3D Cell Culture: A Review

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Abstract

A comprehensive review of methods for 3D cell culture.

Introduction

Cells in vivo are in a three-dimensional environment having characteristic biophysical and biomechanical signals, which influence cell functions like migration, adhesion, proliferation, and gene expression (Figure 1) [2-5]. Some cellular processes of differentiation and morphogenesis for tissue engineering [6, 7] have been shown to occur preferentially in 3D instead of 2D. Mesenchymal stem cells (MSCs) significantly upregulate the expression of smooth muscle-specific proteins such as αSMA and myosin when cultured in 3D polyethylene glycol (PEG) hydrogels as compared to on a tissue culture plastic surface [8]. The differentiation of human embryonic stem cells (hESC) derived cardiomyocytes had much higher efficiency in 3D cultures as compared to 2D, with significant upregulation of functional heart-specific markers like MLC-2A/2V, cTnT, ANP, α-MHC and KV4.3 in 3D [9]. Human neural stem cells with familial Alzheimer's disease mutations, only when cultured in 3D, can recapitulate both amyloid-β plaques and neurofibrillary tangles [10]. Pluripotent stem cells from brains can generate neural organoids (organ spheroids, or brain assembloids) when cultured appropriately [11, 12].

More recently, magnetic iron oxide (MIO)-containing hydrogel has been used to treat cultured cells, and the cells are levitated in the culture media with magnets. In this way, the cultured cells detach entirely from the gravity-based substratum [1]. Magnetic

[enlarge]

levitation has been used to develop an in vitro model for breast tumor [13]. Since cells in vivo remain subjected to gravity force, the effect of such artificial suspension on cellular metabolism and behavior needs to be examined. In 2015, DNA-programmed assembly of cells was used to organize cells into desired three-dimensional tissues [14].

[enlarge]

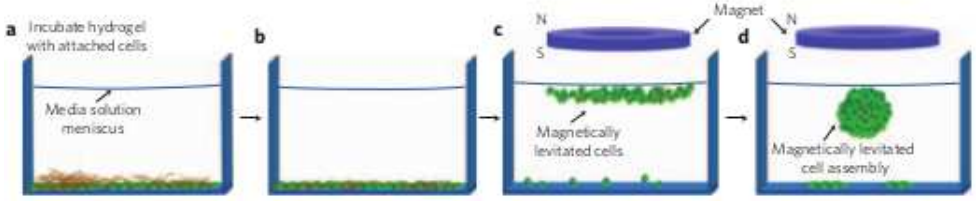

Figure 2. Schematic representation of the 3-D cell culture through magnetic levitation. From [1].

Importance of 3D cell culture

Importance of 3D cell culture scaffolds and models is being increasingly realized. 3D cell culture more accurately simulates normal cell morphology, proliferation, differentiation, and migrations. An increasing shift in research is occurring, where 3D cell culture systems are replacing 2D cell culture systems, and in translating 2D in vitro research to 3D before or as an alternative to testing using in vivo animal models [9, 15].

Advantages and applications of 3D cell culture

3D culture systems can be synthesized using methods that allow facile manipulations for modeling cellular microenvironment.

3D culture systems can be used to study disease models by cellular modeling different disease states [16]. This also reduces the need for animal models.

It is more realistic to grow cells in 3D instead of 2D in vitro to study the effect of drug dosages, , as cells variably respond to drugs in 2D models versus in 3D [17]. In 3D cells form natural barriers to drugs such as layers of cells instead of just a monolayer and tight junctions that bind cells tightly together which effects the diffusion of drugs by blocking or slowing [15, 16, 18, 19].

Scaffolds can be synthesized to support 3D cell growth with simultaneous growth factor, drug or gene delivery [20-24].

3D cell culture has direct applications in tissue engineering and regenerative medicine.

Scaffolds

Scaffolds with incorporated biological cues mediate tissue formation by guiding the adhesion, proliferation, and

Optical Clearing of Biological Tissue
Stem Cell Markers
Stem Cell Research Using Mouse Models
Stem Cells
Subcellular Fractionation
The Cell Cycle Analysis

differentiation of the transplanted cells or the native infiltrating cells at the site of tissue regeneration. The 3D scaffold is typically biocompatible, degrading and resorbing at a rate corresponding to tissue growth, thereby defining the shape and function of the assimilated cell structure [25]. Synthetic and natural materials both have advantages as 3D scaffolds over 2D surfaces. While natural materials provide essential cues that mediate biocompatibility they usually lack the mechanical strength of the synthetic scaffold. Often a combination of materials is used to combine their various advantages.

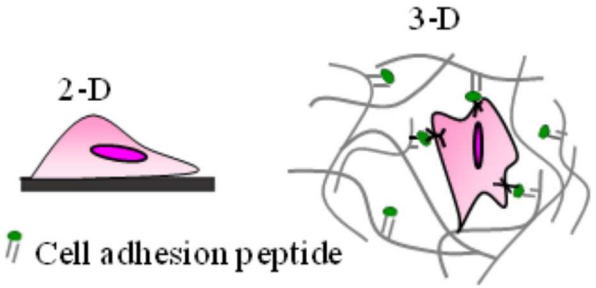

Figure 1. Schematic representation of the cells cultured in 3-D.

