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Diffusion in Musculoskeletal Tissue Engineering Scaffolds: Design Issues Related to Porosity, Permeability, Architecture, and Nutrient Mixing

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Abstract—The field of tissue engineering continues to advance with the discovery of new biomaterials, growth factors and scaffold fabrication techniques. However, for the ultimate success of a tissue engineered construct the issue of nutrient transport to the scaffold interior needs to be addressed. Often, the requirements for adequate nutrient supply are at odds with other scaffold design parameters such as mechanical properties as well as scaffold fabrication techniques, leading to incongruities in finding optimal solutions. The goal of this review article is to provide an overview of the various engineering design factors that promote movement of nutrients, waste and other biomolecules in scaffolds for musculoskeletal tissue engineering applications. The importance of diffusion in scaffolds and how it is influenced by porosity, permeability, architecture, and nutrient mixing has been emphasized. Methods for measuring porosity and permeability have also been outlined. The different types of biomaterials used, scaffold fabrication techniques implemented and the pore sizes/porosities obtained over the past 5 years have also been addressed.

Keywords—Nutrient transport, Pore interconnectivity, Scaffold fabrication techniques.

INTRODUCTION

The majority of tissue engineering techniques currently under investigation utilize a scaffold seeded with cells. These scaffolds are often designed for specific applications and fabricated from a variety of biomaterials such as biopolymers, synthetic polymers, ceramics or metals. Within the realm of musculoskeletal tissue engineering, although scaffolds may be made from different materials, they should possess some common essential characteristics; these include biocompatibility, and certain physical, mechanical, chemical, and structural/architectural properties.[4] Extremely important is the issue of nutrient transport within the scaffold. In the normal *in vivo* scenario vasculature provides most of the nutrients essential for cells to function (an exception is articular cartilage). However, such blood supply is not available for tissue engineered constructs either *in vitro* or during the immediate postimplantation phases *in vivo*. Thus, the ability of a scaffold to enable the adequate delivery of nutrients to resident cells is crucial for the success of any scaffold-based tissue engineering endeavor.

Since transport within the scaffold is mainly a function of diffusion, careful design of the diffusion characteristics of the scaffold is critical. These transport issues relate to oxygen and nutrient delivery, waste removal, protein transport and cell migration, which in turn are governed by scaffold porosity[118] and permeability. The size, geometry, orientation, interconnectivity, branching and surface chemistry of pores and channels directly influence the extent and nature of nutrient diffusion and tissue in-growth.[103,119] Quite often viable tissue formation is observed in the peripheral regions of scaffolds whereas the interior fails to support viable tissue due to lack of adequate diffusion.[43] This may be a manifestation of the fact that as cells within the pores of the scaffold begin to proliferate and secrete extracellular matrix (ECM), they simultaneously begin to occlude the pores and decrease the supply of nutrients to the interior. The formation of this surface layer of tissue with sparse matrix in the interior has been referred to as the "M&M effect", drawing a parallel to the popular brand of candy having a dense crust and soft core.[3]

Porosity

Several studies have emphasized the need for high porosity and high surface area-to-mass ratio for ensuring uniform cell delivery and tissue ingrowth.[46,67] Need has also been expressed for polymeric scaffolds to possess an open pore network for optimal diffusion of nutrients and waste.[107,108] Another study stated that a scaffold should ideally possess a porosity of 90% to allow for adequate diffusion during tissue culture and to provide sufficient area for cell–polymer interactions.[28] However, Goldstein *et al*.[33] have cautioned that polylactic-polyglycolic acid (PLG) scaffolds be prepared with a porosity not exceeding 80% for implantation

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into orthopaedic defects as it would otherwise compromise the integrity of the scaffold. Thus, in the case of polymeric scaffolds there may be a conflict between optimizing the porosity and maximizing mechanical properties. Further complicating matters is the finding by Agrawal *et al*.[2] that lower initial porosity and permeability results in a faster rate of degradation for PLG scaffolds and lower mechanical properties during the initial weeks. Thus, in view of these contradictory factors, there is a need to optimize scaffolds for bone regeneration based on their specific mechanical requirements balanced with their desired useful life and diffusion characteristics. One possible way of achieving this would be to optimize porosity with respect to nutrient availability and match it with biomaterials that can provide adequate mechanical properties.

