greater total number of cells able to be seeded, volumetric as opposed to surface-dependent cell growth, and other benefits specific to each hydrogel. Fibrin is known for its excellent cell attachment characteristics and its ability to induce greater deposition of extracellular matrix (ECM) by cells. Fibrin is also one of the most commonly used hydrogels for growth factor and drug release studies. Alginate is useful for altering the diffusion kinetics of a tissue scaffold, protecting cells from shear stress and fluid flow, growth factor and drug release over short time scales, and culturing specific cell types such as chondrocytes.

BRIEF DESCRIPTION OF SEVERAL VIEWS OF THE DRAWINGS

The invention will be described in conjunction with the following drawings in which like reference numerals designate like elements and wherein:

Fig. 1 is a scheme demonstrating making Super-Sparger microcarrier beads.

Fig. 2 is a scheme demonstrating a structure of a Super-Sparger microcarrier bead.

Fig. 3 is a scheme demonstrating configuration of the PED system.

Fig. 4 is a scheme demonstrating an extruder nozzle.

Fig. 5 is a scheme demonstrating synchronization of positioning and material dispensing system.

Fig. 6 is a scheme demonstrating the information process of the PED system.

Fig. 7 is a scheme demonstrating scaffold layout patterns.

Fig. 8A and 8B are SEM images of scaffolds with different layout pattern wherein 8A is 0°/120° layout pattern and 8B is 0°/90° layout pattern.

Fig. 9 is a scheme demonstrating examples of fabricated scaffolds.

DETAILED DESCRIPTION OF THE INVENTION

The super-sparging bioreactor utilizes a conventional bioreactor but operates with more agitation and oxygen sparging than is normally used. These more stringent conditions would normally be a lethal amount for mammalian cells. In the invention, precision extrusion deposited porous microcarrier beads within the reactor create large amounts of surface area for cell attachment. These pores are uniform with >99% interconnectivity and constructed of a biodegradable polyester, preferably poly-ε-caprolactone. Bubble and fluid kinetic resistance is provided through an outer protective layer such as a thin hydrogel layer at the perimeter of the porous bead. This layer, preferably alginate, allows diffusion of particles smaller than 1300 Daltons while providing mechanical resistance to fluid motion and preventing invasion of bubbles into the bead.

Accordingly, the invention relates to a microcarrier bead which comprises a polymeric porous three-dimensional body having at least 99% interconnected pores and an outer protective layer. The microcarrier bead further comprises a filler material in communication with the polymeric porous three-dimensional body. In certain embodiments, the outer protective layer and the filler material are selected from the same materials. In certain embodiments, the polymeric porous three-dimensional body is made from a scaffold material comprising a biodegradable polyester, fibrin, collagen, hydroxyapatite, and mixtures thereof.

Polycaprolactone (PCL) is a biodegradable polyester with a low melting point of around 60°C and a glass transition temperature of about -60°C.

Advantages of this invention over currently known microcarriers include greater surface area than existing non-porous microcarriers, greater surface area and interconnectivity over existing porous microcarriers, and ability of surviving agitated environment, hence greater nutrient transfer.

The invention will be useful for industrial scale pharmaceutical production of cells and manufacture of biomaterials such as, for example, proteins, hormones, vaccines, antibiotics, insulin, etc. It will also be useful for academic or laboratory scale studies of cellular behavior.

Microcarrier beads are a method for growing anchorage-dependent cells in the conducive environment of a bioreactor. These bioreactors are stirred, and bubbles are passed through them to provide the cells with oxygen and nutrients. The limitation, however, lies in that cells are extremely sensitive to bubbles and the shear force caused by moving liquid. The Super-Sparger microcarrier beads mitigate this limitation by growing the cells in a porous interior and protecting the bead with a layer of hydrogel, which allows nutrients in but protects against shear stress and bubbles. The porous Super-Sparger beads present greater surface area than nonporous products such as Cytodex beads. The internal porosity is more regular and interconnected than existing beads given the method used to manufacture the Super-Sparger beads, precision extrusion deposition. The protective hydrogel layer allows for an environment with higher levels of dissolved oxygen than either existing porous or non-porous alternatives could withstand. The applications for these beads range from laboratory-scale bioreactors to simulate biological responses to mass production of pharmaceutical products such as insulin or antibodies.

The super-sparger bioreactor system includes a standard bioreactor system with a higher agitation rate than that of used in standard reactors and rapid sparging of the system. This creates an environment of shear stress and rapid bubbling hostile to cell growth but also creates

uniform high concentrations of nutrients and oxygen. Anchorage-dependent cells are protected from this environment on macro-carrier beads constructed of porous precision extrusion deposited scaffolds (preferably polycaprolactone) and a thin coating of hydrogel (preferably alginate) on the exterior of the beads. The process of precision extrusion deposition (PED) is described in the articles by Wang, et al., " Precision Extrusion Deposited Poly-Epsilon-Caprolactone Structures for Biological Applications Specification", Rapid Prototype Journal, Vol. 10, No. 1, pp. 42-49, 2004.

The >99% interconnected porespace of the PCL provides surface attachment area for cells, and, as alginate readily diffuses particles of less than 1300 daltons, the alginate coating provides a continuous diffusion of oxygen and nutrients into the scaffold while blocking fluid kinetics and bubbles. This system would allow for high density culture of mammalian cells in bioreactors, cells which are difficult to culture due to shear sensitivity, high oxygen demand, and inability to grow in suspension. The rapid bubbling and agitation of the super-sparger/macrocarrier bead system would fulfill oxygen demand while protecting the cells from shear. The 3-dimensionality of the macrocarrier bead interior would mitigate the inability of the anchorage-dependent cells to grow in suspension. Any number of beads may be used in a given system, providing scaleability.

Precision extrusion deposited porous microcarrier beads within the reactor create large amounts of surface area for cell attachment. These pores are uniform with >99% interconnectivity and constructed of poly-ε-caprolactone in the prototypes. Bubble and fluid kinetic resistance is provided through a thin hydrogel layer at the perimeter of the porous bead. This layer, alginate in the prototypes, allows diffusion of particles smaller than 1300 daltons while providing mechanical resistance to fluid motion and preventing invasion of bubbles into the bead.

A flowchart on Fig. 1 shows the key processes/steps of making the micro-carrier beads of the invention. The micro-carrier bead consists of at least two components, the core and the surrounding film (see Fig. 2). Preferably, the core consists of freeform fabricated poly-epsilon-caprilactone (PCL) deposited in a layered pattern such that > 99% of the porespace is interconnected, and all pores lead to the external surface of the bead. If no filler material is used, the PCL struts of the core constitute the cell attachment surface of the micro-carrier bead. The size and surface area of these struts are variable, based upon freeform fabrication parameters including nozzle aperture size, lateral speed, and pressure behind the nozzle. Strut sizes range from about 100 microns to about 800 microns. For an alternative cell attachment

substrate, the porous PCL core may be filled with a filler material selected for cell type. Example filler materials include fibrin, collagen, and dextran.

Other dimensions of the microcarrier bead include external geometry (size and shape) and internal porosity. The external size and pore size will be variable, based upon the specific usage. For instance, 1 mm cubes with 80-100 micron pores could be used for cells that grow at high density and require great surface area, while 5 mm cubes with 800-1000 micron pores could be used for cells requiring greater rates of nutrient transfer. There is a three order of magnitude difference in the amount of oxygen that different cell types consume, so several types of carrier beads would be marketable.

All super-sparger micro-carrier beads would have the thin (~200 micron thick) layer of hydrogel, for example, alginate, surrounding the exterior of the bead. The function of this layer is to transfer oxygen from the heavily sparged medium to the cell attachment core while protecting against bubble intrusion and mechanical forces. This layer must be added after cells have been seeded within the core, but, once added, it would isolate the cell population.