	Materials	Advantages
Natural	Silk, collagen, gelatin, fibrinogen, hyaluronic acid, alginate	Biodegradable Easily available Bioactive, interact with cells Facilitate restoration of the structure of damaged tissues
Synthetic	PEG, PGA, PMMA, PLGA, polystyrene	Inert Long shelf-life Easily tailored for desired porosity and degradation time Predictable and reproducible mechanical and physical properties

Table 1. Natural and synthetic materials.

Many different types of scaffolds using varied biomaterials have been developed, specifically the following: hydrogels; custom scaffolds; fibrous scaffolds; porous scaffolds; microspheres; native tissue scaffolds.

Various fabrication techniques are available for these scaffolds. However, there is no universal scaffold. The type of scaffold to be used depends on the proposed function of the scaffold and the desired characteristics.

A wide range of characteristics determining design criteria for 3D scaffolds include the following: biomaterial; biocompatibility; biodegradability; porosity, pore size; geometry; co-culture of cells; shape and size; inter-connectivity; orientation; mechanical properties (tensile strength, elastic modulus) [26] ; incorporation of physical signals and ability to entrap soluble signals; incorporation of variants of ECM (extra-cellular matrix).

Porous scaffolds

Sponge or foam porous scaffolds have been widely used in tissue engineering applications. These scaffolds typically have high porosities and a homogeneous interconnected structure. Synthetic biodegradable polymers such as PLLA, PGA, PLGA, PCL, PDLLA, PEE based on PEO, and PBT are used as porous scaffolding materials.

Methods for fabricating porous scaffolds:

- Salt leaching (porogen leaching): Salt crystals such as NaCl (common table salt) are put into a mold. The polymer is then poured over the salt, penetrating into the empty space in-between the salt crystals. After the polymer is hardened, the salt is dissolved in a solvent.
  Sugar and wax can also be used. Its advantage includes simplicity, versatility, control of pore geometry and size. However, it can only produce scaffolds up to 3 mm thick and it is difficult to obtain accurate pore-interconnectivity. Newer generation of salt porogens and modified particle leaching methods are being developed that allow precise control of salt crystal sizes up to a few microns within a narrow size distribution [27], and better control of ensuing pore size and porosity [28].
- Solvent casting: The polymer is dissolved in an organic solvent, mixed with ceramic particles and the solution is then cast into a predefined 3D mold.
  The main advantage of this technique is the ease of manufacturing, simplicity and ability to incorporate drugs (e.g. antibiotics, antioxidants) within the scaffold. The main disadvantages are that only simple shapes, e.g. flat sheets and tubes, can be formed and the pore interconnectivity is low. Moreover, it is a time-consuming technique, and the toxic solvent denatures the protein and can affect other solvents.
- Gas foaming: A polymer is first stirred rapidly to create a foam and then hardened, creating a solid sponge-like material where the foam's air bubbles form the pores of the final scaffold.
  The main advantage of this technique is that it does not require any solvents or solid porogens. The disadvantages are that excessive heat is used during compression molding and that the pores often do not form an interconnected structure. Incorporation of particulate leaching technique can be used to obtain open pore structures in scaffolds formed using gas foaming.
- Melt molding: Polymer and porogen are combined in a mold and heated above the polymer glass transition temperature for amorphous polymers, or melting temperature for semi-crystalline polymers, allowing the polymer to reorganize and bond. The composite material is subsequently cooled, removed from the mold, and soaked in a liquid to leach out the porogen. This technique is advantageous to incorporate bioactive molecules as harsh organic solvents are not avoided, but are limited by the use of high molding temperatures which can denature biomolecules.
- Microsphere sintering: In this process, ceramic/polymer composite microspheres are fabricated first and then

sintered to yield a 3D porous scaffold [29].

A combination of methods is often used for fabricating scaffolds.

Cells	Material	Method	Results	Reference
Vascular smooth muscle cells (VSMCs)	Poly(lactide-co-epsilon-caprolactone) (PLCL)	Extrusion particulate leaching	Cell adhesion and proliferation were proportional to porosity and pore size Useful to engineer smooth muscle containing tissues, under mechanically dynamic culture conditions	[30]
Human osteoblast-like cells	Polylactide-co-glycolide (PLGA) and 45S5 bioactive glass (BG)	Microsphere sintering	Obtained desired mechanical properties Osteointegrative potential Bone tissue engineering applications	[29]
Human mesenchymal stem cells (hMSCs)	Poly(epsilon-caprolactone)	Gas foaming and selective polymer extraction from co-continuous blends	Cell colonization, proliferation and osteogenic differentiation were related to the micro-architecture of the pore structure	[31]
Human hepatoma cells (Hep3B)	Poly(D,L-lactic-co-glycolic acid) (PLGA)	Supercritical gas foaming	PLGA 85:15 sponge supported higher cell infiltration, proliferation and hepatic function, versus PLGA 50:50 sponge	[32]
lymphocytes and SMCs	Poly(D,L-lactic-co-glycolic acid) (PLGA)	Gas foaming/salt leaching	Released dexamethasone, and suppressed proliferation of lymphocytes and SMCs Applicable as an anti-inflammatory porous prosthetic device	[33]
Human chondrocytes	Blend of poly(lactic-co-glycolic acid) (PLGA) and polyvinyl alcohol (PVA)	Melt-molding particulate-leaching method	Supported cell adhesion and growth After implantation, there was better bone in-growth and bone formation inside the scaffold.	[34]

Table 2. 3D cell culture studies using porous scaffolds.

