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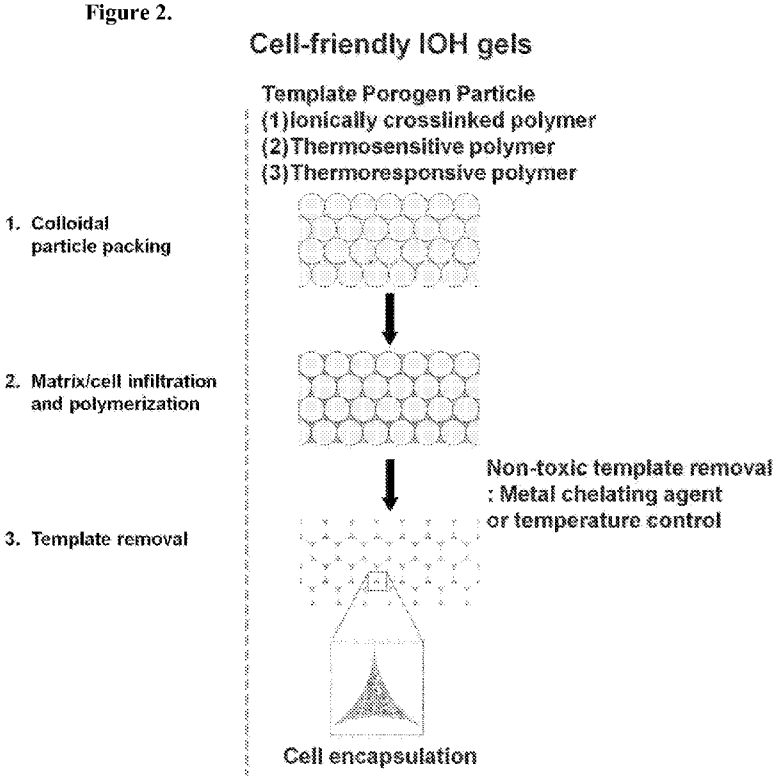
(54) Title: CELL-FRIENDLY INVERSE OPAL HYDROGELS FOR CELL ENCAPSULATION, DRUG AND PROTEIN DELIVERY, AND FUNCTIONAL NANOPARTICLE ENCAPSULATION

Figure 2.



(57) Abstract: The invention provides polymer scaffolds for cell-based tissue engineering.

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CELL-FRIENDLY INVERSE OPAL HYDROGELS FOR CELL ENCAPSULATION, DRUG AND PROTEIN DELIVERY, AND FUNCTIONAL NANOPARTICLE ENCAPSULATION

RELATED APPLICATIONS

This application claims the benefit of priority under 35 U.S.C. § 119(e) to U.S. Provisional Application No: 61/479,774, filed April 27, 2011, which is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

The invention relates to polymer scaffolds for cell-based tissue engineering.

BACKGROUND

Tissue engineering is an approach for regeneration, replacement, and improvement of the functions of damaged tissues by manipulating materials according to the specific structure or function of the desired tissues. Porous and biodegradable polymer scaffolds, *e.g.*, three dimensionally interconnected scaffolds, are utilized as a structural supporting matrix or as a cell adhesive substrate for cell-based tissue engineering. A highly open porous structure with interconnected pores is required to achieve sufficient cell seeding and migration within the scaffold, as well as to facilitate mass transfer of nutrients, oxygen, and metabolite waste for sequential proliferation and differentiation of large quantity of cells. Current approaches to generate porous networks in polymer scaffolds include gas foaming, salt leaching, and freeze drying; however, the limitations of those processes include irregular pore sizes, shapes, and structures, as well as limited interconnectivity. As such, there is a pressing need in the art to develop improved structured polymer scaffolds with interconnected pores.

SUMMARY OF THE INVENTION

The invention described herein provides the fabrication of cell-friendly inverse opal hydrogels that also allow cell-encapsulation in the hydrogel matrix. An inverse opal hydrogel scaffold device comprising a polymer matrix and a sacrificial porogen in which the porogen comprises an ionically-crosslinked polymer, a thermosensitive polymer, a thermoresponsive polymer, a pH-sensitive polymer, or a photocleavable polymer. The polymer matrix is made of a durable polymer relative to the sacrificial porogen such that the

polymer matrix withstands physical or chemical changes that cause porogen sacrifice. For example, polymer matrix is covalently crosslinked, withstands a change (e.g., increase) in temperature, withstands a pH change (e.g., decrease) or change in ionic strength or composition (e.g., contact with a divalent cation chelator), or withstands exposure to light (e.g., UV light).

For tissue engineering and cell scaffold applications, the polymer matrix further comprises an isolated cell, e.g., a eukaryotic cell. By "isolated cell" is meant a cell that has been separated from the other cells, components, and/or environment that naturally accompany it. Alternatively, the matrix contains prokaryotic cells such as bacteria. For example, the polymer matrix is crosslinked and comprises an isolated cell encapsulated in the crosslinked polymer matrix. An exemplary polymer matrix comprises a synthetic polymer such as one that is covalently crosslinked. Examples of polymer matrices include poly(lactide-coglycolide) (PLGA; a copoly lactic acid/glycolic acid polymer), poly(acrylic acid), polyethylene glycol (PEG), poly (vinyl alcohol), or polyphosphazene.

The sacrificial porogen comprises an ionically-crosslinked polymer, a thermosensitive polymer, a thermoresponsive polymer, a pH-responsive polymer, or a photo-cleavable polymer. Exemplary polymers for a porogen include alginate, collagen, gelatin, fibrin, agarose, hyaluronic acid, or chitosan as well as thermosensitive polymer such as agarose, gelatin, or collagen, poly(N-isopropylacrylamide), poly(N-ethylacrylamide), poly(N-cyclopropymethacrylamide), poly(N-methyl-N-ethylacrylamide), poly(N-acryloylpyrrolidine), poly(N-ethylmethacrylamide), poly(N-cyclopropylacrylamide), poly(N-cyclopropylacrylamide), poly(N,N-diethylacrylamide), poly(N-isopropylacrylamide), poly(N-vinylcaprolactam), poly(N-n-propylmethacrylamide), poly(N-methyl-N-isopropylacrylamide), poly(N-n-propylacrylamide), poly(N-methyl-N-n-propylacrylamide), and poly(N-acryloylpiperidine).

Hydrogel (also called aquagel) is a network of polymer chains that are hydrophilic, and are sometimes found as a colloidal gel in which water is the dispersion medium. Hydrogels are highly absorbent (they can contain over 99% water) natural or synthetic polymers. Hydrogels also possess a degree of flexibility very similar to natural tissue, due to their significant water content. Hydrogel shaped as an inverted opal exhibits much higher swelling ratios, and its swelling kinetics is an order of magnitude faster as well. The engineered scaffolds (*i.e.*, inverse opal hydrogels) described herein possess desirable mechanical and optical properties that can facilitate tissue regeneration while allowing for

continuous high-resolution optical monitoring of cell proliferation and cell–cell interaction within the scaffold.

Methods of producing an inverse opal hydrogel with open, interconnected pores are carried out by compressing a plurality of template porogen particles into a mold, and subsequently adding a composition comprising a polymer solution and a plurality of cells to the interstitial space between template porogen particles in the mold to polymerize the template porogen particles. The template porogen particles are removed from the mold, thereby producing an inverse opal hydrogel with open, interconnected pores, wherein the cells are encapsulated in the inverse opal hydrogel. The template porogen particles are removed without using toxic organic solvents or lyophilization. For example, thermosensitive hydrogel beads are removed by controlling the temperature to change the solid phase of the beads. The template porogen particle is an ionically crosslinked polymer, a thermosensitive polymer, a thermoresponsive polymer, a pH-responsive polymer, or a photo-cleavable polymer.

For example, the ionically crosslinked polymer is alginate. The ionically crosslinked polymer is removed by adding a metal-chelating agent selected from the group consisting of citric acid, ethylenediamine, ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTPA), and N,N-bis(carboxymethyl)glycine (NTA).

Suitable thermosensitive polymers include agarose, gelatin, and collagen. The thermosensitive polymer is removed by increasing the temperature of the polymer, thereby altering the phase of the polymer to liquid phase. Examples of photocleavable polymers include chromophore-based crosslinkers for photodegradable hydrogels, (4-vinylpyridine) (P4VP) and poly (methylmethacrylate).