Any hydrogel that allows diffusion of oxygen may be used, but materials that do not allow cell attachment will last longer and are preferred. In preferred embodiments, the hydrogel is a member selected from the group consisting of alginate, collagen, chitosan, fibrin, hyaluronic acid, agar, polyethylene glycol and its copolymers, and acrylamide-based and acrylic acid-based polymers. Alginate was selected for its immediate gelation kinetics, its strong mechanical properties, its ready diffusion of small molecules, and its resistance to attachment by mammalian cells. It's deficit is that it naturally biodegrades on a time-scale of months, limiting the duration of its bioreactor applications. The term "alginate" shall refer to any of the conventional salts of algin, a polysaccharide of marine algae which may be polymerized to form a matrix for use within the growth chamber of the bioreactor. The salts of algin shall include, but are not limited to, any metal salt such as sodium, magnesium, etc. Preferably, the alginate includes, but is not limited to, a composition of gulronic and mannuronic acids and the material has a low viscosity. Porosity of the microcarrier bead of the invention is in a range of 0% to 88%. A hydrogel is defined as a substance formed when an organic polymer (natural or synthetic) is cross-linked via covalent, ionic, or hydrogen bonds to create a three-dimensional open-lattice structure which entraps water molecules to form a gel. Examples of materials which can be used to form a hydrogel include polysaccharides such as alginate, polyphosphazenes, and polyacrylates such as hydroxyethyl methacrylate (HEMA), which are crosslinked, ionically bound, or bound block copolymers such as Pluronics or Tetronics, polyethylene oxide-polypropylene glycol block

copolymers which are crosslinked by temperature or pH, respectively. Other materials include proteins such as fibrin, polymers such as polyvinylpyrrolidone, hyaluronic acid and collagen. Preferred examples of hydrogel include alginate, collagen, chitosan, fibrin, hyaluronic acid, agar, polyethylene glycol and its copolymers, acrylamide-based polymers, and acrylic acid-based polymers.

PCL can also be substituted for other materials that melt at temperatures of less that 200°C without denaturing. A mix of materials can also be used such as, for example, PCL/HA combination.

The super-sparger beads are intended for anchorage-dependent, aerobic cells (cells that both need oxygen and a surface to attach to). The term "anchorage cells" shall refer to any cells particularly mammalian cells, which will grow and multiply when attached to a support material and shall include but are not limited to cells which will only grow when attached to a solid support material. A potential use includes culture of secretory cells such as pancreatic islet cells for large-scale production of drugs such as insulin. Through use of a filler material, non-anchorage dependent cells could also be cultures, including prokaryotes such as E. Coli which are already used in pharmaceutical manufacture.

Further, the invention was driven by a desire to develop an improvement to a method for precision extruding deposition (PED). The inventors have discovered that utilizing an integrated cooling step will provide a method for construction of porous structures with controllable pore sizes and interconnectivity and ability to incorporate biomaterial in a scaffold without affecting the biomaterial's viability due to integrated simultaneous cooling. The use of a coolant with the PED system further enhances scaffold criteria such as strut and pore width. The process is applicable to poly-ε-caprolactone (PCL) as well as other polymers which phase transition can be controlled through the extruding deposition. These porous criteria are important for biological applications. The pore interconnectivity allows nutrient transfer to cells cultured on these structures, porosity effects nutrient transfer and surface area for cell attachment, and pore size affects the behavior of specific cell types. The specific applications for these structures include tissue engineering scaffolds and cell culture microcarrier elements. A filler material within this structural framework must allow passage of nutrients and volumetric cell seeding and attachment. The filler material must be biodegradable to allow replacement by growing extracellular matrix (ECM). The specific applications for these structures include tissue engineering scaffolds.

Precision Extrusion Deposition (as previously described by inventors in the article by

Wang, et al., "Precision Extrusion Deposited Poly-Epsilon-Caprolactone Structures for Biological Applications Specification", Rapid Prototype Journal, Vol. 10, No. 1, pp. 42-49, 2004 and presented at the 14th Annual Solid Freeform Fabrication Symposium, University of Texas, Austin, Texas, 4-6 August 2003) forces powder or pellets of material through a heating element where it is melted and extruded out from a micro-scale nozzle with pressure generated by a rotating screw. The extruded materials are guided by nozzles and solidified as strands of small diameter. Mounted on a 3D positioning system, the extrusion head may deposit these strands at any width, of fill gap, apart from each other. Once a layer is complete, the extrusion head is moved upwards one increment, or layer height, and more strands are deposited at a variable angle to the previous layer. Control of fill gap allows fine control of porosity. Control of fill gap and layer height allows fine control of pore size.

A schematic configuration of the PED system (Bellini, 2002) is shown in Figure 3. The hardware component consists of an XYZ position system, a material extruder system, and a temperature control system. The software component consists of data processing software and system control software. The data processing software slices the STL files and generates the process toolpath. The system control software controls the material deposition according to the process toolpath to form a layered 3D object. University of Singapore reported a fused deposition method utilizing a polymeric filament, which is fed into the extrusion head. There is no coolant used, and the resultant scaffold struts are significantly larger then the ones disclosed in this invention. In the invention, the PED system forces pellets of PCL through a set of heating elements, rather than a filament, the pressure generated by a rotating screw. The pressure generated is greater, allowing for smaller nozzles and smaller strut sizes. The use of PCL pellets as a feed material also negates the necessity of a second machine to create a filament in fused deposition.

The material mini-extruder system delivers the PCL in a fused form through the deposition nozzle. The major difference of the PED process with the conventional FDM process lies in that the scaffolding material can be directly deposited through PED process without involving filament preparation. The pellet formed PCL is fused by a liquefier temperature provided by two heating bands and respective thermal couples and then extruded by a pressure created by a turning precision screw. A schematic of the material mini-extruder system is shown in Figure 4. To achieve deposition accuracy, the positioning system and the material miniextruder are synchronized as shown in Figure 5. The material deposition roads (of both contouring and raster filing) consist of series of line segments, so the extruder movement is

composed of a series of 2D linear interpolations upon which a simultaneous proportional signal to the XY position is extracted. The signal is used to drive the rotating motor of the material extruder. The proportional ratio can be adjusted to coordinate the positioning system and material dispensing system according to its controlled movement, speed, and material extrusion flow rate. Figure 6 shows the information pipeline during the fabrication process. The designed scaffold CAD model is first converted into STL format, and then sliced with each slice patterned stored in the pattern library for toolpath generation. Initialized by a parameters file, the in-house developed system control software provides functions for 3D part visualization, machine and process setup, testing and monitoring during the real-time fabrication process.

PCL (Sigma Aldrich Inc., Milwaukee, Wisconsin) in the form of pellets was used as the scaffolding material. PCL is a semi-crystalline aliphatic polymer that has a slower degradation rate than most biopolymers in its homopolymeric form. It has a low glass transition temperature at 2 608 C, a melting temperature at about 58-608 C, and a high thermal stability. It has a high decomposition temperature *Td* of 3508 C. The mechanical properties of PCL (Mw = 44; 000) with a tensile strength of 16MPa, tensile modulus of 400MPa, flexural modulus of 500MPa, elongation at yield of 7.0 percent, and elongation at break of 80 percent have been reported. A designed scaffold cylinder model, measuring 20mm in diameter and 10mm in height, was first created in a CAD format. This cylinder CAD model was converted to a STL format then sliced into layers. Each layer was then filled with the designed scaffold pattern to generate toolpath file. The strands of PCL were extruded in four distinct layer patterns: 0, 90, 60, and 1208 (designated P1, P2, P3 and P4, respectively), or alternating layers of 608 /1208 with different gap lengths between the strands. The definition of scaffold layout pattern is shown in Figure 7.

A set of PCL scaffolds was fabricated using the PED system. The following processing parameters were used for all scaffold fabrication: the processing liquefier temperature 908 C, the orifice diameter of the tip 0.25mm, and deposition velocity at 20mm/s. The filling gaps of 0.42 and 0.51mm were applied for two different sets of scaffolds. For each scaffold, there are a total of 39 layers with each layer thickness at 0.254mm. The scaffold patterns were either 08 /908 (three samples), or 08 /1208 (three samples), or combined 08 /1 biocompatibility of the scaffold. Descriptions of the evaluations, results and experimental procedures are presented as follows.