Fibrous scaffolds

The main feature of fibrous scaffolds is their fiber structure as it provides large surface area for cell attachment. These scaffolds have comparatively high inter-fiber distances which facilitate nutrition and gas exchange, and cell infiltration. Both natural and synthetic polymers have also been explored for synthesizing fibrous scaffolds. These include collagen, hyaluronic acid (HA), silk, chitosan, PLA, PLGA, PCL, PU, and PEVA.

Methods

Fiber mesh: These scaffolds consist of individual fibers either woven or knitted into three-dimensional patterns of variable pore size. However, they might lack structural and mechanical stability for in vivo applications

Fiber bonding: This technique was developed to overcome the limitations of fiber meshes, by binding fibers at the point of intersection by using specific solvents, raising temperatures above polymer melting points etc. The main advantages of this technique are its simplicity, use of biocompatible materials and structural advantages over felt and tassel arrangements, while the disadvantages are lack of control over pore size and porosity, unavailability of suitable solvents, immiscibility of the two constituent polymers in melt state, and the required relative melting temperatures of the polymers.

Electrospinning: This technique is used to produce continuous fibers from submicron to nanometer diameter, by electrostatic spraying of polymer coatings. Electrospun scaffolds have wide applications in neural tissue engineering [35, 36]. They are mainly used to provide structural guidance for neurite growth and axonal extension. Moreover, the fibrous nerve conduits can be introduced at lesion sites by implantation.
Electrospinning is advantageous for the production of ultrafine fibers with special orientation, high aspect ratio, high surface area, and controlled pore size, but is mainly limited by cell seeding.

Phase separations [37] : Typically a solvent with a low melting point, that is easy to sublime, is used to dissolve a polymer. Water is added to induce phase separation, and a polymer-rich phase and a polymer-poor phase are formed. A scaffold is then obtained by cooling below the solvent melting point and vacuum drying to sublime the solvent.

Self-assembly [38] : Here designer peptide fibers self-assemble to form stable and highly ordered scaffolds on the nanoscale.

Cells	Material	Method	Results	Reference
Rat marrow stromal cells	Titanium	Fiber mesh	Bone tissue engineering	[39]
Chondrocytes	Poly(epsilon-caprolactone)-block-poly(L-lactide) (PCL-b-PLLA)	Thermally induced phase separation and salt leaching	Obtained nano-fibrous scaffolds with interconnected pore structures (144±36 μm in diameter) Applicable for cartilage tissue engineering	[37]

PC12 cells	Poly(ε-caprolactone) (PCL)	Electrospinning	Fibrous tubular scaffolds supporting neurite growth and extension	[40]
Embryonic stage nine (E9) chick dorsal root ganglia (DRGs) and rat Schwann cells (SCs)	Poly (L-lactic acid)	Electrospinning	Facilitated guided neurite and SC growth along the aligned fibers	[41-43]
Hepatocytes, human adipose-derived stem cells (hASC) and endothelial cells (HUVEC)	Silk fibroin/chitosan (SFCS)	Self-assembly [44], Dielectrophoresis [45]	Facilitated cell attachment Low inflammatory response on implantation [44] Cell adhesion, migration [45]	[44, 45]
Endothelial cells	Blend of a hydroxyl functionalized polyester (poly(hydroxymethylglycolide-co-ε-caprolactone), pHMGCL) and poly(ε-caprolactone) (PCL)	Coaxial electrospinning	Used for delivering VEGF Maintained functionality and facilitated cell adhesion	[21]
Human dental pulp stem cells (DPSCs)	Poly(L-lactic acid)	Phase-separation technique and porogen-leaching	Nanofibrous scaffolds supported in vitro and in vivo osteogenic differentiation	[46]

Table 3. 3D cell culture studies using fibrous scaffolds.

Hydrogels

There is a lot of interest in tissues like hydrogels as scaffolds since they embody tissue-like flexibility while possessing viscoelastic properties, interstitial flow and diffusive transport characteristics similar to native tissues. Natural polymers used to form hydrogels include fibrinogen [24, 47], hyaluronic acid (HA) [19, 48], collagen, aginate [49], gelatin and chitosan. Synthetic polymers used to form hydrogels include PEG and PEG derivatives, PLA and PVA. More recently, Lou J et al covantly linked hyaluronic acid and collagen to generate hydrogels with simlar features of viscoelasticity and fibrillarity as extracellular matrix [50].

Methods

Solvent casting and particulate leaching: These methods with slight modifications have been applied to fabricate hydrogels [51]. see above.

Gas foaming: Fabrication of hydrogels using foaming methods has been well characterized and studied [52]. see

above.

Freeze drying: This technique is based on sublimation, where the polymer is first dissolved in a solvent to the desired concentration, frozen and subsequently lyophilized to remove the solvent. It does not require high temperatures or a separate leaching step, scaffolds have high porosity and interconnectivity, and the pore size can be controlled by adjusting the freezing rate and pH. However, it is limited by long processing time and produces smaller pore sizes.