Thermoresponsive polymers include poly(N-isopropylacrylamide), poly(N-ethylacrylamide), poly(N-cyclopropymethacrylamide), poly(N-methyl-N-ethylacrylamide), poly(N-acryloylpyrrolidine), poly(N-ethylmethacrylamide), poly(N-cyclopropylacrylamide), poly(N-cyclopropylacrylamide), poly(N,N-diethylacrylamide), poly(N-isopropylacrylamide), poly(N-vinylcaprolactam), poly(N-n-propylmethacrylamide), poly(N-methyl-N-isopropylacrylamide), poly(N-n-propylacrylamide), poly(N-methyl-N-n-propylacrylamide), and poly(N-acryloylpiperidine). Thermoresponsive polymers are removed by increasing the temperature above a lower critical solution temperature (LCST) to reduce the size of the template particles.

The hydrogels described herein have open, interconnected pores of various diameters, e.g., 1 μm pores, 10 μm pores, 50 μm pores, 100 μm pores, 250 μm pores, 500 μm

pores, 750 μm pores, 1,000 μm pores, 1,500 μm pores, 2,000 μm pores, 2,500 μm pores, or 3,000 μm pores. Exemplary hydrogels have pores that are 600 μm, 1000 μm, or 1,500 μm in diameter.

Cell-adhering peptides such as Arg-Gly-Asp (RGD) are optionally used to modify the hydrogels described herein. In some cases, the hydrogels described herein comprise a first and a second plurality of cells. For example, the first plurality of cells is comprised within the hydrogel matrix, and the second plurality of cells is added to the open, interconnected pores. The first plurality of cells and the second plurality of cells are selected from the group consisting of mesenchymal stem cells, stromal cells, cancer cells, dendritic cells, macrophages, neutrophils, natural killer cells, or fibroblast cells. Preferably, the first plurality of cells and the second plurality of cells are different cell types.

Methods of producing an inverse opal hydrogel with open, interconnected pores are carried out by compressing a plurality of template porogen particles into a mold, adding a composition comprising a polymer solution and an agent to the interstitial space between template porogen particles in the mold to polymerize the template porogen particles, and removing the template porogen particles, thereby producing an inverse opal hydrogel with open, interconnected pores. The agent is selected from the group consisting of a drug, a nanoparticle (e.g., magnetic nanoparticles or gold nanoparticles), a growth factor (e.g., vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), brain derived neurotrophic factor (BDNF), epidermal growth factor (EGF), or fibroblast growth factor (FGF)), a cytokine (e.g., interferon gamma (IFN-γ), erythropoietin (EPO), thrombopoietin (TPO), interleukin-1 (IL-1), IL-4), a chemokine (e.g., a CC chemokine, a CXC chemokine, a C chemokine, or a CX3C chemokine), a hormone (e.g., insulin, growth hormone, vasopressin, testosterone, or cortisol), a protein, a nucleic acid, or a small molecule. In one example, nanoparticles are encapsulated within the hydrogel matrix, and cells are dispersed within the open-interconnected pores.

Compositions comprising an inverse opal hydrogel with open, interconnected pores are produced by the methods described above.

For example, provided is a composition comprising an inverse opal hydrogel with open, interconnected pores comprising a first plurality of cells encapsulated in a hydrogel matrix and a second plurality of cells in the open, interconnected pores, wherein the first plurality of cells encapsulated in the hydrogel matrix occupy an interstitial space between the open, interconnected pores. The first plurality of cells and the second plurality of cells are selected from the group consisting of mesenchymal stem cells, stromal cells, cancer cells,

dendritic cells, macrophages, neutrophils, natural killer cells, or fibroblast cells. For example, the hydrogel comprises gelatin or poly(ethylene glycol) (PEG).

By "substantially pure" is meant a nucleic acid, polypeptide, or other molecule that has been separated from the components that naturally accompany it. Typically, the polynucleotide, polypeptide, or other molecule is substantially pure when it is at least 60%, 70%, 80%, 90%, 95%, or even 99%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. For example, a substantially pure polypeptide may be obtained by extraction from a natural source, by expression of a recombinant nucleic acid in a cell that does not normally express that protein, or by chemical synthesis.

A small molecule is a compound that is less than 2000 daltons in mass. The molecular mass of the small molecule is preferably less than 1000 daltons, more preferably less than 600 daltons, *e.g.*, the compound is less than 500 daltons, 400 daltons, 300 daltons, 200 daltons, or 100 daltons.

The transitional term "comprising," which is synonymous with "including," "containing," or "characterized by," is inclusive or open-ended and does not exclude additional, unrecited elements or method steps. By contrast, the transitional phrase "consisting of" excludes any element, step, or ingredient not specified in the claim. The transitional phrase "consisting essentially of" limits the scope of a claim to the specified materials or steps "and those that do not materially affect the basic and novel characteristic(s)" of the claimed invention.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims. Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All published foreign patents and patent applications cited herein are incorporated herein by reference. Genbank and NCBI submissions indicated by accession number cited herein are incorporated herein by reference. All other published references, documents, manuscripts and scientific literature cited herein are incorporated herein by reference. In the case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic of the utilization of conventional inverse opal hydrogels (IOHs) prior to the invention described herein.

Figure 2 is a schematic showing "cell-friendly" IOHs produced by the methods described herein.

Figure 3 is a series of photomicrographs demonstrating that ethylenediaminetetraacetic acid (EDTA) efficiently dissolves alginate beads in IOHs.

Figure 4a is a series of photomicrographs showing alginate/gelatin composites with different shapes. Figure 4b is a series of photomicrographs showing disc-shaped porous IOHs with different pore sizes. Figure 4c is a photomicrograph showing poly(ethylene glycol) (PEG) IOHs.

Figure 5a is a series of photomicrographs showing cell viability after treatment with EDTA for up to 3 hours. Figure 5b is a bar chart showing about 98% viability of cells after 3 hour incubation in EDTA.

Figure 6a is a series of photomicrographs showing cell viability after treatment with EDTA for up to 7 days. Figure 6b is a bar chart demonstrating the proliferation of cells encapsulated in IOHs.

Figure 7a is a series of photomicrographs demonstrating that the density of cells on the surface of IOHs increased 4 days post seeding. Figure 7b is a bar chart showing that cell number in cell-seeded IOHs increased over time.

Figure 8 is a series of photomicrographs showing the proliferation of cells on the surface of PEG without (Figure 8a) or with (Figure 8b) Arg-Gly-Asp (RGD) modification.

DETAILED DESCRIPTION

The hydrogel compositions described herein provide a mechanically robust, defined micro-environment for ex vivo cell loading and/or in vivo cell infiltration, as well as adhesion and motility cues to support cell migration and interactions. The cell-compatible or cell-friendly polymer structures, macroporous "inverse opal" hydrogels, comprises interconnected arrays of pores to accommodate the size of a eukaryotic cell. "Opal" refers to the crystalline array of close-packed spheres and "inverse" denotes that this array contains negative space, or pores.

The invention provides the fabrication of inverse opal hydrogels, e.g., composed of gelatin or poly(ethylene glycol) (PEG), that allow not only seeding of cells on porous hydrogels, but also encapsulation of cells in a hydrogel matrix. The elasticity of inverse

opal hydrogels was controlled and the hydrogels were modified with the cell-adhering peptide, Arg-Gly-Asp (RGD). The use of the inverse opal hydrogel as 3D scaffolds was evaluated in a culture of mesenchymal stem cells encapsulated in matrix and seeded on pores of the hydrogel. Furthermore, the hydrogel systems described herein are used for the examination of tumor-stromal interactions.

Tissue engineering is a promising approach for regeneration, replacement, or improvement of the functions of damaged tissues by manipulating materials according to the specific structure or function of the desired tissues (R. Langer, Adv Mater 2009, 21, 3235). Porous and biodegradable polymer scaffolds, especially three-dimensionally interconnected scaffolds, has been examined for use as a structural supporting matrix or as a cell adhesive substrate for cell based tissue engineering (S. J. Hollister, Adv Mater 2009, 21, 3330). A highly open porous structure with well interconnected pores is required to achieve sufficient cell seeding and migration within the scaffold as well as to facilitate mass transfer of nutrients, oxygen, and metabolite waste for sequential proliferation and differentiation of large quantity of cells. Various approaches have been proposed to generate porous networks in polymer scaffolds, including gas foaming (D. J. Mooney et al., Biomaterials 1996, 17, 1417; L. D. Harris, et al., J Biomed Mater Res 1998, 42, 396; Y. S. Nam, et al., Journal of Biomedical Materials Research 2000, 53, 1), salt leaching (M. H. Sheridan, et al., J Control Release 2000, 64, 91; L. Lu, et al., Biomaterials 2000, 21, 1837; C. J. Liao, et al., Journal of Biomedical Materials Research 2002, 59, 676), and freeze drying (P. X. Ma, R. Zhang, J Biomed Mater Res 1999, 46, 60; K. Whang, et al., Biomaterials 2000, 21, 2545; A. J. Thornton, et al., Transplantation 2004, 77, 1798). However, prior to the invention described herein, the substantial limitations of current methods include irregular pore sizes, shapes, and structures, as well as limited connectivity.