FEI/Phillips XL-30 fi eld emission environmental scanning electron microscope (ESEM) was used to evaluate the microstructural formability and internal morphologies of as-fabricated scaffolds. The SEM images were taken using a beam intensity at 20.0 keV and the gaseous secondary electron detectors at 1.3Torr. The SEM images of scaffolds are shown in Figure 8A

(for 08 /908 deposition pattern) and Figure 8B (for 08 /1208 deposition pattern), along with the images of the as-fabricated scaffolds. Both SEM images clearly present that the fabricated microarchitecture of the scaffolds could be achieved at about 250 μm scale level. The good uniformity of the fill gaps and the depositing struts shown in Figures 8A and 8B, and the internal pore connectivity demonstrate the applicability of using the PED process to fabricate PCL scaffolds at micro-scale level.

Micro-CT enables 3D characterization of the salient features, the structural formability and the morphologies of the as-fabricated PCL scaffolds. The micro-CT was set at 19.1 μm resolution. 2D analyses and 3D reconstructions of core regions of the sample scaffolds were performed. These results illustrate that qualitative and quantitative analysis of polymer scaffolds is possible through micro-CT and 3D reconstruction techniques.

SkyScan 1072 micro-CT desktop scanner (Skyscan, Belgium) was used to scan the internal architecture of the scaffold. The output format for each sample was approximately 500 serial 1,024 x 1,024 bitmap images. These slice images were analyzed in SkyScan's Tview software. Initially, length measurements were taken around the sample to determine the degree to which the sample conformed to the cylindrical template. Volume analyses were performed on the center of each sample. Volume fraction and surface per unit volume were determined in 3D analysis, and relative area was measured in ten randomly selected slice images in 2D analysis. Ten strut and pore widths were measured in 2D images from each sample.

3D reconstruction was performed using Mimics software (Materialise, Belgium) with re-processing using ImageJ. Sixty-two sequential 200 x 200 pixel images were cropped from the serial images from the center of each sample. Imported into Mimics, these serial core images were reconstructed into 3D volumetric models. Thresholds were inverted to allow measurement of the volume of all pore spaces within the model. Subsequently, a region-growing operation was performed, creating a mask consisting only of interconnected pore spaces. Volume for this region-grown mask was determined and the ratio of region-grown volume to the total volume was calculated. The percentage of this ratio is defined as the degree of interconnectivity. A summary of data retrieved from the analysis through Tview is displayed in Table I. The results also include the measurements of individual strut and pore widths based on the 2D serial slice imaging. Ten measurements were taken for each sample in this analysis. 3D reconstructed models by using Mimics are displayed in Table I with a sample of corresponding2D images and the characterized porosity and interconnectivity of each sample.

The Instron 5800R machine was used for the test of as-fabricated scaffold. The initial strain rate of the tests was adjusted to 10 percent per minute at the beginning of the test and no preload was applied before initiating compression testing. Standard solid compression platens were used for testing. Stress-strain data were computed from load is placement measurements. Compressive modulus was determined based on the slope of the stress-strain curve in the elastic region. The data were corrected on the strain axis to a value of approximately 0.025 for calculating the compressive elastic modulus of the scaffold. The stiffness of the machine was also put into the calculations to decrease machine error.

Three SP-2 specimens were tested under compression to a limit of specific compressive displacements. The PCL specimens were measured for their dimensions for accurate area calculations. The scaffolds were cylindrically shaped with minute irregularities on the circumference wall due to specimen processing. The samples were tested at a speed of 0.1mm/ min at a room temperature of 24°C with a relative humidity of 15 percent.

The invention, Precision Extrusion Deposition and Cooling System, is an improvement to the previously disclosed Precision Extrusion Deposition system and provides an in-situ cooling step, wherein a coolant is used to speed up the physical solidification of scaffolding materials and provide milder temperatures for incorporating biomaterials simultaneously with depositing scaffolding materials.

Inventors have discovered that biomaterial sensitive to elevated temperatures (i.e., above 37°C) can be deposited together with the scaffolding material (e.g., PCL) without compromising its viability if the scaffolding material is cooled off during the deposition step. Without the additional cooling step, the scaffolding material is deposited on a surface at a room temperature as described in the article by Wang, et al., " Precision Extrusion Deposited Poly-Epsilon-Caprolactone Structures for Biological Applications Specification", Rapid Prototype Journal, Vol. 10, No. 1, pp. 42-49, 2004. Since the scaffolding material is being extruded at its glass transition temperature (about 90-60°C), it will take time to cool off to 37°C. Advantageously, the additional cooling step involving using a coolant addresses these shortcomings in several ways and provides a method of making more viable biomaterial containing scaffolds.

In certain embodiments, the scaffolding material can be deposited in a layer of a coolant, which is kept at a temperature below 20°C, wherein the coolant is, for example, a liquid, a foam or any other surface capable of being chilled. An example of a liquid is water and an example of a suitable foam is a surfactant. The coolant is preferably mixed with biomaterial such as, for example, cells, proteins, pharmacologically active agents, a component of a substrate-ligand

pair, etc.

The coolant is provided as a layer of a constant depth or multiple layers which can be added as needed during the deposition of the scaffolding material. As the scaffolding material is being dropped into the coolant, it forms a drop or a round shape rather than an irregular shaped drop formed without using a coolant. This is another advantage of using a coolant.

In certain embodiments, the scaffolding material can be deposited simultaneously with the coolant, wherein the coolant is preferably mixed with biomaterial such as, for example, cells. During such deposition, the scaffolding material is being chilled along the outer parts of its extruded mass. A nozzle for depositing the scaffolding material is positioned adjacent to two or more nozzles for depositing a coolant. In another variant, the nozzle for depositing the scaffolding material is nested inside a larger nozzle for depositing the coolant.

The above described ways of introducing a coolant can be combined. More than one type of the scaffolding material can be deposited. The scaffolding material can be deposited with other non-scaffolding material such as, for example, a filler material (e.g., hydrogel) which can also act as a coolant in some embodiments.

Non-limiting examples of the scaffold material are poly(capralactones), poly(lactic acid), PLGA, fibrin, collagen, hydroxyapatite, and other biologically useful materials. The scaffold materials are preferably biocompatible.

In preferred embodiments, the hydrogel is a member selected from the group consisting of alginate, collagen, chitosan, fibrin, hyaluronic acid, agar, polyethylene glycol and its copolymers, and acrylamide-based and acrylic acid-based polymers, and the scaffold material is a member selected from the group consisting of polycaproactone, tricalcium phosphate, hydroxyapatite, polyglycolic acid, polylactic acid, and their co-polymers, polyhydroxybutyrate, and polypropylene fumarate. The scaffold material can have a wide range of biodegradability, depending on the desired properties and purpose of the scaffold.

The scaffold material can also be combined with various additives to better suit the type of cell or tissue that is being used. For example, hydroxyapatite could be used when working with osteoblasts to create bone implant scaffolds. The scaffold could also be coated with proteins and receptors that facilitate cellular adhesion or migration onto the scaffold surface. Growth factors and other biologically active agents could also be included within the scaffold material.