Copolymerization/cross-linking methods: Other methods include polymer gels, free radical polymerization, in situ polymerization and photo-polymerization. For example, Nam S et al generated hydrogel-based 3D cell culture by mixing a cell alginate solution with various concentrations of calcium sulfate [49].

Microfluidics: The use of microfluidics to fabricate hydrogels is an upcoming technology. The main advantages of this technique are that hydrogels with uniform pore size, porosity and complex patterns can be produced [53]. Reproducible 3D cell culture systems for hydrogels can be achieved using microfluidics [54].

Cells	Material	Method	Results	Reference
Neural stem/progenitor cells	D-mannitol crystals mixed with photo-cross-linkable chitosan, Macroporous	Photo-polymerization	Promoted 3D differentiation	[55]
Neural cancer stem cells	Collagen, 80 um pore size	Gelation	Enhanced attachment, viability, and differentiation (neurite outgrowth)	[56]
Rabbit marrow mesenchymal stem cells	Oligo(poly(ethylene glycol) fumarate) (OPF) with encapsulated cells and gelatin microparticles loaded with TGF-β1	Injectable, in situ cross-linkable and biodegradable oligomer. Radical-polymerization, 37°C for 8 minutes	Maintained viability of cells for 14 days Differentiation of cells into chondrocyte-like cells	[57]
HepG2 liver cells	Alginate	Gelation	Supported cell viability for more than 14 days, functionality and metabolism of prodrug EFC	[18]
Prostate cancer cells	Hyaluronic acid (HA)	Covalent cross-linking of HA derivatives, HAALD and HAADH in PBS and the formation of hydrazone bond	Applicable for in vitro anti-cancer drug screening like camptothecin, docetaxel, and rapamycin	[19]
Pancreatic islets	Chitosan	Freeze-drying	Sustained 3D islet culture Cells retained initial morphology for at least 53 days	[58]

			Cells showed constant insulin secretory capacities for at least 49 days	
Aortic SMCs	Conjugates of Poly(ethylene glycol) (PEG) and collagen or fibrin	Photopolymerization	Sustained cell adhesion and proteolytic degradation, enabling 3D cell spreading and migration	[59]
Chondrocytes	Nitrogen gas, aqueous alginate solution, Pluronic® F127 surfactant	Microfluidics	Fabricated scaffolds of uniform pore size Maintained cell proliferation Can be used to study the effect of pore and porosity on various tissue engineering parameters Can produce complex patterns	[53]

Table 4. 3D cell culture studies using hydrogels.

Custom scaffolds / rapid prototyping / solid free-form technique

This is also known as rapid prototyping or solid free-form fabrication. Custom scaffolds can be made using computer-aided design (CAD), which is the use of computer systems to assist in the creation, modification, analysis, or optimization of a design. CAD output is often in the form of electronic files for print or machining operations. The output of CAD consists of specific information about the shape, materials, processes, and dimensions. There are many competing methods that make use of digital models from CAD, for additive manufacturing. This basically is achieved using additive processes where an object is created by laying down successive layers of material.

Methods

Stereo-lithography [60] : Stereolithography was patented in 1987 by Chuck Hall. cross-linkable liquid resin is photopolymerized using ultraviolet or visible light, one 2D cross-section layer at a time using either a laser rastering or dynamic photomask approach. Layer by layer stereolithography using photo-cross-linkable poly(ethylene glycol) dimethacrylate has been shown to facilitate precise patterning of ligands, extracellular matrix (ECM) components and growth factors, and controlled release particles [61]. This technique allows fabrication of predesigned internal architectures and porosities.

Other advantages of this technique are shorter build times, and using dynamic masking approach the equipment is

lower cost both in terms of machine purchase and running costs. Grigoryan B et al employed commonly used food dye additives as biocompatible yet potent photoabsorbers for projection stereolithography to build vascular networks within biocompatible hydrogels [62].

Three-dimensional printing: This technology uses special printers for solid object creation. The 3D printers jet binders onto powdered, composite materials one layer at a time, enabling the fabrication of fully printed prototypes. Up till now most of the scaffolds only allow 3D cell culture for the development of simple tissue constructs. However, custom scaffolds using 3D printing can assist the development of more complex structures essential for biofunctional tissues [63, 64].

Selective laser sintering: This technique uses a high power laser to fuse small particles of plastic, metal, ceramic or glass powders into a mass that has a specific three-dimensional shape.

Fused deposition modeling: Stratasys invented Fused Deposition Modeling in the late 1980s. It is commonly used for modeling, prototyping, and production applications. In this process, a thermoplastic or wax material is extruded through a heated nozzle that traces the part's cross-sectional geometry layer by layer. The plastic hardens immediately after flowing from the nozzle and bonds to the layer below.

The main advantages of FDM are low maintenance costs, no toxic materials, easy material change and no supervision is required. While the disadvantages include longer build times for more area in slices, supports, continuous movement of extrusion head to avoid bumping material and possible occurrence of delamination due to temperature fluctuations.

Organ printing: This technology makes use of the natural cells and substances (growth factors) instead of ink on special printers, for creating organs.

Membrane lamination: This is another SFF fabrication techniques where porous scaffold membranes prepared using methods like solvent casting and salt leaching are cut, stacked and bonded chemically using a solvent such as chloroform. Using CAD templates polymeric foam scaffolds having specific shape can be produced, where the bulk properties of the scaffold are identical to those of the individual membrane.