A list of scaffold fabrication techniques and related scaffold information from the past five years (since 1998) is provided in Table 1. Salt leaching is a popular technique used for fabricating scaffolds, wherein the pore size of the resulting scaffold is controlled by the size of the porogen, and porosity is controlled by the porogen/polymer ratio. However, natural porogen dispersion allows little control over pore interconnectivity and this has led to the modification of this technique to produce greater pore interconnectivity in some cases,[1,58,69,113,114] and to new techniques like rapid prototyping (RP), also known as solid free form fabrication (SFF), in others.[5,103,123] RP techniques involve building 3D objects using layered manufacturing methods. The process, in general, comprises the design of a scaffold model using computer-aided design (CAD) software, which is then expressed as a series of cross-sections.[88] Corresponding to each cross-section, the RP machine lays down a layer of material starting from the bottom and moving up a layer at a time to create the scaffold. Each new layer adheres to the one below it, thereby providing integrity to the finished product. The different types of techniques that fall under the umbrella of SFF techniques include fused deposition modeling (FDM), selective laser sintering (SLS), stereolithography (STL) and 3D printing (3DP).[19,103] FDM[16,22,41,85,123] utilizes a moving nozzle that extrudes a polymeric fiber in the horizontal plane and once a layer is completed, the model is lowered and the procedure is repeated. SLS[82,94,104] involves building objects by sintering powder on a powder bed using a beam of infrared laser. The laser beam interacts with the powder to increase the local temperature to the glass transition temperature of the powder, causing the particles to fuse to each other as well as the layer underneath.[119] STL[21,26,40,50,57,71,78,100] uses an ultraviolet (UV) laser beam to selectively polymerize a liquid photocurable monomer, a layer at a time.[88] The CAD data guides the UV beam onto the liquid surface, which is then lowered to enable the liquid photopolymer to cover the surface. 3DP[89] involves ink jet printing of a binder onto a ceramic,[23,87] polymer[25,31,74,88,117,119,124] or composite[86,93,97] powder surface, one layer at a time. The movement of the jet head, which dispenses the binder, is controlled by the CAD cross-sectional data. Adjacent powder particles join as the binder dissolves.[88] The main advantage of RP techniques is their ability to finely control the microstructure and macrostructure of scaffolds and thus produce complex topographies from a computer model; their main drawbacks are the low resolutions achievable by the current systems and the types of polymeric materials that can be used.[119] Agrawal *et al*.[4] and Yang *et al*.[120] have provided comprehensive reviews weighing the pros and cons of traditional scaffold materials and fabrication methods. Yang *et al*.[119] have also reviewed the advantages and limitations of various RP techniques. Sachlos *et al*.[88] have not only discussed the conventional scaffold fabrication techniques and their drawbacks but have also described various SFF techniques and how they can overcome current scaffold design limitations.

Another scaffold fabrication technique receiving increasing importance is that of electrospinning for the production of scaffolds from nanofibers.[66] Electrospinning is the process by which nanometer-scale diameter polymer fibers are produced using electrical forces.[81] When an applied electric field creates forces at the surface of a polymer solution large enough to overcome the surface tension, an electrically charged jet is ejected that solidifies into an electrically charged fiber, which can be manipulated into various shapes by electrical forces.[81] Li *et al*.[53] studied the interaction of fibroblasts and bone-marrow derived mesenchymal Stem Cells on an electrospun 500–800 nm diameter PLG nanofibrous structure. Since pores in the structure were formed by randomly oriented fibers lying loosely upon one another, the cells while migrating through the pores could possibly push aside the surrounding unresisting, but mechanically strong, fibers thereby causing the pore to expand.[53] The authors hypothesized that this type of dynamic scaffold architecture allowed cells the freedom to adjust the pore diameter according to their liking and also let them pass through relatively small pores but cautioned that their theory needed further investigation. Li *et al*.[54] also evaluated electrospun 700 nm diameter poly(ϵ-caprolactone) (PCL) nanofibrous scaffolds for their ability to retain the functionality of chondrocytes and proposed their use as suitable scaffolds for cartilage tissue engineering. Yoshimoto *et al*.[122] too successfully cultured rat mesenchymal stem cells on electrospun 400 nm ($\pm$200 nm) diameter PCL scaffolds to show their potential as suitable scaffolds for bone tissue engineering. However, they found the fibers to have varying diameters along their lengths and irregular surfaces. In spite of these minor drawbacks, nanofibers hold great promise as potential scaffolds owing to their high porosity and high surface area-to-volume ratio, which are favorable parameters for cell attachment, growth and proliferation in addition to possessing favorable mechanical properties.[53]

Researchers have used a variety of techniques to achieve different porosities. For example, Wintermantel *et al*.[116]

TABLE 1. Biomaterials, fabrication techniques and porosities used for musculoskeletal tissue engineering scaffolds since 1998.