Prior to the invention described herein, inverse opal structured polymer scaffolds were proposed to provide uniform pore size and 3-dimensional pore interconnectivity for cell culture (N. A. Kotov, Y. F. Liu, S. P. Wang, C. Cumming, M. Eghtedari, G. Vargas, M. Motamedi, J. Nichols, J. Cortiella, Langmuir 2004, 20, 7887; Y. F. Liu, S. P. Wang, J. W. Lee, N. A. Kotov, Chemistry of Materials 2005, 17, 4918; Y. J. Zhang, S. P. Wang, M. Eghtedari, M. Motamedi, N. A. Kotov, Advanced Functional Materials 2005, 15, 725; A. N. Stachowiak, D. J. Irvine, Journal of Biomedical Materials Research Part A 2008, 85A, 815; S. W. Choi, J. W. Xie, Y. N. Xia, Advanced Materials 2009, 21, 2997).

Prior to the invention described herein, solid beads, such as polystyrene (PS), poly (methyl metalcrylate) (PMMA) or poly(caprolactone) (PCL) were used as sacrificial

templates, while silicate, polyacrylamide (PAM), chitosan, poly(ethylene glycol) (PEG), or poly(lactic-co-glycolic acid) (PLGA) were used as the polymer matrix (Figure 1). However, in the conventional inverse opal hydrogel systems described previously, it was necessary to use toxic organic solvents or acidic solutions to remove the template beads and/or to use a freeze drying process, which preclude the possibility of cell encapsulation in the inverse opal polymer matrix (Figure 1). The resulting conventional hydrogels were only used for cell-seeding on the surface of pores after fabrication.

Prior to the invention described herein, cells could not be encapsulated in porous scaffolds prepared by other conventional fabrication methods due to the use of organic solvents, freeze drying of scaffolds, or high pressure of gas (D. J. Mooney, D. F. Baldwin, N. P. Suh, J. P. Vacanti, R. Langer, Biomaterials 1996, 17, 1417; L. D. Harris, B. S. Kim, D. J. Mooney, J Biomed Mater Res 1998, 42, 396; Y. S. Nam, J. J. Yoon, T. G. Park, Journal of Biomedical Materials Research 2000, 53, 1; M. H. Sheridan, L. D. Shea, M. C. Peters, D. J. Mooney, J Control Release 2000, 64, 91; L. Lu, S. J. Peter, M. D. Lyman, H. L. Lai, S. M. Leite, J. A. Tamada, S. Uyama, J. P. Vacanti, R. Langer, A. G. Mikos, Biomaterials 2000, 21, 1837; C. J. Liao, C. F. Chen, J. H. Chen, S. F. Chiang, Y. J. Lin, K. Y. Chang, Journal of Biomedical Materials Research 2002, 59, 676; P. X. Ma, R. Zhang, J Biomed Mater Res 1999, 46, 60; K. Whang, T. K. Goldstick, K. E. Healy, Biomaterials 2000, 21, 2545; A. J. Thornton, E. Alsberg, M. Albertelli, D. J. Mooney, Transplantation 2004, 77, 1798).

Taken together, prior to the invention described herein, porous polymer scaffolds were used as supports for subsequent cell seeding and growth in pores. As described herein, the encapsulation of cells in the matrix of scaffolds and the seeding of other cells in the pores allows the interior of the matrix and the pores within the matrix to provide extracellular environments to the cells. Furthermore, manipulating different cells in controllable environments in hydrogels allows for the examination of cancer cell-stromal cell interactions and paracrine effects on stem cell proliferation and differentiation. The methods described herein provide new material systems for ex vivo cell production and manipulation.

The invention described herein provides the fabrication of cell-friendly inverse opal hydrogels that also allow cell-encapsulation in the hydrogel matrix (Figure 2). As described herein, this is achieved by using sacrificial templates (usually polymers) that are removed through cell-friendly routes without using toxic organic solvents. The cell-friendly routes of removing the sacrificial polymer template are determined by the type

of polymer template. Suitable sacrificial templates include ionically crosslinked polymers (e.g., alginate), thermosensitive polymers (e.g., agarose, gelatin, collagen) and thermoresponsive polymers (e.g., poly(N-isopropylacrylamide), poly(N-ethylacrylamide), poly(N-cyclopropymethacrylamide), poly(N-methyl-N-ethylacrylamide), poly(N-acryloylpyrrolidine), poly(N-ethylmethacrylamide), poly(N-cyclopropylacrylamide), poly(N-cyclopropylacrylamide), poly(N,N-diethylacrylamide), poly(N-isopropylacrylamide), poly(N-vinylcaprolactam), poly(N-n-propylmethacrylamide), poly(N-methyl-N-isopropylacrylamide), poly(N-n-propylacrylamide), poly(N-methyl-N-n-propylacrylamide), poly(N-acryloylpiperidine)).

Ionically crosslinked hydrogel beads (e.g., alginate) are removed by using various metal-chelating agents including citric acid, ethylenediamine, ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTPA), N,N-bis(carboxymethyl)glycine (NTA), etc. The chelating agents bind with metal ions used as the crosslinker of templating beads, which results in the generation of pores via the dissociation of metal ions and polymers forming beads. Thermo-sensitive hydrogel beads (e.g., agarose, gelatin, collagen) are removed by increasing the temperature to change the solid phase of polymer beads to liquid phase. Thermo-responsive polymer beads (*e.g.*, poly(N-isopropylacrylamide), poly(N-ethylacrylamide), poly(N-cyclopropymethacrylamide), poly(N-methyl-N-ethylacrylamide), poly(N-acryloylpyrrolidine), poly(N-ethylmethacrylamide), poly(N-cyclopropylacrylamide), poly(N-cyclopropylacrylamide), poly(N,N-diethylacrylamide), poly(N-isopropylacrylamide), poly(N-vinylcaprolactam), poly(N-n-propylmethacrylamide), poly(N-methyl-N-isopropylacrylamide), poly(N-n-propylacrylamide), poly(N-methyl-N-n-propylacrylamide), poly(N-acryloylpiperidine)) are removed by controlling the temperature above lower critical solution temperature (LCST) to reduce the size of template beads, and the resulting smaller beads readily escape from the outer polymer matrix to generate pores. Other stimuli-responsive polymers (*e.g.*, pH-responsive polymer, photo-cleavable polymer, etc.) are also suitable in similar routes.

A variety of materials including natural polymers (*e.g.*, collagen, gelatin, alginate, fibrin, agarose, hyaluronic acid, chitosan, etc.) and synthetic polymers (*e.g.*, PEG, PLGA, poly(acrylic acid), poly(vinyl alcohol), polyphosphazene, etc.) are used for making the polymer matrix between template beads.

To prepare the inverse opal hydrogel encapsulated with cells, the template polymer beads are close-packed in molds of varying shapes/sizes, and the polymer solution mixed

with cells is infiltrated into the interstitial space of template beads (Figure 2). After polymerization of polymer matrix via photopolymerization, redox polymerization, or other polymerization to form crosslinked matrix encapsulating cells, template beads are dissolved and removed to generate pores through appropriate routes mentioned above. This removal of template beads occurs in aqueous solution without using toxic organic solvents or lyophilization, as those processes prohibit cell encapsulation in conventional inverse opal hydrogel systems. The prepared inverse opal hydrogels are rinsed with buffer solution to wash out the residual polymers of templates, and cultured in cell culturing condition (37 ^{o}C, 5% CO_2) to maintain the viability of cells encapsulated in the inverse opal hydrogel matrix.

The structures of inverse opal hydrogels are controllable by using different sizes and different geometry of template polymers. For example, spherical pores are generated from polymer bead template. Polymer templates with an elongated shape are prepared through electrospinning, which results in elongated pores in the hydrogel after removal of the template particles.

The inverse opal hydrogels described herein are also used as the supporters for different types of cells. The inverse opal hydrogels are separated from cell culture media, and a second type of cell dispersed in appropriate cell culture media are seeded onto the hydrogels by adding the cells into the inverse opal hydrogels dropwise. The second type of cells attach on the surface of the inverse opal hydrogel without any additional cell culture media. After the cells have attached to the hydrogel, the excess unattached cells around the inverse opal hydrogel are removed. Finally, the inverse opal hydrogels encapsulating one cell type and seeded with another cell type are cultured for further study.

The system described above is utilized to examine the paracrine effect between cells and the cell-cell interaction between different cells. Suitable combinations of cells include cancer cell-dendritic cell, cancer cell-mesenchymal stem cells, cancer cell-fibroblast, dendritic cell-mesenchymal stem cell, and other various cells.