Precision extrusion deposition (PED) of poly-ε-caprolactone (PCL) is a freeform fabrication technology allowing for the construction of tissue engineering scaffolds and other bioactive

structures with customizable macro- and microarchitectural characteristics. The ability to control porosity, pore size, and external shape is not found with the same flexibility in any alternative scaffold manufacturing technology. These capabilities are valuable in a number of research and commercial applications. In terms of research, the ability to create tissue engineering scaffold specimens with precise geometry is ideal for cell experimentation, in vivo experimentation, and studies of nutrient transfer. The market for research tissue engineering scaffolds is estimated at 1.05 million dollars for 2005. Potential clinical applications of such scaffolds include a range of regenerative medicine treatments including bone repair, cartilage repair, plastic surgery, dental repair, and eventually transplantation of complex tissues and organs. In terms of the bone market alone, 5-10% of all fractures are delayed healing or non-union and hence candidates for scaffold-based regenerative medicine. With the aging of the population, that is expected to rise to 20% by 2025. All gross tissue/organ replacement therapies in the world currently constitute a $300 billion annual market, with an estimated increase of 10-11% per year. PED fabricated biocompatible structures present a unique approach applicable to a broad range of these therapies. In another commercial application, PED fabricated PCL structures are ideal for mass production of anchorage dependent cells as microcarrier beads. Currently, microcarrier beads such as CYTODEX and CULTISPHER are readily available, but no single product possesses the rigidity and surface area offered by PED-manufactured PCL structures. The technology is at an advanced state of development, with architectural abilities of the prototype system well characterized, in vitro cell culture demonstrated, in vivo culture under way, and microcarrier bead research pending. Sales of scaffolds to the academic research market could begin with three months preparation.

Hydrogels such as fibrin and alginate present the potential for volumetric cell immobilization and attachment, but their mechanical properties are inherently weak, and the structures buildable through mold fabrication of hydrogels are limited. Suspension in a rigid framework of makes hydrogels much more versatile for biological applications, but the framework itself must have finely controlled internal and external architecture. Precision extrusion deposition (PED) of poly-ε-caprolactone (PCL) is a freeform fabrication technology allowing for the construction of tissue engineering scaffolds and other bioactive structures with customizable macro- and microarchitectural characteristics. The ability to control porosity, pore size, and external shape is not found with the same flexibility in any alternative scaffold manufacturing technology. Once the rigid framework is constructed, alginate or fibrin may be gelled within the scaffold pores, allowing for volumetric cell immobilization and cell attachment. These capabilities are valuable in a number of research and commercial applications. In terms of research, the ability to create tissue

engineering scaffold specimens with precise geometry is ideal for cell experimentation, in vivo experimentation, and studies of nutrient transfer. The ability to immobilize cells 3-dimensionally is ideal for uniformly seeding tissue constructs for clinical implantation. Potential clinical applications of such scaffolds include a range of regenerative medicine treatments including bone repair, cartilage repair, plastic surgery, dental repair, and eventually transplantation of complex tissues and organs. In terms of the bone market alone, 5-10% of all fractures are delayed healing or non-union and hence candidates for scaffold-based regenerative medicine. With the aging of the population, that is expected to rise to 20% by 2025. All gross tissue/organ replacement therapies in the world currently constitute a $300 billion annual market, with an estimated increase of 10-11% per year. Hydrogel-filled PED fabricated biocompatible structures present a unique approach applicable to a broad range of these therapies. The scaffold manufacture technology is at an advanced state of development, with architectural abilities of the prototype system well characterized, in vitro cell culture demonstrated, and in vivo culture pending. Sales of scaffolds to the academic research market could begin with three months preparation.

Preliminary biological experiments have been conducted to study the basic scaffold biocompatibility. These experiments were intended to address the issue of free radicals caused by heating of the polymer and whether these radicals would be detrimental to cell growth. Another question was whether the pore size of approximately 250 μm would be conducive to cell growth alone or would require a filler material.

Cardiomyoblasts (H9C2) were seeded onto three sets of 908 scaffolds, one set with no filler material, one with collagen filling the pores, and one with fibrin gel filling the pores. Initial seeding size was approximately 105. All samples showed cell attachment to the scaffold on the fifth day, and a monolayer of cells atop the scaffold sample. The confluence of the monolayer atop the scaffold on the fifth day indicates uninterrupted cell growth.

The presence of a material greatly enhanced proliferation and differentiation of cells, as indicated by the visible cellular processes. This would indicate that a 250 μm pore size may be too large enough to optimally enhance cardiomyoblast growth. While 250 μm is ideally suited for the free diffusion of oxygen, the cells were unable to cross the pore spaces without the presence of a fibrin gel filler material. Currently, quantitative proliferation and attachment studies are being performed for three cell types with cytofluorimetry, fluorescence microscopy, and immunofluorescence as methods of analysis.

For the comparative proliferation study, 10^4 cardiomyoblasts, fibroblasts, and smooth muscle cells were seeded upon 90°PCL scaffolds of 1mm thickness without filler material. Cell

numbers were measured using Alamar blue staining and cytofluorimetry. Initial results showed that anchorage-dependent cells such as fibroblasts are better able to take advantage of the scaffold microarchitecture without a filler material. The fibroblasts attach to the surfaces preferentially compared to polystyrene and progressively grow into the pore space, narrowing the channel. Qualitatively, the ingrowth of the fibroblasts is such that it may close off pores completely. While the sample scaffolds for this proliferation study were thin (1mm), the fibroblast ingrowth may reduce nutrient flow in thicker scaffolds.

A study on using PED process to freeform fabricate cellular PCL scaffolds and on using SEM, micro-CT and the experimental testing to characterize the morphology, internal geometry, mechanical property and biological compatibility of the as-fabricated scaffolds were conducted. Both hardware and software configuration of the PED process system were described and the PCL scaffolds with controlled internal architectures were produced. Results of the characterization demonstrated the capability of the PED fabrication process in manufacturing the PCL scaffolds with microstructure and pore size at about 250 μm scale. This process directly fabricates tissue scaffolds by converting designed architecture into layered deposition pattern without involving the material preparation and in-direct casting, and thus opens opportunities for complex scaffold fabrication. Results of the characterization also show that micro-CT is a capable tool for nondestructive evaluation of PCL scaffolds. The use of 2D analysis and 3D reconstruction software allows the examination of morphologies, internal architecture, the interconnectivity of as fabricated tissue scaffolds, and provides a quantitative measurement of porosity and micro-architecture. As shown in the analysis, a typical pore size of the fabricated scaffold range from 200 to 300 μm, near the optimal size suggested for bone tissue scaffold application. In addition, strut width is consistent between samples, all samples showed greater than 98 percent interconnectivity. The scaffold compression modulus obtained from the test is in the range between 150 and 200MPa. The preliminary result of biological experiments demonstrated the biocompatibility of the process and material. All these suggest the viability of the fabrication and the characterization process, as well as its potential applications in tissue engineering.

The dominant approach in 3D tissue engineering is to construct a scaffold of biocompatible material, to seed the scaffold with an appropriate cell type, to culture these cells in a bioreactor, and to implant the resulting tissue construct. Numerous individual materials have been investigated, but no single material has proven ideal for tissue culture. Inventors propose the use of multiple materials within a single scaffold. Such scaffolds can be produced

using a 3D positioning system possessing multiple heads, capable of both fused deposition and droplet deposition of multiple materials.

While the invention has been described in detail and with reference to specific examples thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof.

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CLAIMS

WHAT IS CLAIMED IS:

1. A microcarrier bead comprising a porous three-dimensional core having at least 99% interconnected pores and an outer protective layer.

2. The microcarrier bead of claim 1, further comprising a filler material in communication with the polymeric porous three-dimensional core.

3. The microcarrier bead of claim 1, wherein the porous three-dimensional core is made from a scaffold material comprising a biodegradable polyester, fibrin, collagen, hydroxyapatite, and mixtures thereof.

4. The microcarrier bead of claim 3, wherein the scaffold material is at least one of poly-caprolactone, polylactic acid, polyglycolic acid, and polylactide co-glucolide.

5. The microcarrier bead of claim 1, wherein the filler material is at least one of fibrin, collagen, and dextran.

6. The microcarrier bead of claim 1, wherein the outer material is hydrogel.

7. The microcarrier bead of claim 1, wherein the scaffold material comprises a biodegradable polyester and the outer material comprises alginate.