Authors	Year	Scaffold material	Method of fabrication	Channel/Pore size, Porosity	Claims/Conclusions
Scaffolds fabricated via porogen extraction techniques					
Thomson *et al.*[105]	1998	HA short fibers in PLG matrix	Solvent casting and compression molding followed by particulate leaching	23–52 μm (median pore diameter), 47–50%	HA short fibers serve as reinforcements and are also osteoconductive. Porosity up to 85% could be obtained but the scaffolds had minimal compressive yield strength.
Widmer *et.al.*[115]	1998	PLG, PLLA	Solvent casting, extrusion and salt leaching	5–30 μm, 60–90%	Useful for regeneration of tissues that require tubular scaffolds e.g., long bone
Agrawal *et al.*[1]	2000	PLG	Vibrating particle technique	$>$90%	Higher permeability and more even distribution of pores compared to salt leaching technique.
Ma *et al.*[58]	2001	PLLA, PLG	Solvent casting/paraffin leaching	100–500 μm, 96%	Well-controlled interpore connectivity. Scaffold can be molded into desired shape. No sophisticated equipment required. Process can be expanded for large-scale production.
Liao *et al.*[55]	2002	PLG	Solvent merging/particulate leaching	250–500 μm, $>$85%	Polymer mixed directly with salt particles in solid state, through which solvent is passed under negative pressure. This allows for efficient wash out of salt by water later on.
Murphy *et al.*[69]	2002	85:15 PLG	Solvent casting/particulate leaching or gas foaming/particulate leaching	97 ± 1%	Enhanced pore interconnectivity due to partial fusing of NaCl crystals via treatment in 95% humidity.
Suh *et al.*[102]	2002	PLG	Solvent casting/particulate (gelatin) leaching	95%	Scaffolds using gelatin particles performed better than ones using salt due to better pore interconnectivity.
Vehof *et al.*[109]	2002	PPF coated with rhTGF-β_1	Photocrosslinking-porogen leaching	66%	Photocrosslinking eliminates the potential toxicity associated with the use of crosslinking monomer and accelerator and releases low levels of heat.
Holy *et al.*[37]	2003	PLG	Combined phase inversion and particle extraction without use of toxic solvents	1.44 ± 0.30 mm (average pore size), 92%	Macroporous geometry of scaffold allowed diffusion of acidic products and did not induce inflammatory response.
Hou *et al.*[38]	2003	PDLLA, PCL	Coagulation, compression molding and particulate leaching	106–710 μm, 70–95%	Thermal processing allows flexibility in shape and size of porous specimens. Precipitation of polymer during the process results in its purification.
Lin *et al.*[56]	2003	poly(L-lactide-co-DL-lactide)	Solution coating, porogen decomposition	58–80%	Initial mechanical properties of scaffold comparable to trabecular bone.
Oh *et al.*[72]	2003	PLG/PVA	Melt-molding particulate-leaching	200–300 μm, 90%	Improved cell adhesion and growth on PLG/PVA compared to PLG. No organic solvents involved during fabrication process.

TABLE 1. Continued.