Prior to the invention described herein, previous 3-dimensional cell culture systems using matrigel or other 3 dimensional biomaterials usually used the same matrix without any physical separation between cells, i.e., the cells were co-encapsulated in the same matrix or co-seeded on the same surface of matrix or culture dish. The inverse opal hydrogels described herein make it possible to culture different cell types in different physical space, thereby mimicking the natural cellular microenvironment. By physically

separating cells, the paracrine effect (e.g., the effect of soluble factors from one type of cells on another type of cells) is examined. For example, the paracrine effect of cancer cells and stromal cells (fibroblast, immune cells, stem cells, etc.) is examined. The inverse opal hydrogel system described herein is also utilized to study previous culture systems.

In addition to cell encapsulation, the methods described herein are used for general encapsulation and delivery of drugs, proteins, or growth factors, etc. Prior to the invention described herein, when the organic solvent was used to generate pores in the conventional inverse opal systems, the encapsulated molecules of drugs, proteins, or growth factors lost their function due to exposure to the organic solvent. Also, the remaining organic solvent would have a toxic effect when placed in a living organism. As described herein, the mild conditions for pore removal in the current invention circumvents the problems derived from using organic solvents.

For example, various chemical drugs including small molecules and functional proteins (growth factors, cytokines, chemokines, hormones, etc) are mixed with the polymer precursor solution to be added into the template beads in the mold. After polymerization of the polymer precursors and removal of the template beads, the encapsulated molecules are released slowly. The release profiles depend on crosslinking density, the affinity of molecules to the polymer chain, the size of molecules, etc. In this context, the inverse opal hydrogels are used as delivery systems for the cells encapsulated in the hydrogel or the cells outside the hydrogel.

The methods described herein also encapsulate functional nanoparticles to actuate the porous hydrogel systems to release cells, drugs, proteins, and growth factors on demand. The nanoparticles are encapsulated in the hydrogel in a similar manner in which cells and drugs are encapsulated. Specifically, the functional nanoparticles are mixed with polymer precursors and added into the template beads in the mold. For example, magnetic nanoparticles or gold nanoparticles are encapsulated in the polymer matrix and the resulting porous hydrogels are responsive to an external magnetic field or light, respectively. The guest molecules are released upon detection of the external stimulus. In the case of magnetic nanoparticles, the external magnetic force modulates the volume of pores in the inverse opal hydrogel due to its high porosity. The guest molecules encapsulated or seeded in the inverse opal hydrogels are released by the mechanical forces via convection. In addition, both magnetic nanoparticles and gold nanoparticles are used as hyperthermic moieties. Magnetic nanoparticles and gold nanoparticles

generate heat by alternating magnetic fields and irradiation with lasers, respectively. Thus, both magnetic and gold nanoparticles allow thermal motion of the polymer matrix and the encapsulated guest molecules, which accelerates the release rate of guest molecules.

Example 1: Alginate Beads as a Sacrificial Template

Described herein is an example of the fabrication of a cell-friendly inverse opal hydrogel. Alginate beads, formed using Ca2+-crosslinking were used as the porogen, and 50 mM EDTA solution, a metal chelating agent, was used as the template removal solution. To evaluate if EDTA can dissolve the alginate beads efficiently, three different sized alginate beads were prepared using 2% alginate solution in 100 mM Ca2+ solution (Figure 3, upper row). Rhodamine-labeled bovine serum albumin (BSA) was encapsulated in alginate beads to visualize the beads and their dissolution. The resulting alginate beads were incubated in 50 mM EDTA solution under shaking. After 20 min, all alginate beads dissolved in EDTA solution and lost their spherical morphology, and this resulted in a pink solution due to released rhodamine-labeled BSA from alginate beads (Figure 3, lower row). This demonstrates that the alginate beads were used as sacrificial template by using EDTA as leaching solution.

Example 2: Fabrication of Inverse Opal Hydrogels

Inverse opal hydrogels were fabricated using alginate beads as the template and methacrylated-gelatin as the hydrogel precursor (Figure 4). Alginate beads were close-packed in molds with different shapes. Methacrylated-gelatin (10 wt%) solution was infiltrated by adding to the top of the packed alginate beads, and was subsequently polymerized under UV (365 nm) irradiation for 20 min. Figure 4a shows alginate/gelatin composites with different shapes, such as disc, cubic, and cylinder. The interstitial space was filled with opaque gelatin hydrogels. Finally, the resulting alginate beads/hydrogel composites were incubated in 50 mM EDTA solution for 1h at 37°C under shaking to remove alginate beads. Figure 4b shows disc-shaped porous IOHs with different pore sizes: 1500, 1000, and 600 um, respectively. The IOH maintained the original structure after removal of templates. The pore size was uniform and 3-dimensionally interconnected pores were clearly observed in each inverse opal hydrogel. The pore size was easily controlled by using different size of alginate beads as templates. As described herein,

synthetic polymers are also used for inverse opal hydrogel system. PEG inverse opal hydrogels were prepared using the same protocol (Figure 4c).

Example 3: EDTA is Non-Toxic to Cells Encapsulated in IOHs

To evaluate if incubation in EDTA solution is toxic to cells, cell viability was checked after incubation in 50 mM EDTA solution. Mouse mesenchymal stem cells (MSCs) were cultured in a flask, and incubated in a 50 mM EDTA solution for 10, 30, 60, 120, or 180 min. Subsequently, the viability of cells was measured with a live/dead cell assay by using calcein AM and ethidium homodimer-1. Although the cell morphology changed to a round shape, the representative fluorescent images of the live/dead assay showed that there was no significant toxicity of the EDTA solution to the cells for up to 3 h incubation (Figure 5a). Quantitative analysis also showed ~98% viability even after 3h incubation in EDTA solution (Figure 5b). Based on these observations, incubation of the gels in 50 mM EDTA solution for up to 3 h to remove alginate beads was determined as a nontoxic process to cells encapsulated in IOHs.

Example 4: Proliferation of Cells in a Hydrogel Matrix

The encapsulation of cells in IOHs was investigated. Mouse MSCs were dispersed in gelatin-MA at a concentration of 5 x 10^6 cells/ml, and added to packed alginate beads in a mold. The gelatin was polymerized under 365 nm UV lamp for 20 min, and the alginate beads were subsequently removed in EDTA solution. The cells were observed on a fluorescent microscope using a fluorescent live/dead assay (Figure 6a). The cells were alive (stained green) after removal of template alginate beads and were uniformly distributed over the entire scaffolds and the IOHs maintained 3- dimensionlly interconnected pore structures. The morphology of cells changed over time. The cells showed a spherical morphology at day 1, started to spread at day 4, and most of cells were spread in the hydrogel matrix at day 7. The proliferation of cells encapsulated in IOHs (Figure 6b) was demonstrated using an alamar blue assay in which the fluorescence of the dye increases proportionally to the live cell number. There is an increase of cell number, which means the cells are proliferating in the hydrogel matrix.

Example 5: Proliferation of Cells on the Surface of IOHs

Cell seeding on the surface of IOHs after the template was removed was also investigated. Gelatin IOH was prepared and mouse MSCs were seeded on IOHs. After 4 days culture, the cells were well-attached on the inner pore surfaces, and the density of cells increased (Figure 7a). The cell number in cell-seeded IOHs (Figure 7b) increased over time, which indicates that the cells proliferated on the gel.

Example 6: Proliferation of Cells on the Surface of PEG IOHs

PEG without (Figure 8a) and with (Figure 8b) RGD modification were prepared, and mouse mesenchymal cells (Figure 8a) or breast cancer cells (Figure 8b) were seeded on IOHs. As cells cannot attach to the intact PEG hydrogel, the cells formed spherical aggregates in intact PEG IOHs (Figure 8a). However, the cells were well-attached on the inner pore surfaces of RGD-modified IOH (Figure 8b) (upper: bright filed images, lower: fluorescent images). Thus, the attachment of seeded cells was controlled by changing the surface functionality.

OTHER EMBODIMENTS

While the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

The patent and scientific literature referred to herein establishes the knowledge that is available to those with skill in the art. All United States patents and published or unpublished United States patent applications cited herein are incorporated by reference. All published foreign patents and patent applications cited herein are hereby incorporated by reference. Genbank and NCBI submissions indicated by accession number cited herein are hereby incorporated by reference. All other published references, documents, manuscripts and scientific literature cited herein are hereby incorporated by reference.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

WHAT IS CLAIMED IS:

1. An inverse opal hydrogel scaffold device comprising a polymer matrix and a sacrificial porogen, wherein said porogen comprises an ionically-crosslinked polymer, a thermosensitive polymer, a thermoresponsive polymer, a pH-sensitive polymer, or a photocleavable polymer.