8. The microcarrier bead of claim 1, wherein the porous three-dimensional core is made by depositing poly-caprolactone using in a layered pattern such that at least 99% of pores is interconnected, and substantially all pores lead to the external surface of the microcarrier bead.

9. The microcarrier bead of claim 1, further comprising cells.

10. An assembly of microcarrier beads of claim 1.

11. A method for making an artificial scaffold, the method comprising:

providing a scaffolding material;

providing a coolant having a temperature of at most 20°C, provided that the scaffolding material has a temperature of at least 25°C; and

extruding the scaffolding material into the coolant and thereby making the artificial scaffold.

12. The method of claim 11, wherein extruding the scaffolding material into the coolant is performed in a layered pattern such that the artificial scaffold has a porous three-dimensional core.

13. The method of claim 12, wherein at least 99% of pores in the porous three-dimensional core are interconnected and substantially all pores lead to the external surface of the porous three-dimensional core.

14. The method of claim 11, wherein the artificial scaffold comprises an artificial tissue.

15. The method of claim 11, wherein the scaffolding material comprises a biodegradable polyester, fibrin, collagen, hydroxyapatite, and mixtures thereof.

16. The method of claim 11, wherein the coolant is at least one of a liquid, a foam, and a gel.

17. The method of claim 11, wherein the coolant comprises hydrogel.

18. The method of claim 11, wherein at least one of the scaffolding material or the coolant comprises a biomaterial.

19. A microcarrier bead made by the method of claim 11.

20. An artificial tissue made by the method of claim 11.

21. In a process for manufacturing complex parts and devices comprising (a) utilizing a computer aided design program to design a part or device to be created; (b) converting the computer aided design program designed part or device into a heterogeneous material and multi-part assembly model which can be used for multi-nozzle printing; and (c) printing the designed part or device using different, specialized nozzles, the improvement comprising extruding a scaffolding material into a coolant having a temperature of at most 20°C.

22. A method of growing cells, the method comprising:

providing the microcarrier bead of claim 1; and

providing cells.

FIG. 1

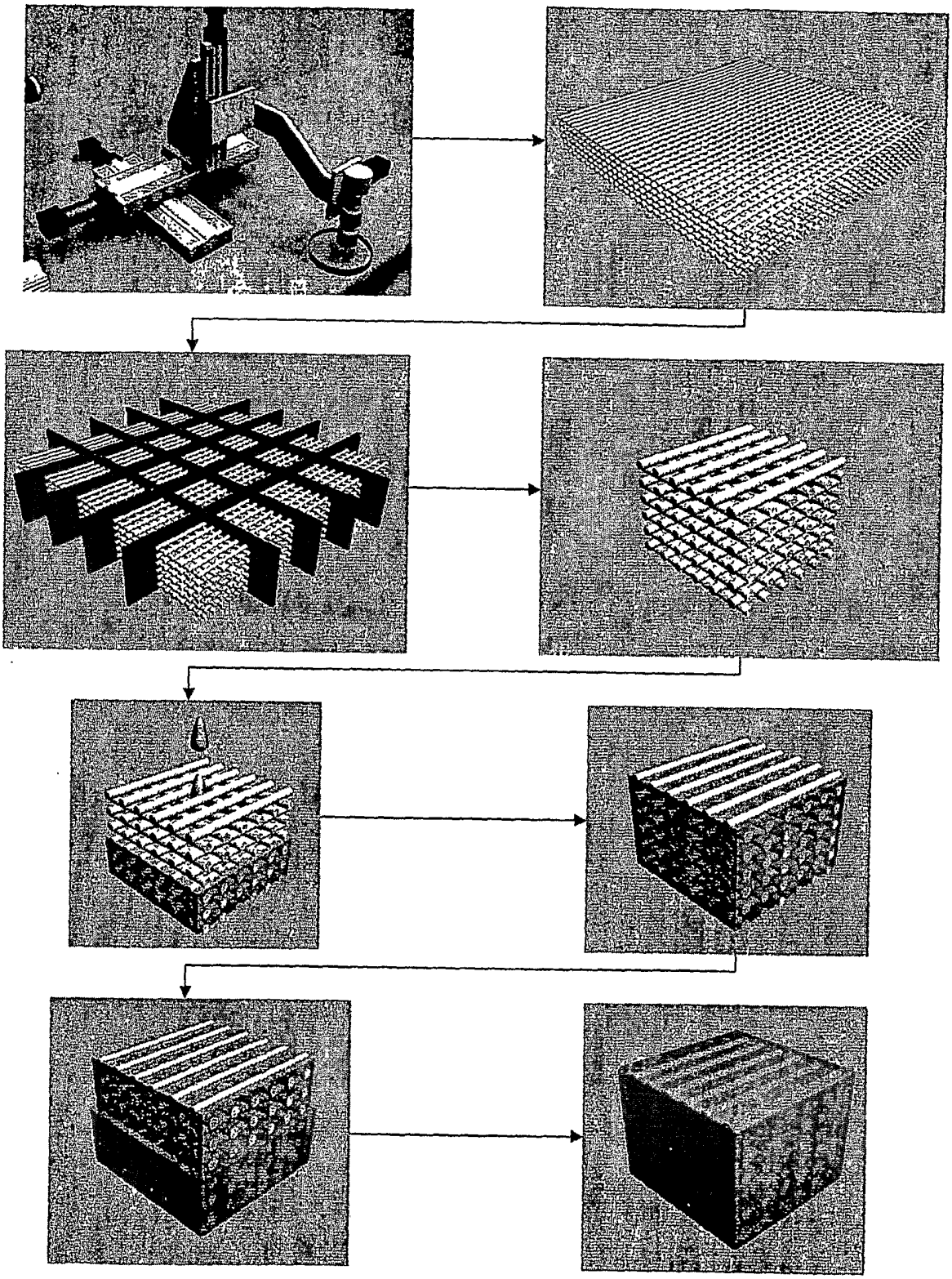


FIG.1 Continued

Porous block of PCL is freeform fabricated using PED system

Precision Extrusion Deposition of Block
Parameters:

- Strut size
- Strut orientation
- Porosity
- Bead height
- Material (PCL, PCL/HA, etc.)

The porous block is cut into individual microcarrier beads
Cut-out of Individual Beads
Parameters:

- Bead external geometry (cylinder, cube, etc.)

A filler is introduced to the bead either through gelation of a liquid such as fibrin with thrombin or direct introduction of a low viscosity material such as collagen. If no filler is used, cells must still be seeded at this stage.

Filler Material (optional)

Parameters:

- Filler material for cell attachment (e.g. fibrin or collagen)
- Filler material for cell immobilization(e.g. fibrin with embedded cells)

A film of alginate is created over the bead (filled or unfilled) by sequential dipping in sodium alginate solution and calcium chloride.

Alginate Coating (optional but recommended)

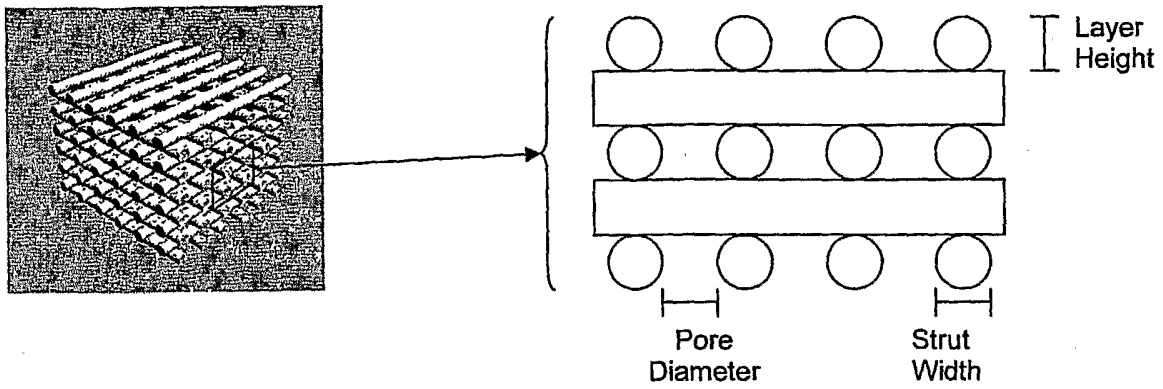
Parameters:

- Thickness of alginate coating
- Alginate concentration

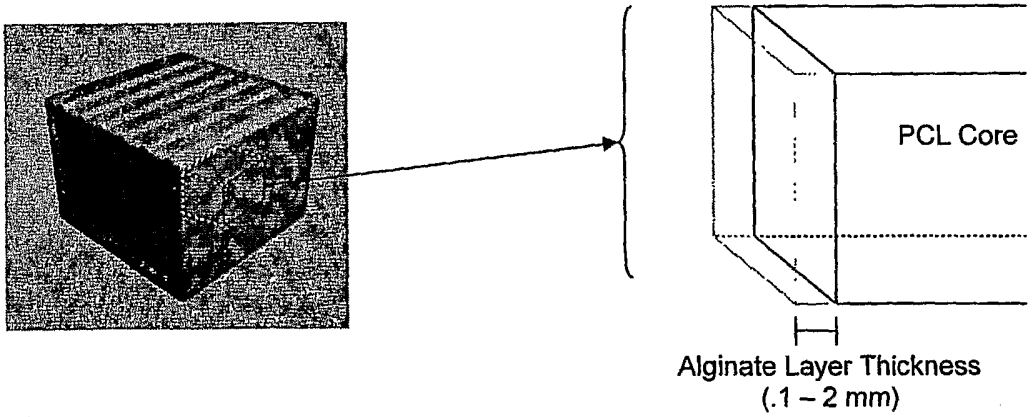
FIG. 2

Super-Sparger Micro-Carrier Bead Structure

Rigid PCL component



Protective Alginate Film



4/11
Fig.3

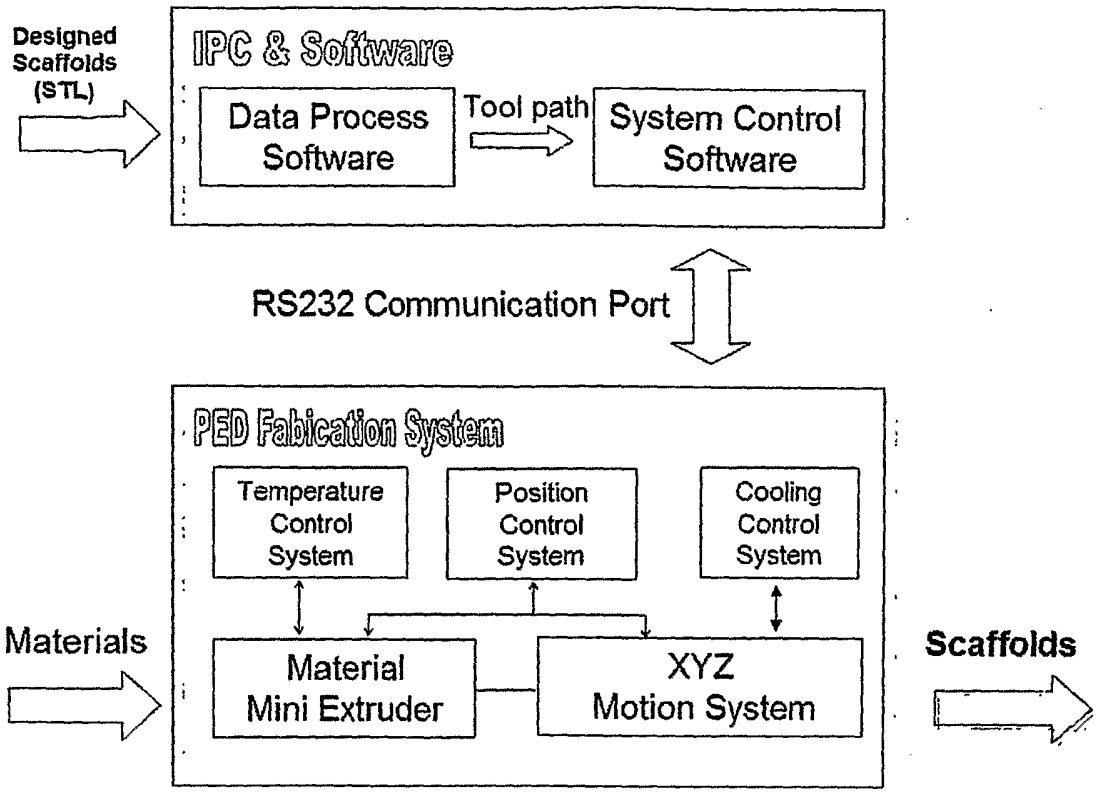

Designed Scaffolds (STL)
IPC & Software
Data Process Software
Tool path
System Control Software
RS232 Communication Port
PED Fabication System
Temperature Control System
Position Control System
Cooling Control System
Materials
Material Mini Extruder
XYZ Motion System
Scaffolds

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Fig.4

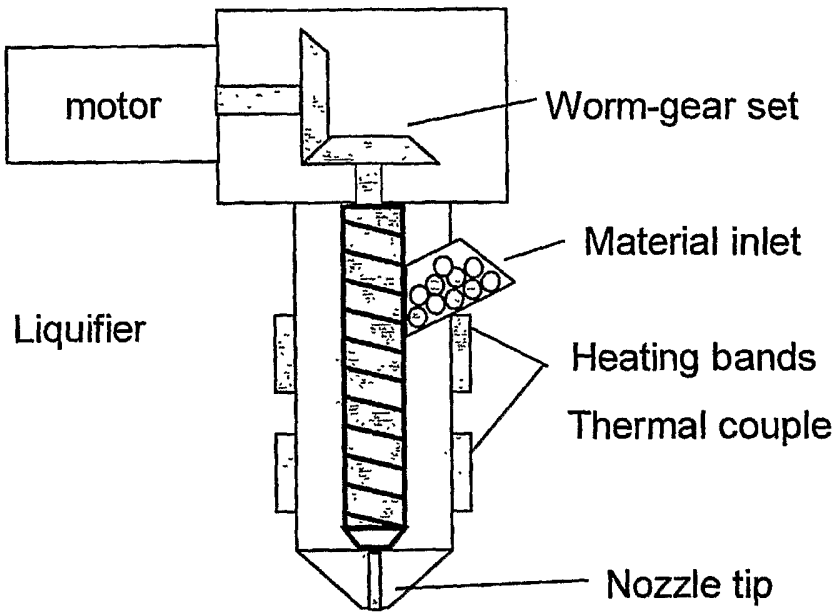
motor
Worm-gear set
Material inlet
Liquifier
Heating bands
Thermal couple
Nozzle tip

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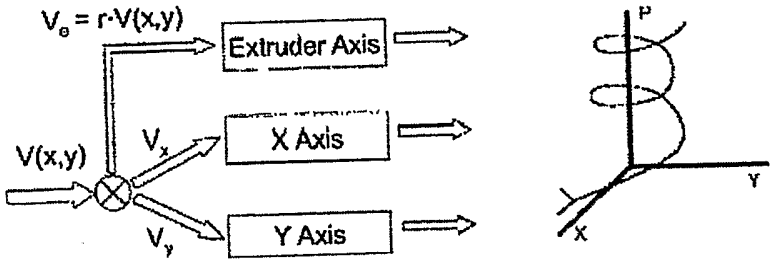
Fig. 5