Authors	Year	Scaffold material	Method of fabrication	Channel/Pore size, Porosity	Claims/Conclusions
Scaffolds fabricated via freeze drying techniques					
Ma *et al.*[60]	1999	PLLA, PLG, PDLLA	Gelation, solvent exchange and freeze drying.	98.5%	Average fiber diameter: 160–170 nm. Surface-to-volume ratio much larger than scaffolds fabricated with particulate-leaching technique or textile technology.
Whang *et al.*[114]	1999	PLG	Emulsion freeze-drying	16–32 μm (median pore size), 90%	Scaffolds promote osteoinduction by hematoma stabilization (analogous to induction phase in fracture healing).
Whang *et al.*[113]	2000	PLG	Emulsion freeze-drying	7–70 μm (median pore size), 90%	Scaffolds with controlled microarchitecture. Ability to incorporate and deliver proteins.
Scaffolds fabricated via phase separation					
Zhang *et al.*[125]	1999	PLLA-HA, PLG-HA	Thermally induced phase separation	50–600 μm, 85–95%	Improved mechanical properties of composite scaffold over pure polymer foams. Osteoconductive properties of HA provide for better cell seeding and growth. Acidic degradation byproducts from polyesters may be buffered.
Hu *et al.*[39]	2001	PDLLA, PLG	Phase separation followed by sublimation	100–350 μm, $>$90%	Interconnected open-pore foam structure.
Ma *et al.*[59]	2001	PLLA, PLG	Phase separation	88–97%	Parallel array of microtubular architecture useful for making tissues with anisotropic structures and anisotropic mechanical properties e.g., tendon, ligament, muscle, bone, dentin.
Zhao *et al.*[126]	2002	HA/chitosan-gelatin network (HA/CS-Gel) composite	Phase separation and subsequent sublimation of solvent	300–500 μm, 85–95%	Biomimetic approach adopted to simulate ECM of hard tissues, which mainly comprise HA and type I collagen.
Maquet *et al.*[64]	2003	poly(L-lactide-co-ε-capro-lactone)	Thermally induced phase separation/freeze drying	Tubular macropores ($\geqslant$100 μm) and micropores (10–100 μm), 72.7–96.3%	Bimodal pore distribution and pore anisotropy make this technique suitable for regeneration of highly oriented tissues.
Scaffolds fabricated via SFF/RP techniques					
Hutmacher *et al.*[42]	2000	PCL	Fused deposition modeling	T16 tip: 240–690 μm, 47.6–60.7%. T10 tip: 330–670 μm, 68.6–74.4%	Scaffolds with a 0/90° lay-down pattern had a significantly higher compressive stiffness and yield strength than those with a 0/60/120° lay-down pattern, mostly due to orientation of pore edges/ struts with respect to loading direction.
Hutmacher *et al.*[41]	2001	PCL	Fused deposition modeling	61%	PCL is a soft- and hard-tissue-compatible, semicrystalline, bioresorbable polymer with favorable properties for thermoplastic processing.

TABLE 1. Continued.

Authors	Year	Scaffold material	Method of fabrication	Channel/Pore size, Porosity	Claims/Conclusions
Ang *et al.*[5]	2002	Chitosan and chitosan-HA	Three-dimensional printing	400–1000 μm (chitosan) 200–400 μm (chitosan-HA)	Technique can use a wide variety of polymers. No heating required
Chu *et al.*[18]	2002	Porous HA	Combined image-based design and SFF	Orthogonal design: 444 μm, 44%. Radial design: 366 μm, 38%	Technique allows control over the morphology of regenerated bone tissue inside HA implant.
Fisher*et al.*[26]	2002	PPF	Photocrosslinking via UV laser stereolithography	300–500 μm, 600–800 μm, 57–75%	PPF, which is mechanically strong, biocompatible and biodegradable, can be crosslinked using a thermal or photo initiator.
Sherwood *et al.*[93]	2002	D,L-PLG/L-PLA (cartilage) and L-PLG/TCP (bone) composite	Three-dimensional printing	Cartilage: 90%, Bone: $>$125 μm, 55%	Scaffolds built a layer at a time, allowing for production of multiphasic devices with biologically and anatomically relevant features. Presence of a transition region between bone and cartilage, with a gradient of materials and porosity to prevent delamination.
Zein *et al.*[123]	2002	PCL	Fused deposition modeling	160–700 μm, 48–77%	No solvent required. Ease and flexibility in material handling and processing. Fully interconnected channel network. Controllable porosity and channel size.
Roy *et al.*[86]	2003	PLG-β-TCP	Three-dimensional printing	125–150 μm, 80–87.5%	Technique allows for incorporation of complex geometries, porosity gradients and heat-sensitive biomolecules during scaffold manufacturing.
Taboas *et al.*[103]	2003	PLLA	Indirect solid free form fabrication (SFF)	Global pores (500–800 μm) and local pores (50–100 μm wide voids or 5–10 μm length plates)	Global pores for augmenting diffusion and for anchoring scaffold in host. Local pores for tissue growth. Indirect SFF via casting can produce composite scaffolds having mechanically interdigitated regions.
			Scaffolds fabricated via foaming technique		
Sheridan *et al.*[92]	2000	PLG	High pressure gas foaming using CO_2	360 ± 85 μm (average pore size), up to 95%	Successful incorporation and controlled release of angiogenic factors from scaffold. The released factors retained over 90% of their bioactivity.
Spaans *et al.*[101]	2000	50/50 ε-caprolactone/L-lactide soft segments and polyurethane based hard segments	Combination of salt leaching and in-situ formation of CO_2	100–380 μm, 70–80%	Technique used is solvent-free. Adipic acid used as chain extender to regularize and limit pore size. Ultrasonic waves enhance pore regularity and pore interconnectivity. Scaffolds fulfill requirements for meniscal reconstruction.
Yoon *et al.*[121]	2001	PLG	Gas-foaming/salt-leaching	200 μm (mean pore size), $>$90%	Varying concentrations of citric acid in aqueous medium used to control porosities and mechanical strength of scaffolds by controlling the amount of gas evolved.