2. The inverse opal hydrogel of claim 1, wherein said polymer matrix further comprises an isolated eukaryotic cell.

3. The inverse opal hydrogel of claim 1, wherein said polymer matrix is crosslinked and wherein scaffold device further comprises an isolated cell encapsulated in said crosslinked polymer matrix.

4. The inverse opal hydrogel of claim 1, wherein said polymer matrix comprises a synthetic polymer.

5. The inverse opal hydrogel of claim 1, wherein said polymer matrix comprises covalent crosslinking.

6. The inverse opal hydrogel of claim 1, wherein said polymer matrix comprises a poly(lactide-coglycolide) (PLGA), poly(acrylic acid), polyethylene glycol (PEG), poly (vinyl alcohol), or polyphosphazene.

7. The inverse opal hydrogel of claim 1, wherein said sacrificial porogen comprises alginate, collagen, gelatin, fibrin, agarose, hyaluronic acid, or chitosan.

8. The inverse opal hydrogel of claim 1, wherein said ionically crosslinked polymer comprises alginate.

9. The inverse opal hydrogel of claim 1, wherein said ionically crosslinked polymer is removed by adding a metal-chelating agent selected from the group consisting of citric acid, ethylenediamine, ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTPA), and N,N-bis(carboxymethyl)glycine (NTA).

10. The inverse opal hydrogel of claim 1, wherein said thermosensitive polymer comprises agarose, gelatin, or collagen.

11. The inverse opal hydrogel of claim 1, wherein said thermoresponsive polymer is selected from the group consisting of poly(N-isopropylacrylamide), poly(N-ethylacrylamide), poly(N-cyclopropymethacrylamide), poly(N-methyl-N-ethylacrylamide), poly(N-acryloylpyrrolidine), poly(N-ethylmethacrylamide), poly(N-cyclopropylacrylamide), poly(N-cyclopropylacrylamide), poly(N,N-diethylacrylamide), poly(N-isopropylacrylamide), poly(N-vinylcaprolactam), poly(N-n-propylmethacrylamide), poly(N-methyl-N-isopropylacrylamide), poly(N-n-propylacrylamide), poly(N-methyl-N-n-propylacrylamide), and poly(N-acryloylpiperidine).

12. A method of producing an inverse opal hydrogel with open, interconnected pores comprising:

compressing a plurality of template porogen particles into a mold;

adding a composition comprising a polymer solution and a first plurality of cells to the interstitial space between template porogen particles in said mold to polymerize said template porogen particles;

removing said template porogen particles;

thereby producing an inverse opal hydrogel with open, interconnected pores.

13. The method of claim 12, wherein said template porogen particles are removed without using toxic organic solvents or lyophilization.

14. The method of claim 12, wherein said cells are encapsulated in said inverse opal hydrogel.

15. The method of claim 12, wherein said template porogen particle is an ionically crosslinked polymer, a thermosensitive polymer, a thermoresponsive polymer, a pH-responsive polymer, or a photo-cleavable polymer.

16. The method of claim 15, wherein said ionically crosslinked polymer is alginate.

17. The method of claim 15, wherein said ionically crosslinked polymer is removed by adding a metal-chelating agent selected from the group consisting of citric acid, ethylenediamine, ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTPA), and N,N-bis(carboxymethyl)glycine (NTA).

18. The method of claim 15, wherein said thermosensitive polymer is agarose, gelatin, or collagen.

19. The method of claim 18, wherein said thermosensitive polymer is removed by increasing the temperature of said polymer, thereby altering the phase of said polymer to liquid phase.

20. The method of claim 15, wherein said thermoresponsive polymer is selected from the group consisting of poly(N-isopropylacrylamide), poly(N-ethylacrylamide), poly(N-cyclopropymethacrylamide), poly(N-methyl-N-ethylacrylamide), poly(N-acryloylpyrrolidine), poly(N-ethylmethacrylamide), poly(N-cyclopropylacrylamide), poly(N-cyclopropylacrylamide), poly(N,N-diethylacrylamide), poly(N-isopropylacrylamide), poly(N-vinylcaprolactam), poly(N-n-propylmethacrylamide), poly(N-methyl-N-isopropylacrylamide), poly(N-n-propylacrylamide), poly(N-methyl-N-n-propylacrylamide), and poly(N-acryloylpiperidine).

21. The method of claim 20, wherein said thermoresponsive polymer is removed by increasing the temperature above a lower critical solution temperature (LCST) to reduce the size of the template particles.

22. The method of claim 12, wherein said open, interconnected pores are 600 μm, 1000 μm, or 1,500 μm.

23. The method of claim 12, wherein said hydrogel is modified with a cell-adhering peptide, wherein said cell-adhering peptide is Arg-Gly-Asp (RGD).

24. The method of claim 12, further comprising adding a second plurality of cells to said open, interconnected pores.

25. The method of claim 12, wherein said first plurality of cells and said second plurality of cells are selected from the group consisting of mesenchymal stem cells, stromal cells, cancer cells, dendritic cells, macrophages, neutrophils, natural killer cells, or fibroblast cells.

26. The method of claim 25, wherein said first plurality of cells and said second plurality of cells are different cell types.

27. A method of producing an inverse opal hydrogel with open, interconnected pores comprising:

compressing a plurality of template porogen particles into a mold;

adding a composition comprising a polymer solution and an agent to the interstitial space between template porogen particles in said mold to polymerize said template porogen particles;

removing said template porogen particles;

thereby producing an inverse opal hydrogel with open, interconnected pores.

28. The method of claim 27, wherein said agent is selected from the group consisting of a drug, a nanoparticle, a growth factor, a cytokine, a chemokine, a hormone, a protein, a nucleic acid, or a small molecule.

29. The method of claim 28, wherein said nanoparticles are magnetic nanoparticles or gold nanoparticles.

30. A composition comprising an inverse opal hydrogel with open, interconnected pores produced by the method of claim 12.

31. A composition comprising an inverse opal hydrogel with open, interconnected pores comprising a first plurality of cells encapsulated in a hydrogel matrix and a second plurality of cells in said open, interconnected pores, wherein said first plurality of cells encapsulated in said hydrogel matrix occupy an interstitial space between said open, interconnected pores.

32. The composition of claim 31, wherein said first plurality of cells and said second plurality of cells are selected from the group consisting of mesenchymal stem cells, stromal

cells, cancer cells, dendritic cells, macrophages, neutrophils, natural killer cells, or fibroblast cells.

33. The composition of claim 31, wherein said hydrogel comprises gelatin or poly(ethylene glycol) (PEG).

Figure 1.