Cells	Material	Method	Results	Reference
Human mesenchymal stem cells	Poly(ethylene glycol)-based resin	3D microstereolithography	Simple method Offers control of features in X, Y and Z-planes Maintained cell viability for upto 7 days	[60]
Human umbilical vein endothelial cells (HUVECs)	Gelatin methacrylate (GelMA)	Projection stereolithography (PSL)	Mechanical properties can be tailored Uniform cell distribution and	[65]

			proliferation Maintained cell phenotype	
Bone marrow stem cells	Polyglycolic acid (PGA), poly (lactic acid) (PLA)	Three dimensional printing, positive-negative mold interchange technique	Cell infiltrated the scaffold Good cellular compatibility Applicable to repair craniomaxillofacial bone defects	[66]
	13-93 glass mixed with stearic acid (as the polymer binder)	Selective laser sintering (SLS)	Excellent for non-load bearing applications Capable of supporting robust cell growth	[67]
C2C12 myoblast cells	Polycaprolactone (PCL)	Selective laser sintering (SLS)	Maintained cell culture for 21 days Mechanical properties can be tailored for soft tissue engineering Scaffolds with different macropore sizes can be fabricated	[68]
Endothelial progenitors and bone marrow stromal cells (BMSCs)	Hydrogels	Organ or tissue printing (3D fiber deposition with a plotting device, Bioplotter)	Cell viability was not affected by extrusion conditions used Cells were able to undergo osteogenic differentiation Applicable for the development of bone grafts constituting multiple types of cells	[69]
Osteoblasts and chondrocytes	Polycaprolactone (PCL)	Fused deposition modeling	Sustained osteochondral co-culture Applicable for osteochondral defect repair	[70]
Human mesenchymal stem cells (MSCs)	Fibrin, polymer (PCL), ceramic (CaP)	Fused deposition modeling	Supported cell attachment, migration and osteogenic differentiation 27 fold increased degradation of compared to PCL scaffolds.	[71]

Table 5. 3D cell culture studies using custom scaffolds.

Microspheres

Methods

Solvent evaporation: Microspheres of biodegradable polymers and copolymers of hydroxy acids are prepared using

this technique, where the hydrophobic solvent is removed by evaporation [72].

Single and double emulsification technique: In single emulsification technique, polymers are first dissolved in an aqueous medium, followed by dispersion in a non-aqueous medium. cross-linking of the dispersed globule is then carried out by using heat or chemical cross-linkers. Double emulsification technique involves the formation of multiple emulsions. Double emulsion of aqueous/non-aqueous/aqueous type is mostly used to incorporate water-soluble proteins, drugs or peptides.

Particle aggregated scaffold: The technique is based on the random packing of prefabricated particles of microspheres, with subsequent further physical or thermal aggregation to form a three-dimensional porous structure.

Microspheres can also be fabricated using freeze-drying and phase separation coacervation.

Cells	Material	Method	Results	Reference
Chondrocytes	Gelatin microparticle aggregates, +/- TGF-β1	Particle aggregation, diameter: 10 μm	Supported viability and function of chondrocytes Applications in cartilage-engineering	[73]
Rabbit nucleus pulposus (NP) cells	Collagen microspheres		Maintained round morphology and phenotype Cells remodeled the template collagen matrix	[74]
Mesenchymal stem cells isolated from adipose tissue	Chitosan	Particle aggregation	Non-cytotoxic, supported cell in-growth Conducive for osteogenic and chondrogenic differentiation Osteochondral bilayered scaffolds could be developed	[75]
Y7 retinoblastoma (RB) cells	Poly(D,L)-lactide-co-glycolide (PLGA) microparticles	Solvent evaporation, diameter: 145-162 μm	Applicable as an in vitro model for evaluating chemotherapeutic drugs	[17]
Breast cancer cells	PLGA/PLA microparticles	Solvent evaporation	PLA microparticles containing poly(vinyl alcohol) (PVA) in the matrix structure (PLA-PVA) and pretreated with serum supported good cell adhesion and cell growth Applicable for evaluating anticancer agents	[17]
Adipose-derived stem cells	Chitosan	Water-in-oil emulsification process along with an ionic coacervation technique	Supported cell attachment and migration Applicable for use as cell carriers to collagen gels	[76]

Cancer cells (MCF-7 cells) (stirred suspension bioreactor culture)	Poly(lactic acid-co-glycolic acid) (PLGA)	Water/oil/water double emulsion method	Maintained cancer cell culture Can be used for cancer cell transplantation vehicle for tumor construction in vivo	[77]

Table 6. 3D cell culture studies using microsphere scaffolds.

Native scaffolds

The extracellular matrix (ECM) is a constituent of all tissues and organs [78]. It can be harnessed from various sources such as the dermis of the skin, submucosa of the small intestine and urinary bladder, pericardium, basement membrane, the stroma of the decellularized liver, and decellularized Achilles tendon [79]. The composition, structure and mechanical properties of the ECM depends on its location within the tissue and organs, the age of the host and the physiologic requirements of the particular tissue. The advantages of an intact ECM over other scaffold materials is its constituent combination of diverse structural proteins, bioactive molecules, native three-dimensional ultrastructure and their unique spatial distribution, which interact with cells and direct cell fate.