TABLE 1. Continued.

Authors	Year	Scaffold material	Method of fabrication	Channel/Pore size, Porosity	Claims/Conclusions
Li *et al.*[52]	2002	HA	Dual-phase mixing with foaming	450–524 μm (mean pore size), up to 70%	Increase in porosity from 50% to 70% with inclusion of foaming. Better pore interconnectivity.
Maspero *et al.*[65]	2002	PLG	Rapid consolidation of PLG particles in a mould using subcritical CO_2	100 μm (mean pore diameter), $63 \pm 3\%$	Technique permits manufacturing of defect analogous scaffolds without any time consuming solvent extraction step.
			Scaffolds fabricated via sintering technique		
Kuboki *et al.*[47]	2001	Honeycomb-shaped HA	HA extrusion, condensation, hole drilling and sintering	300–400 μm	Pore size of 300–400 μm was most effective for bone formation. Geometry of BMP carrier controlled differentiation into cartilage or bone.
Lee *et al.*[49]	2001	Calcium metaphosphate (CMP)	Burning of CMP-coated polyurethane sponge and sintering of resulting inorganic material	200 μm	CMP is osteoconductive, biocompatible and its degradation rate is easily controllable. It is cheaper and its processing temperature is low compared to other calcium phosphate ceramics.
Pilliar *et al.*[77]	2001	CPP	Gravity sintering	100 μm (mean pore size), 30–45%	CPP possessing a much higher tensile strength than other calcium-phosphate-based materials of similar porosity obtained by unique process.
Bancroft *et al.*[8]	2002	Titanium fiber mesh scaffolds	Die-punching discs from sheet of sintered nonwoven titanium fiber mesh	250 μm (mean pore size), 86%	Flow perfusion of scaffolds increased mineralized matrix production over statically cultured constructs.
Grynpas *et al.*[36]	2002	CPP	Gravity sintering of particles	10–7250 μm (55–75 μm: median pore size), 35–45%	CPP rods implanted in distal femur of rabbits promoted rapid bone ingrowth and their rate of degradation can be partially controlled by appropriate selection of original particle size.
Botchwey *et al.*[13]	2003	PLG	Sintered microspheres	200 μm (median pore size), 30%	Modeled diffusion of glucose into scaffold under static and dynamic culture conditions and found static and inappropriately designed dynamic culture conditions to be detrimental to cell viability.
Borden *et al.*[12]	2003	85/15 PLG	Sintered microspheres	83–300 μm (210 μm: median pore size), 35%	Porosity similar to percent bone in cancellous bone. Thus, matrix can serve as a negative template for cancellous bone.
			Scaffolds fabricated via miscellaneous techniques		
Barralet *et al.*[9]	2002	Calcium phosphate cement (CPC)	Cement compaction/porogen melting	0.006–350 μm, 31–63%	Cement matrix denser than CPC formed from slurry systems. Process carried out below room temperature.
Li *et al.*[53]	2002	PLG	Electrospinning	2–465 μm with majority in 25–100 μm range, 91.63%	Electrospun nanofibers of diameter 500–800 nm have a morphological similarity to ECM, including large pore size distribution, high porosity and good mechanical properties.

TABLE 1. Continued.