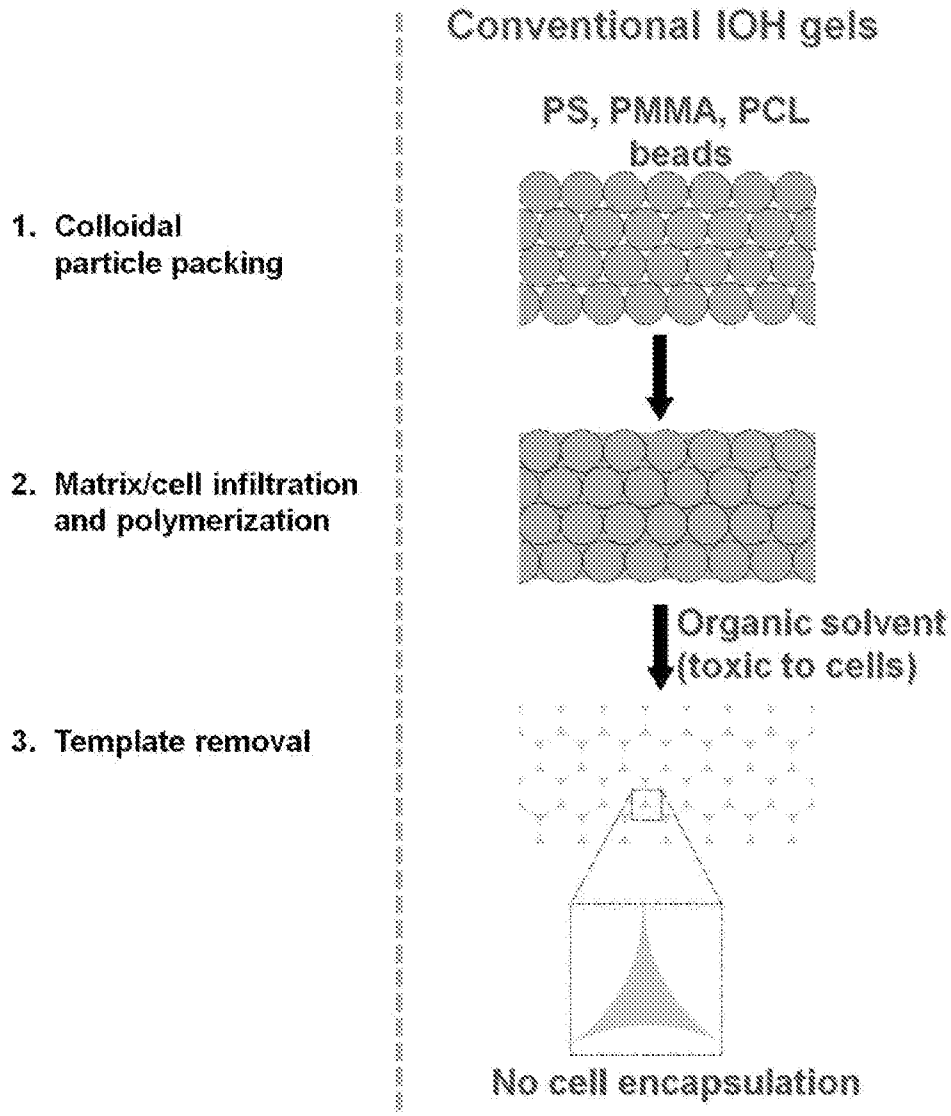

1. Colloidal particle packing
2. Matrix/cell infiltration and polymerization
3. Template removal
Conventional IOH gels
PS, PMMA, PCL beads
Organic solvent (toxic to cells)
No cell encapsulation

Figure 2.

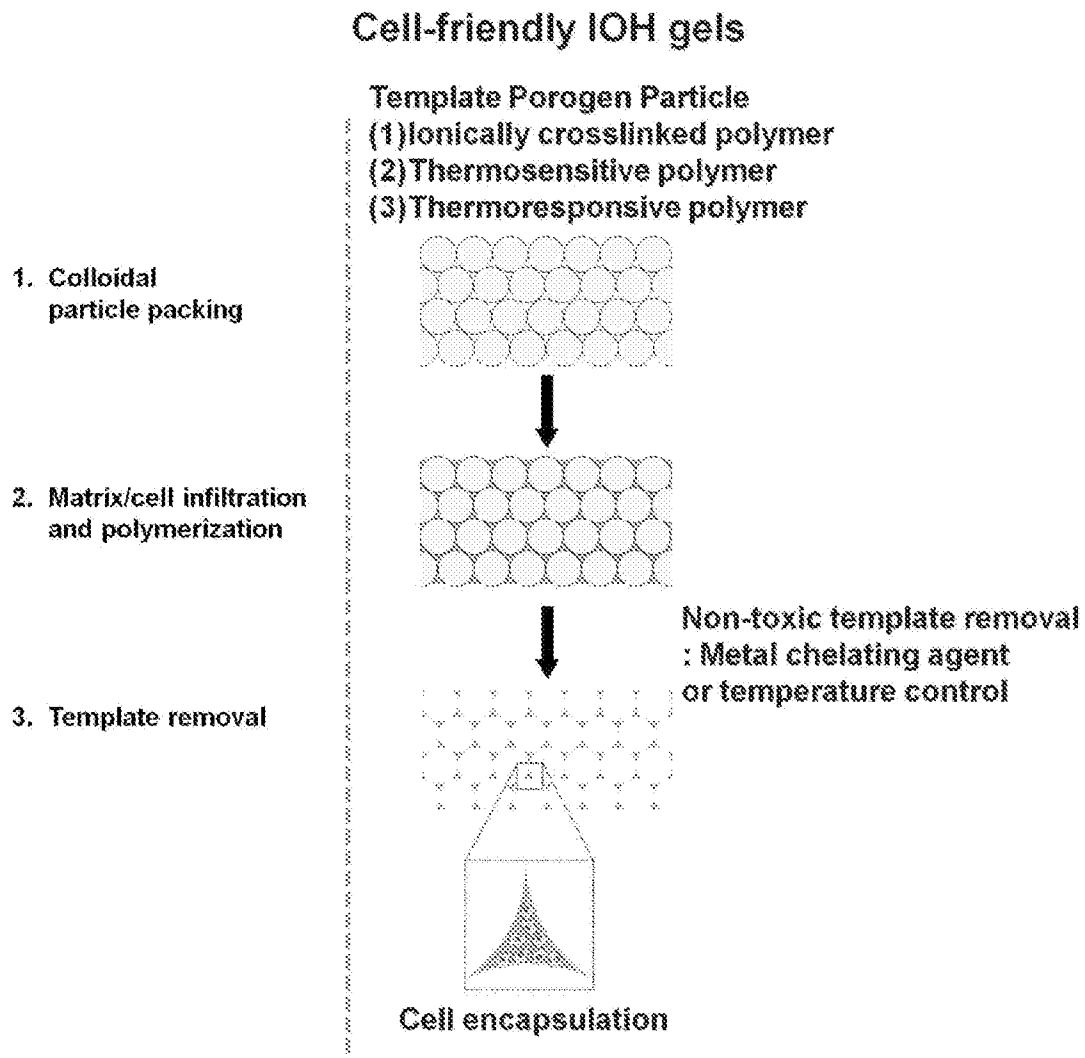

Cell-friendly IOH gels
Template Porogen Particle
(1)Ionically crosslinked polymer
(2)Thermosensitive polymer
(3)Thermoresponsive polymer
1. Colloidal particle packing
2. Matrix/cell infiltration and polymerization
3. Template removal
Non-toxic template removal
: Metal chelating agent
or temperature control
Cell encapsulation

Figure 3.

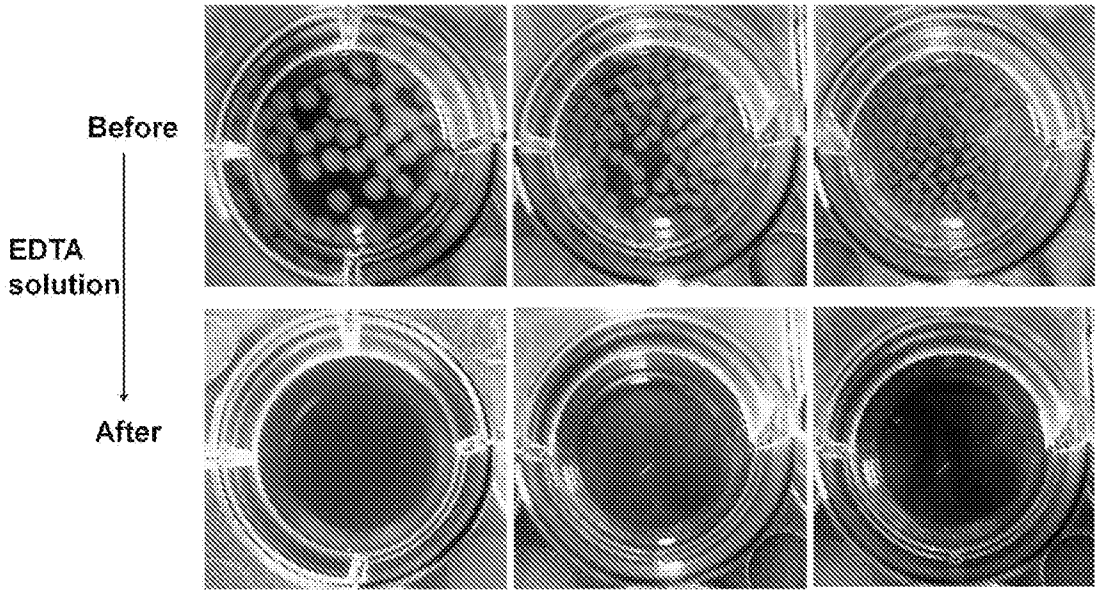


Figure 4.