Whole ECM can be used as an initial material, instead of a scaffold. Decellularized ECM degraded using nuclease solution and re-suspended, has been used for fabricating scaffolds by freeze-drying and other cross-linking techniques for chondrogenic bone marrow-derived mesenchymal stem cells [80].

Methods

Decelluralization: Extracellular matrix scaffolds are mainly prepared by decellularization of the native tissue, using enzymes, detergents and hypertonic solutions. Decellularized extracellular matrix is applicable for tissue engineering of heart valve [81, 82], muscle [83] etc.

Cells	Material	Method	Results	Reference
Human adipose-derived stem cells	Genipin-cross-linked cartilage-derived matrix		Using genipin resulted in contraction free biomaterial Chondrogenesis	[84]
Co-culture of differentiated urothelial cells (UC) and smooth muscle cells (SMCs) from urine-derived stem cells (USC)	Small intestinal submucosa (SIS) scaffold	Decellularization with 5% peracetic acid (PAA)	3D cell matrix in-growth and development of a multilayer mucosal structure	[85]

Human umbilical vein endothelial cells (HUVECs), SMCs (Dynamic co-culture in perfusion bioreactor)	Small caliber porcine carotid artery	Enzymatic and detergent decellularization	Applicable for small caliber vascular graft reconstruction therapies	[86]
Porcine aortic endothelial cells (Pulsatile bioreactor system)	Porcine aortic heart valve roots	Decellularized by chemical extraction, treated with penta-galloyl glucose (PGG) for stabilization	Applicable for developing tissue engineered heart valves (TEHVs), capable of remodeling and cellular repopulation	[87]
hESC-derived mesenchymal progenitor cells (medium perfusion bioreactor)	Trabecular bone	Enzymatic and detergent decellularization	Formation of large and compact bone constructs, which were stable in vivo for 8 weeks	[88]
Human fetal hepatocytes (FH-hTERT), primary human hepatocytes	Whole mouse liver	Decellularized using detergents	Applicable as a carrier for hepatocyte transplantation	[89]
Late second/early third-trimester fetal kidney explants	Adult rhesus monkey kidney	Decellularized using detergents	Supported Pax2+/vimentin+ cell attachment and migration Need to study recellularization with a range of other cells Applicable for renal tissue engineering	[90]

Table 7. 3D cell culture studies using native scaffolds.

Comparison of scaffolds

Scaffold	Advantages	Disadvantages
Porous scaffolds	High porosityInterconnected structure Simple and easy to manufacture	Use of highly toxic solvent Low pore interconnectivity Difficulty in homogeneous cell seeding after scaffold fabrication Highly porous scaffolds can have weak mechanical properties Lack of control over scaffold thickness
Fibrous scaffolds	Fiber meshes and fiber bonding are simple techniques A large surface area-volume ratio High inter-fiber distances for nutrition and gas exchange Can form stable and highly ordered scaffolds using self-assembly	Fiber meshes lack mechanical integrity Fiber bonding lacks control over porosity and pore size Small pore sizes produced during fabrication processes such as electrospinning limit cell infiltration and 3D cellular integration with host tissue after implantation
Hydrogels	Tissue-like flexibility Viscoelasticity	Higher cost Non-adherent and usually need to be secured by a

	Intestinal flow and diffusive transport	secondary dressing, for in vivo testing Natural polymer hydrogels like collagen gelatin, alginate and agarose may evoke inflammatory responses
Custom scaffolds (CAD technique)	Controlled matrix architecture: size, shape, interconnectivity, branching, geometry and orientation Can control pore and pore size Controlled mechanical properties and degradation kinetics Reproducible architecture and compositional variations	Low resolution of current systems Selective polymeric materials can only be used
Microspheres	Used as cell carriers, when fabricated using biodegradable and non-toxic materials [76] Large surface area for cell attachment and growth Applicable for 3D cell culture in a stirred suspension bioreactor	Difficult to remove once injected or implanted Unknown toxicity associated with microsphere/beads
Native/ECM scaffolds	Simulate the cell's natural microenvironment in terms of composition, bioactive signal and mechanical properties	Difficult to control the degree of decellularization and retain all ECM Non-uniform distribution of cells Immunogenicity upon incomplete decellularization [91]

Table 8. Overview of the advantages and disadvantages of various scaffolds.

Organoid Cultures

Organoids, organized 3D structures, are grown in an extracellular matrix (ECM) and mimic their tissue of origin. Stem cells, adult or embryonic, have been used to generate organoids [92]. Gastric [93], intestinal [94] and retinal [95] organoids have been developed from stem cells. 3D organoid systems have been generated from liver [96], pancreata [97], mammary glands [98], fallopian tubes [99], taste buds [100] and salivary glands [101]. Engle DD et al generated pancreatic ductal organoids to study the promotion of pancreatitis and pancreatic cancer by glycan CA19-9 in mice [97]. Serra D et al cultured crypts of the murine small intestine on Matrigel in STEMCELL Technologies IntestiCult Organoid Growth Medium to generate organoids for investigating intestinal stem cell self-organization and symmetry breaking during development [102]. Such organoids may eventually be constructed through synthetic biology [103].