Authors	Year	Scaffold material	Method of fabrication	Channel/Pore size, Porosity	Claims/Conclusions
Rodriguez-Lorenzo *et al.*[83,84]	2002	HA	Starch consolidation	45–70%	Simple technique providing the possibility of forming complex shapes using nonporous molds with different porosities and pore sizes. Linear relationships between planned and measured porosity and between flexural strength and pore volume fractions allow tailoring of microstructure.
De Oliveira *et al.*[73]	2003	HA/TCP	Mixing synthetic calcium phosphates with organic materials followed by pressing and calcination	Mainly 300–400 μm, 50–78%	Ca/P ratio of calcium phosphate determines its dissolution properties. Thus, choice of raw materials and processing parameters makes it possible for mono- or multi-phase structures with variable solubilities to be produced.
Gomes *et al.*[34]	2003	SEVA-C, SPCL	SEVA-C: extrusion SPCL: fiber bonding	SEVA-C: 60% SPCL: 75%	Starch-based polymers are biocompatible, biodegradable, cheap, modifiable, and easily reinforcable. Flow perfusion significantly enhanced mineralization.
Ramay *et al.*[79]	2003	HA	Combined gel-casting and polymer sponge methods	200–400 μm, 70–77%	New technique combines the advantages of both gel-casting and polymer sponge methods, resulting in the creation of HA scaffolds with high mechanical strength and controlled porosity.
Ramay *et al.*[80]	2004	β-TCP matrix and HA nanofibers	HA nanofibers: biomimetic precipitation. Composite: gel casting and polymer sponge technique	300–400 μm, $73 \pm 0.4\%$	Mechanical properties of scaffold significantly enhanced by addition of HA nanofibers.

HA, hydroxyapatite; PLG, poly(lactic-co-glycolic acid); PLLA, poly(L-lactic acid); PDLLA, poly(D,L-lactic acid); PCL, poly(ε-caprolactone); PPF, poly(propylene fumarate); TCP, tricalcium phosphate; PVA, poly(vinyl alcohol); CPP, calcium polyphosphate; SEVA-C, 50/50% weight blend of starch with ethylene vinyl alcohol; SPCL, 30/70% weight blend of starch with polycaprolactone.

developed a two-phase liquid system comprising immiscible liquids to produce an injectable interbody fusion element for the spine. PMMA was chosen as the hardening phase since it had to mimic mechanical properties of bone, where as PVA was selected to be the viscoelastic second phase that would produce a porous structure on separating from PMMA. The pore structure of the resulting element was similar to that of trabecular bone with the mean pore diameter varying from 300 to 1500 μm depending on processing parameters like mixing time and mixing intensity. Pore volumes of 50% were obtained and mechanical properties similar to that of cancellous bone were achieved.

Sherwood *et al.*[93] developed a 3D osteochondral composite scaffold for articular cartilage repair. To prevent delamination between the cartilage and bone regions, having a porosity of 90% and 55%, respectively, a transition region with a gradient of materials and porosities was introduced. This technology may have potential for repair of articular joints and treatment of osteoarthritis.

Mankani *et al.*[63] transplanted mixtures of hydroxyapatite/tricalcium phosphate (HA/TCP) particles of different sizes and shapes and human bone marrow stromal cells (BMSCs) subcutaneously in mice and found that transplants incorporating spherical HA/TCP particles of 100–250 μm size showed the most bone formation. Larger or smaller particle sizes resulted in less bone formation, with 44 μm representing the particle size limit below which no bone formation was observed. Moreover, flat-sided HA particles of the same size (100–250 μm) formed no bone. The authors thus concluded that the size and shape of carrier particles

determined the extent of bone formation. It is not clear how particle size would translate to pore size in scaffolds, although it is reasonable to speculate that the space between particles, which would be analogous to pores, played a role in bone formation.

There is no consensus regarding the optimal scaffold pore size for bone regeneration in scaffolds, although a range of 50–400 μm was found to be optimum for the development of highest fixation strength in metallic implants possessing porous surfaces.[10,11] Kuboki *et al.*[47] found that a pore size of 300–400 μm was optimal to efficiently promote bone formation in porous, honeycomb-shaped hydroxyapatite (HCHAP). They absorbed a solution of rh-BMP-2 on HCHAP scaffolds containing longitudinal tunnels of different diameters and implanted these subcutaneously into the backs of rats. The scaffolds with smaller diameter (90–120 μm) tunnels were found to promote chondrogenesis first, followed by vascular invasion leading to subsequent osteogenesis, whereas with larger diameter (350 μm) tunnels there was direct osteogenesis without any cartilage formation, probably owing to vascularization from the onset. Thus, the authors concluded that the geometry of the BMP carrier controlled differentiation of cells to form bone or cartilage. However, there are studies that argue that smaller pore sizes may be adequate for bone growth[45] and that pore sizes in the 150–710 μm range do not have any significant effect on osteoblast behavior.[43,44,75] Although results vary, an important underlying trend is the need for scaffolds to have a high porosity.