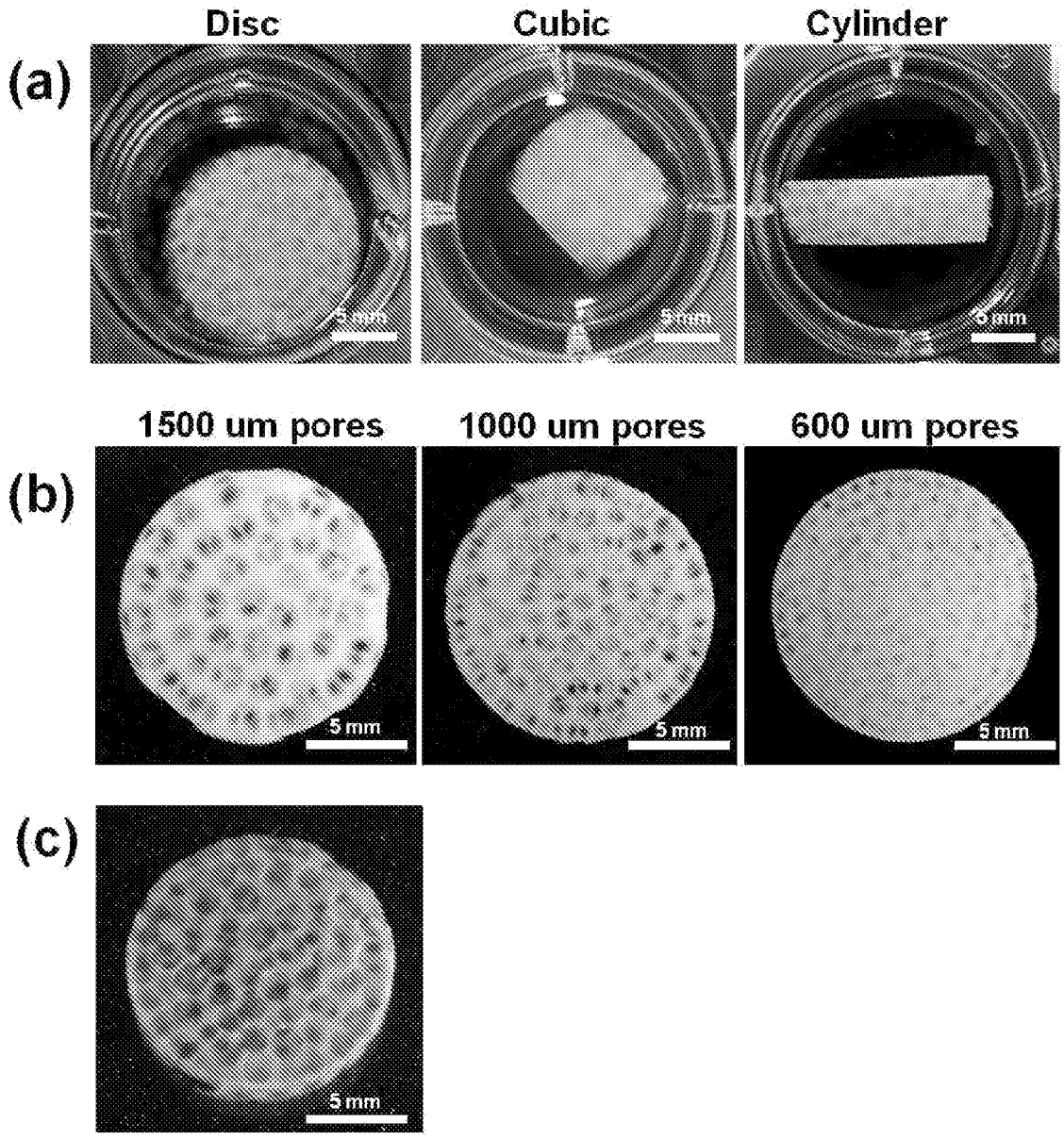

(a)
Disc
Cubic
Cylinder
5 mm
5 mm
5 mm
(b)
1500 um pores
1000 um pores
600 um pores
5 mm
5 mm
5 mm
(c)
5 mm

Figure 5.

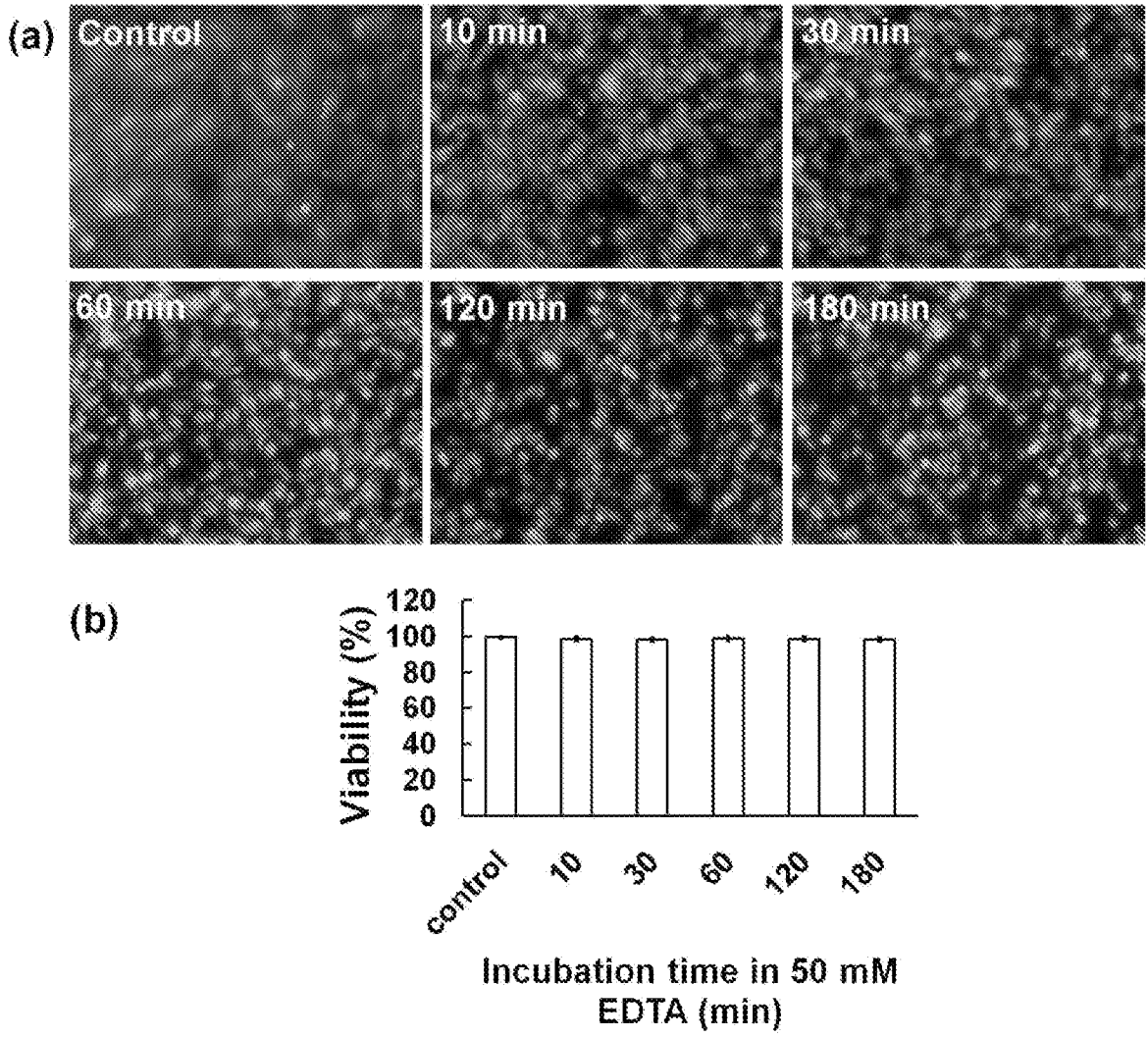

(a)
Control
10 min
30 min
60 min
120 min
180 min
(b)
Viability (%)
120
100
80
60
40
20
0
control
10
30
60
120
180
Incubation time in 50 mM EDTA (min)

Figure 6.