The applications of 3D organoids include studies of the local immune responses to infections, in vitro modeling of genetic pathologies, toxicity analysis, transcriptomics and proteomics technologies and personalized therapeutic approaches. For example, cerebral organoids, generated from embryonic stem cells, have been used to study interactions between Zika virus and Toll-like receptor 3 [104].

3D organoids are used to model different genetic diseases. For example, a rectal organoid model of cystic fibrosis has been applied to evaluate the effects of the transmembrane conductance regulator-modulating compounds [105]. Hepatic organoids, developed from human stem cells and with differentiated hepatocytes and cholangiocytes, appear to be histologically and functionally similar to the human embryonic liver [106]. In addition, to model polycystic kidney disease, Cruz et al have developed tubular organoids, which indicated a crucial role of the microenvironment in the cyst formation [107].

Stem cell therapies would also benefit from 3D organoid cultures, since active precursor cells may be isolated from organoids. For instance, organoids, generated from colon stem cells, have been applied to studies of chronic colitis in mouse models [108]. Furthermore, various organoids have been generated by co-culture of pluripotent lineage-specific and mesenchymal stem cells with endotheliocytes [109].

One limitation of organoids from stem cells is the variability of organoids from the same stem cell preparation. However, Velasco S et al showed that dorsal forebrain organoids were highly reproducible, and recapitulated the individual endogenous brains [110].

For the study of neurodegenerative diseases, organoids can be useful models to study Alzheimer (AD) and Parkinson (PD) diseases. For instance, brain organoids, which have been generated using induced pluripotent stem cells obtained from patients with AD, have been used for the development of new therapeutic strategies [111, 112]. Promising therapeutic effect has been observed, when brain organoids have been treated with β- and γ-secretase inhibitors [113]. There are ethical dilemmas concerning brain organoids, including the ethical quandaries related to embryonic stem cells, issues of informed consent and potential engraftment of human cerebral organoids into mice [114], and one of the most vexing questions is the conscious experiences or subjective phenomenal states of the brain organoids [115].

Bioreactors

These are devices that have been developed to simulate the in vivo physiological environment to promote cell growth and differentiation. Various aspects of the physiological environment provided by bioreactors include: temperature; oxygen concentration; carbon-dioxide concentration; mechanical stimuli (fluid shear stress); chemical stimuli; electrical current.

Moreover, perfusion and fluid shear stress have been shown to affect the developmental potential of stem cells in 3D cell culture [116, 117], underlying the importance to simulate the in vivo dynamic culture conditions. Bioreactors have

been employed for suspension culture of embryonic stem cells as aggregates while maintaining their undifferentiated state [118]. Perfusion bioreactor culture has also been shown to be vital for forming stable bone tissue grafts from hESCs [88].

Cells	Material and method	Results	Reference
Mouse testicular sperm cells	Soft Agar matrix and perfusion bioreactor	Complete spermatogenesis	[119]
Primary human hepatocytes	Multi-compartment capillary membrane-based bioreactor Perfusion culture	Maintained stable cell function for 10 days. Applicable system for pharmacological studies based on hepatic drug metabolism.	[120]
Human mesenchymal stem cells	3D perfusion bioreactor Poly (ethylene terephthalate) (PET) fibrous matrices. Four chambers with three matrices each. Integrated seeding operation	Applicable for multiple tissue-engineered construct production Scalable, easy to operate. Preserved multi-lineage differentiation potential of hMSCs	[121]
Embryonic stem cells (ESCs)	Two-stage perfusion fibrous bed bioreactor system	Facilitated the production of billions of ESCs in a small bioreactor, using the 2-stage process without the support of ECM proteins and growth factors Scalable, applicable for mass production of ESCs	[122]
Human smooth muscle cells (SMCs)	Tubular elastic poly(trimethylene carbonate) (PTMC) scaffolds Pulsatile flow conditions in a bioreactor	Dynamically cultured SMC constructs performed better than the statically cultured constructs Applicable to prepare a medial layer for tissue-engineered vascular grafts	[123]
Cartilage progenitor cells (CPCs)	Rotating wall vessel (RWV) bioreactor pC-HAp/ChS (porous material consisted of collagen, hydroxyapatite, and chondroitin sulfate) scaffolds	CPCs formed elastic cartilage-like tissue	[124]
Embryonic stem cells (ESCs)	Stirred suspension bioreactors (SSBs)	Formed embryoid bodies Differentiated into cardiomyocytes	[125]

Table 9. 3D cell culture studies using bioreactors.

Micro-fluidic Systems

Increasingly, microfluidic devices are utilized in 3D cell culture.

Hydrogel fabrication technique: As mentioned for hydrogels, microfluidic systems are being used to fabricate hydrogels (section 2.3.5) [53, 55, 126]. Randomly assembling endothelial cell-seeded submillimeter-sized collagen cylinders (modules) into a microfluidic perfusion chamber, led to the formation of constructs containing tortuous

endothelial cell-lined perfusion channels, for tissue engineering [127].

Integrated within scaffolds: Additionally, the use of microfluidic channels/networks within scaffolds is a new research platform for fabricating tissue engineering scaffolds. Three-dimensional microfluidic networks preseeded with endothelial cells have been incorporated in scaffolds to facilitate vascularization, for uniform flow of nutrients and oxygen throughout the scaffold [128, 129]. Recently, Poly(1,3Diamino-2-hydroxypropane-co-polyol sebacate) (APS) was used to fabricate microfluidic channels using a modified replica-molding technique with SU-8 photolithography. These were shown to have more suitable mechanical, degradation and elastomeric properties than microfluidic channels made using PDMS, PLGA and PGA, for forming micro-vascular networks using endothelial cells. The advantages are that this is a rapid, inexpensive, reproducible and scalable fabrication technique [128].