Porosity Measurement

Characterization of porosity is an important aspect of any scaffold fabrication protocol. There are several methods to determine the porosity of a scaffold and quite often more than one method is used to verify results obtained by the other method.[39,64,113] A popular technique is the use of mercury intrusion porosimetry (MIP)[12,33,39,51,65,70,72,83,105,113,115] in which void volume is determined by forcing mercury into the pores under pressure. This technique is based on the principle that the pressure required to force a nonwetting liquid such as mercury into pores, against the resistance imparted by liquid surface tension, is indicative of pore size and void volume.[3]

Porosity values may also be derived from scanning electron microscopy of cross-sections of scaffolds.[10,11,39,42,51,73,98,102,113,126] Two-dimensional images and image analysis can yield the pore to polymer area, which is then extrapolated to 3D to obtain estimates of porosity.

A rather simple technique involves the use of the Archimedes' Principle.[1,18] This method requires initial measurement of the dry mass of the scaffold following which the scaffold is saturated with water by prewetting with ethanol under negative pressure. The scaffold is then removed from water and weighed to determine its wet mass. The scaffold is then totally immersed in water and its submerged mass is recorded. Percent porosity can then be expressed as: $\%\text{Porosity} = (M_{\text{wet}} - M_{\text{dry}})/(M_{\text{wet}} - M_{\text{submerged}})$.

A modification of this technique is the liquid displacement method.[79,125] Here a scaffold of weight W is immersed in a graduated cylinder containing a known volume (V_1) of ethanol. The cylinder is then placed in vacuum to force ethanol into the pores of the scaffold till no air bubbles emerge from the scaffold. V_2 is designated as the total volume of ethanol and ethanol-saturated scaffold. The volume difference ($V_2 - V_1$) is the volume of the scaffold skeleton. On removing the ethanol-saturated scaffold from the cylinder, the residual ethanol volume (V_3) is recorded. Now, the void volume of the scaffold is the volume of ethanol held in the scaffold, which is given by $V_1 - V_3$. Hence, the total volume (V) of the scaffold is given by: $V = (V_2 - V_1) + (V_1 - V_3) = V_2 - V_3$. The density ($d$) of the scaffold is expressed as $d = W/(V_2 - V_3)$ and the porosity (p) of the scaffold, $p = (V_1 - V_3)/(V_2 - V_3)$.

A simpler method used to obtain the porosity of a scaffold also requires the use of its density,[38,41,73,92] and is referred to by some as gravimetry.[65] Once the dimensions and mass of the scaffold are measured, the density (d) of the porous structure is obtained from $d = m/v$, where m is the mass and v the volume of the porous structure. The porosity of the porous structure, p_o, is calculated using $p_o = 1 - d/d_p$, where d_p is the density of the nonporous polymer, obtained using pycnometry.

Ma *et al.*[59] calculated scaffold porosity (p) from measured scaffold density (D_m) and polymer skeletal density (D_s): $p = (D_s - D_m)/D_s$. D_m was calculated using the mass and volume measurements of the porous structure as shown above and D_s was determined by $D_s = 1/\{[(1 - X_c)/D_a] + [X_c/D_c]\}$, where X_c was the degree of crystallinity determined with differential scanning calorimetry, D_a was the density of amorphous polymer and D_c was the density of 100% crystalline polymer.

Maspero *et al.*[65] differentiated between open and closed porosity in their structural characterization of PLG scaffolds using CO_2 as a solvent. Closed pores are generally created due to the formation of a gas phase in polymers and correspond to regions that are inaccessible to mercury during porosity measurements. The scaffold porosity and pore size distribution were studied using gravimetry, MIP, confocal laser scanning microscopy (CLSM) and computed micro tomography (CMT). Gravimetry does not distinguish between open and closed pores and the volume measurements are often inaccurate, leading to an overestimation of total porosity. MIP was found to be unreliable for certain conditions like when the inner pores in a scaffold were inaccessible or for measuring pores greater than 800 μm.[51] The presence of large pores was reported to cause mercury penetration into the scaffold without any pressure application,