7/11
Fig. 6

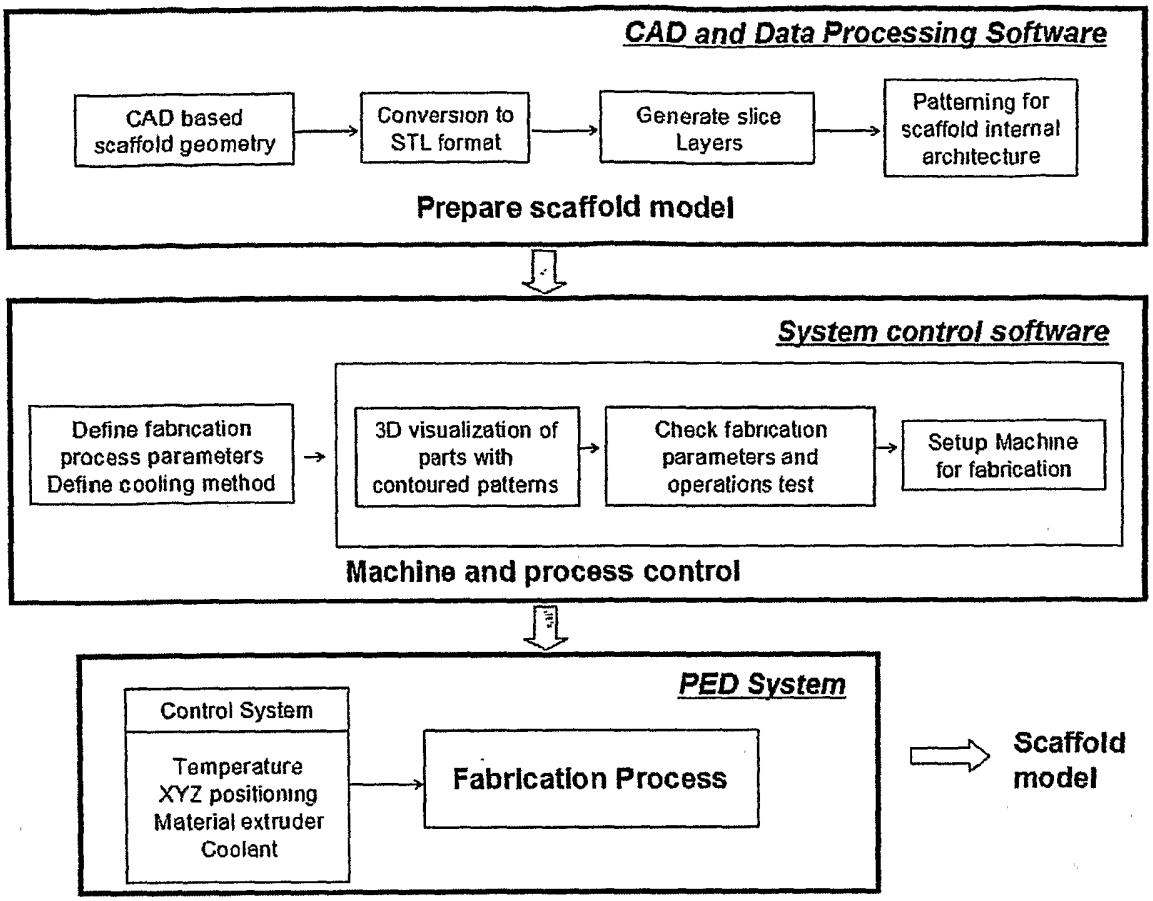
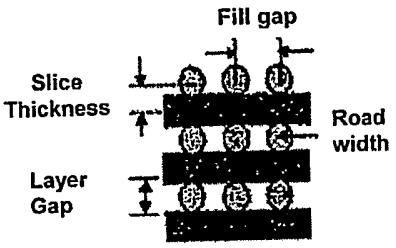
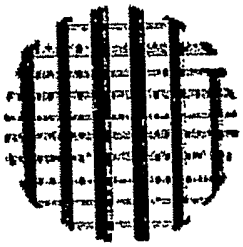

CAD and Data Processing Software
CAD based scaffold geometry
Conversion to STL format
Generate slice Layers
Patterning for scaffold internal architecture
Prepare scaffold model
System control software
Define fabrication process parameters
Define cooling method
3D visualization of parts with contoured patterns
Check fabrication parameters and operations test
Setup Machine for fabrication
Machine and process control
PED System
Control System
Temperature
XYZ positioning
Material extruder
Coolant
Fabrication Process
Scaffold model