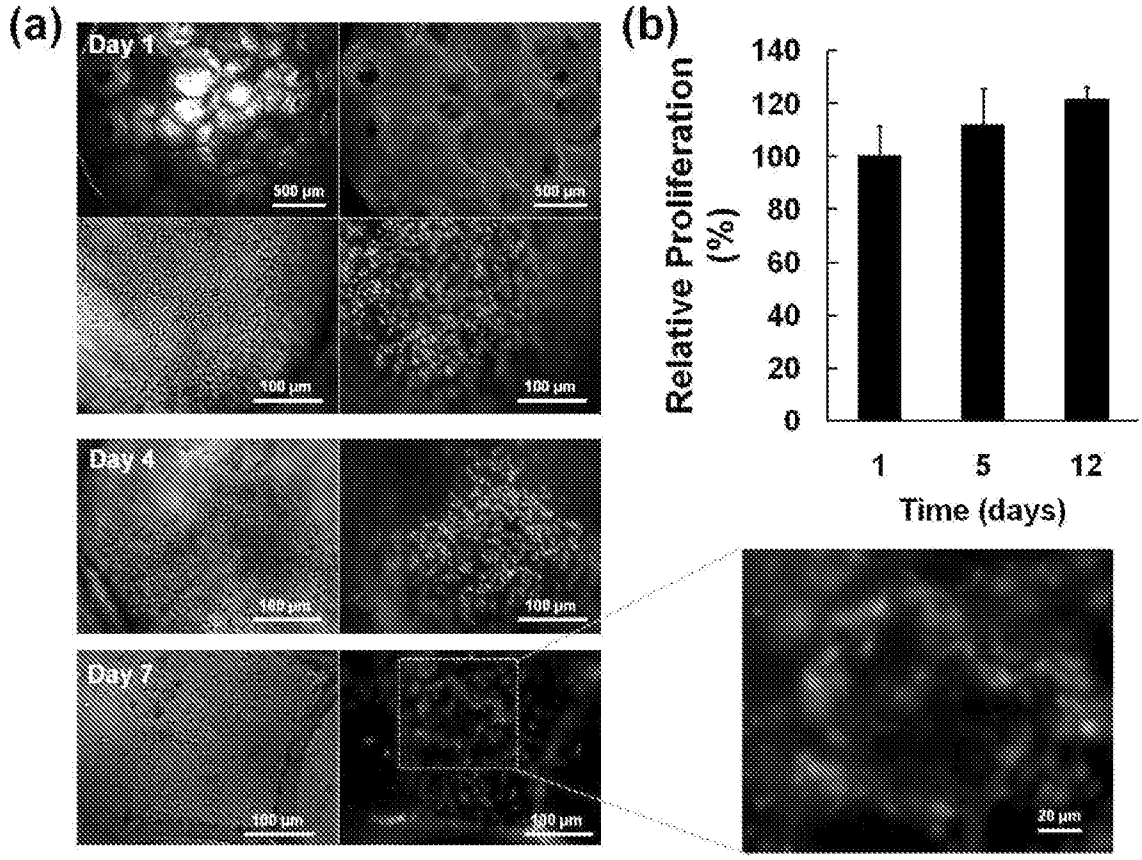

(a)
Day 1
500 μm
500 μm
100 μm
100 μm
Day 4
100 μm
100 μm
Day 7
100 μm
100 μm
(b)
Relative Proliferation (%)
140
120
100
80
60
40
20
0
1
5
12
Time (days)
20 μm

Figure 7.

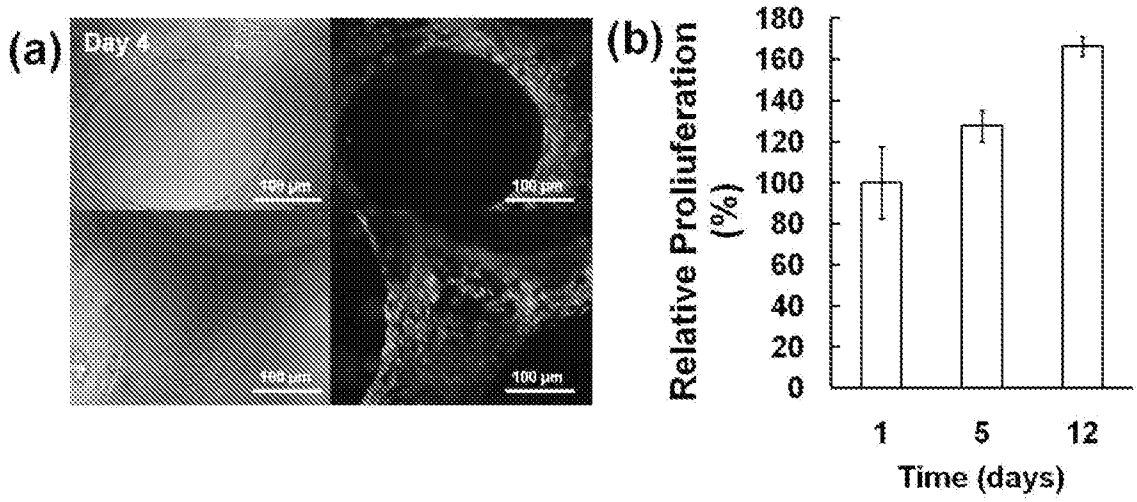
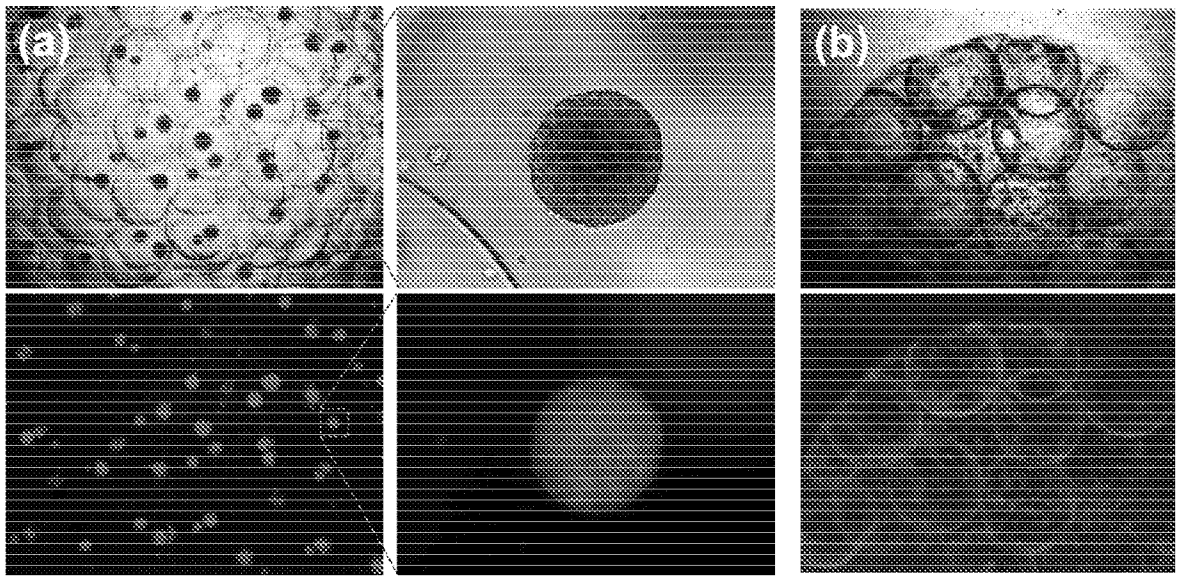

(a)
Day 4
100 µm
100 µm
100 µm
100 µm
(b)
Relative Proliuferation (%)
180
160
140
120
100
80
60
40
20
0
1
5
12
Time (days)

Figure 8.



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 2012/033208

A. CLASSIFICATION OF SUBJECT MATTER

A61L 27/14 *(2006.01)*
A61L 27/52 *(2006.01)*
A61L 27/56 *(2006.01)*
C12N 5/00 *(2006.01)*
B82Y 5/00 *(2011.01)*
A61K 9/51 *(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)

A61L 27/00, 27/14, 27/52, 27/56, C12N 5/00, C08L 101/16, B82Y 5/00, A61K 9/51

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)

PatSearch, RUPAT, Esp@cenet, PubMed, ScienceDirect, SpringerLink

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y A	US 2007/003595 A1 (SHAOPENG WANG et al.) 04.01.2007, abstract, paragraphs [0010] - [0013], [0015], [0062], p.3-4, example 1	30, 31 1-8, 10, 12-16, 18, 22-29, 32, 33 9, 11, 17, 19-21
Y	QINGLIANG ZHOU et al. Microstructure and Mechanical Properties of Poly(L-lactide) Scaffolds Fabricated by Gelatin Particle Leaching Method. Journal of Applied Polymer Science, 2005, Vol. 98, pp. 1373– 1379	1-8, 10, 12-16, 18, 22-29, 32, 33
Y	US 2008/0044900 A1 (DAVID J. MOONEY) 21.02.2008, abstract, p. 2 –3, paragraphs [0017], [0041], [0044], [0047]	2, 23, 25, 26, 32

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

* Special categories of cited documents:

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"E" earlier document 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	Date of mailing of the international search report
28 June 2012 (28.06.2012)	09 August 2012 (09.08.2012)
Name and mailing address of the ISA/ FIPS Russia, 123995, Moscow, G-59, GSP-5, Berezhkovskaya nab., 30-1 Facsimile No. +7 (499) 243-33-37	Authorized officer M. Kostyushenkova Telephone No. (495)531-65-15

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 2012/033208

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2007/0178159 A1 (ALZA CORPORATION) 02.08.2007, abstract, paragraphs [0002], [0030], [0031], [0034]	8, 16, 22
Y	XUANHE ZHAO et al. Active scaffolds for on-demand drug and cell delivery. PNAS, January 4, 2011, Vol. 108, No.1, pp. 67-72	5, 29
A	US 2010/0055186 A1 (MAHROKH DADSETAN et al.) 04.03.2010	1-33