Cell culture devices: Microfluidic devices have been developed for automation of 3D culture [130]. These devices produce complex patterning of different cell types and have been used to study the molecular regulation of perivascular stem cell niches [131].

Generation of gradients in scaffolds: Microfluidic techniques are also used to generate gradients of stiffness and adhesive ligands while fabricating scaffolds. Durotactic gradients of mechanical stiffness [132], and haptotactic gradients of adhesive ligands including bioactive peptide sequences from laminin, YIGSR, or IKVAV through the collagen gel formed using microfluidic systems were shown to enhance directional neurite growth [133].

Commercial 3D Cell Culture Systems/Scaffolds

Many scaffolds are available as commercial products for use as research tools and tissue regeneration therapies, including the following:

Matrigel, Tisseel, Qgel™, ECMgel, Corgel™ BioHydrogel, and Nano Dox™ are various kinds of commercially available hydrogel scaffolds. For example, Matrigel from Corning (#356231) was used to maintain intestinal crypts in a minigut organoid culture to study the synaptic formation between neuropod cells with sensory neurons in the small intestine [134]. Matrigel contains laminin-111 (60%), collagen type IV (30%), and entactin (8%) [135].

Various commercially available bioceramic scaffolds of calcium phosphate, calcium sulfate and bioactive glass cements include BoneSource®, Calcibon®, ChronOS®, Eurobone®, HydroSet™, Norian SRS®, Ostim®, MIIG® X3), and Cortoss® [136].

Other bone substitutes include Lactosorb, Calciresorb, CCerasorb, Cerasorb M, Tutogen bovine, Tutobone human, Biobon, and Perossal [137].

Restore™ device and CellPatch™ are two different materials, composed of porcine small intestinal submucosa, commercially available for treating musculotendinous defects.

Other native scaffolds consist of GraftJacket® and Alloderm obtained from human dermis, TissueMend® obtained

from fetal bovine skin and Permacol™ composed from the porcine dermis.

Various fibroblast populated scaffolds available as skin substitutes include Dermagraft™, Apligraf™, Orcel™, Polyactive™ and Hyalograf 3D™.

Synthetic porous materials, such as polystyrene (as Alvetex Scaffold). Ombrato L et al co-cocultured primary MMTV–PyMT actin–GFP cells and mouse lung cells in collagen-solution-coated Alvetex Scaffold plates from ReproCELL [138].

Material	Commercial supplier	Composition	Cell line	Results	Reference
Matrigel	Corning and BD	Reconstituted, laminin-rich basement membrane	Cancer cell lines Endothelial cells	Useful culture system for testing of anti-cancer and anti-metastatic compounds Endothelial tube formation [139]	[97, 140]
Tisseel	Baxter Healthcare Corp., BioScience	Fibrin sealant kit	Human mesenchymal stem cells (hMSCs)	Cell proliferation, osteogenic differentiation	[141]
GraftJacket	Wright Medical Technology, Inc.	Human dermis		Biocompatible Incorporated well with host tissue Applicable for tendon repair augmentation	[142]
Corgel™ BioHydrogel	Lifecore Biomedical	Hyaluronic acid-based hydrogel	Chondrocytes	Hydrogels are stable to hyaluronidase digestion Biocompatible Maintained the metabolic activity of the encapsulated cells	[143]
ChronOS®	Synthes	β-tricalcium phosphate (100%)	Human peripheral blood mononuclear cells (PBMCs)	Significant correlation existed between initial material-dependent changes in the pH of culture supernatants, osteoclast multinuclearity, and biomaterial resorption	[137]
Mozaik®	Integra Orthobiologics	20% type I collagen + 80% tricalcium phosphate	Osteogenic cells	Osteoconductive scaffold for bone regeneration	[144]
Absorbable Collagen Sponge (ACS)	Medtronic Sofamor Danek	Type I Collagen	Osteogenic cells	Applicable for bone regeneration and orthopedic therapies	[145]

AlgiMatrix™	Invitrogen	Alginate	Human embryonic stem cells (hESCs) Cardiomyocytes	Formation of vascularized embryoid bodies Applicable for the development of cardiac co-cultures	[146]

Table 10. Examples of 3D cell culture studies using commercially available scaffolds.

Future Challenges

Much work remains to be done in biomaterial development for tissue engineering and regeneration. For 3D culture, the choice of a scaffold is a pivotal consideration as it provides a site for cell attachment and proliferation. Biomaterials can affect cell differentiation significantly [147]. An important aspect remains to determine the effect of biomaterial and scaffold itself, on cell proliferation and differentiation, as independent of cell culture conditions (dimension, geometry, growth factors). Controlling the dynamics and spatial presentation of various signals remains a challenge. 3D cell culture systems need to be designed to more realistically assimilate the organizational levels present in vivo . Combination of systems biology and bioinformatics to simulate cell dynamics which can be applied in the design of more complex 3D cell culture systems, is one of the approaches to overcome these challenges.

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