Fig. 7



Top: pattern definition
Top right: 0/90 pattern
Bottom Right: 0/120 pattern



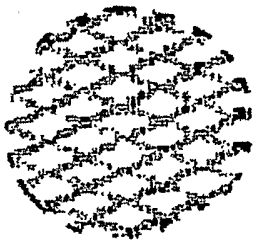


Fig. 8A

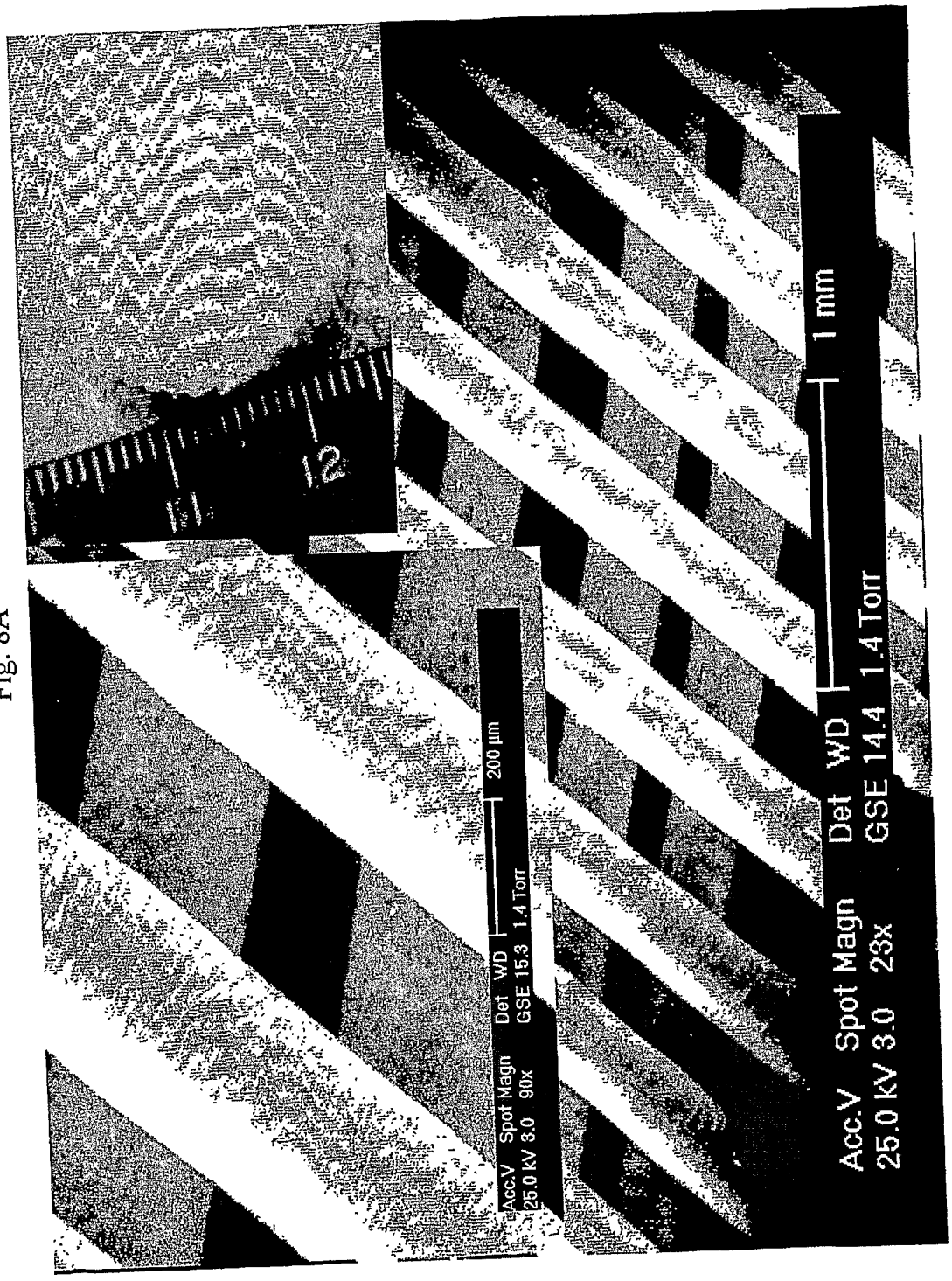
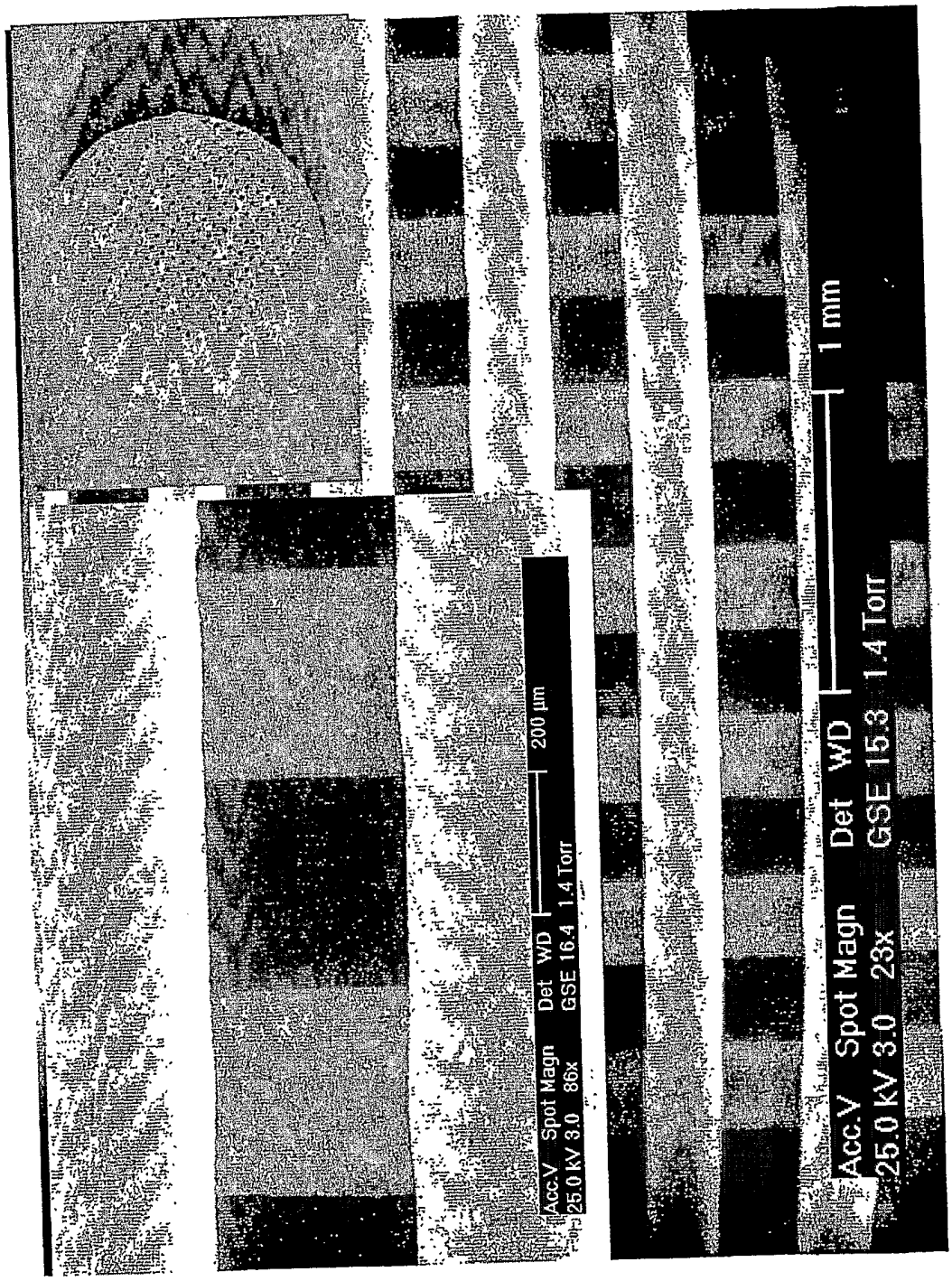

Acc.V Spot Magn Det WD
25.0 kV 3.0 23x GSE 14.4 1.4 Torr
1 mm
Acc.V Spot Magn Det WD
25.0 kV 3.0 90x GSE 15.3 1.4 Torr
200 µm

Fig. 8B


1 mm
Acc.V Spot Magn Det WD
25.0 kV 3.0 23x GSE 15.3 1.4 Torr
200 µm
Acc.V Spot Magn Det WD
25.0 kV 3.0 86x GSE 16.4 1.4 Torr

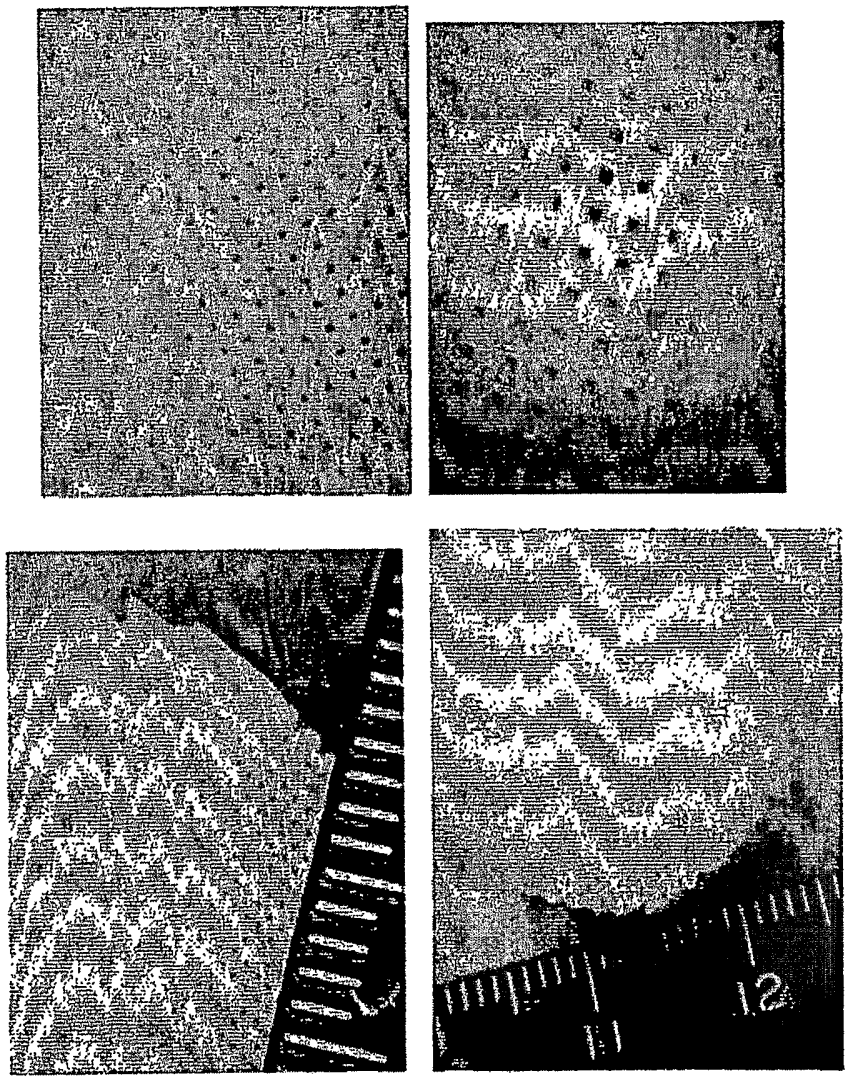


Fig. 9