thereby leading to an underestimation of scaffold volume and total porosity.[64,65] CLSM is a 2D imaging technique requiring the destruction of the sample to prepare planar sections. CLSM images allow one to distinguish between the PLG scaffold, epoxy resin and holes based on the range of values occupied by these phases on a gray scale. Here, the resin, which is used to fill the open pores and solidifies to maintain their shape while sectioning of the scaffold, corresponds to open pores and holes correspond to regions where material is absent and no light is reflected, indicating closed pores. However, this method is plagued by difficulty in obtaining uniform brightness on the whole image. The resin and polymer matrix can exhibit same gray values if the surface and laser are not exactly aligned, leading to an underestimation of open porosity. Nonuniform wetting of scaffold with epoxy resin resulting in irregular filling of interconnected pores could also pose a potential problem. Moreover, some bubble-like pores having thin orifices, which correctly classify as open porosities by 3D analysis may do so as closed porosities in 2D analysis. Although CLSM has high resolution in the horizontal plane, its penetration depth is only up to a few hundred microns, thereby necessitating slicing of scaffold for visualization of pores. Thus, CMT proved to be most advantageous due to its precise, nondestructive 3D evaluation of open and closed porosities. The use of CMT, however, is generally limited by its resolution, which in this case was high (5.4 μm). However, CMT is not widely available and hence its use in tissue engineering is limited.

Pore Interconnectivity

Tienen *et al.*[106] have emphasized the need for high porosity and interconnectivity in a scaffold. Suh *et al.*[102] compared the proliferation of chondrocytes on equally porous (95%) PLG scaffolds prepared by the solvent casting and particulate leaching technique using two different porogens: salt and gelatin. The scaffolds produced using gelatin exhibited better cell attachment and proliferation, and this was attributed to better pore interconnectivity at the same porosity. Hou *et al.*[38] suggested that extraction of salt particles in a salt leaching process implied that the resulting pores were interconnected. However, complete removal of the salt does not necessarily ensure a permeable structure as there might be dead-end spaces with only a single opening thereby not permitting end-to-end interconnectivity of the whole structure.

Li *et al.*[52] appreciated the difficulty in obtaining 3D information about pore interconnectivity through 2D images and devised a rather simple, but novel experiment to verify the same. They soaked porous HA in black pigment dispersion and centrifuged it. After removal of pigments, they sectioned, dried and pictured the sample. Black colored pores were found to have been accessible either directly or via adjacent pores.

Permeability

The terms permeability and porosity are sometimes incorrectly used interchangeably in tissue engineering. These terms have inherently different meanings where porosity indicates the amount of void space within a structure while permeability is a measure of the ease with which a fluid can flow through the structure.[2] To measure the permeability of a scaffold direct permeation experiments can be performed. These involve the measurement of the rate of flow of water through the scaffold under a known hydrostatic pressure head.[1,2] Darcy's Law can then be used to calculate the permeability of the specimen: $k = QL/(hAt)$, where k is the permeability constant, Q is the quantity of discharge, A is the cross-sectional area of the sample, L is the length of the sample in the direction of flow, h is the hydraulic head, and t is the time. Alternatively, for a viscous fluid, $k = qL\mu/pA$, where q is the volumetric flow rate, μ is the fluid viscosity and p is the pressure drop across the specimen.[51] Emperical relations for permeability and porosity have been described by Scheidegger[91] using various simplistic capillaric models to represent the porous structure, for example the straight capillaric model comprising a bundle of straight parallel capillaries, where the permeability $k = P^3/T^2S^2$, where P is the porosity, T is the tortuosity, i.e., the ratio of the flow path length to the model length and S is the average specific surface area i.e., the ratio of the area of the capillaries to the volume of the model. The tortuosity term is a reflection of the internal architecture of the scaffold and refers to the twisted path that a fluid would take through the interconnected pores in order to get from one end of the scaffold to the opposite end, divided by the straight line distance between the opposite faces. The fluid will take the path of least resistance though the scaffold, thus changing its direction whenever its path is obstructed by the polymer. Tortuosity is, thus, the result of the hindrance offered to fluid flow by the polymer. Greater the hindrance, greater the tortuosity and lesser the permeability. The Kozeny equation describing permeability in a system by the equation $k = cP^3/S^2$, has also been modified to cP^3/TS^2 in order to incorporate the tortuosity term.[20,91] Here c is a dimensionless constant, called the Kozeny constant, whose value depends on the geometry of the cross-section of the capillary tube. However, T and S are difficult to determine independently and c varies considerably from one sample to the next as it is an empirical factor.[20]

Although it is true that quite often an increase in porosity leads to an increase in permeability, this happens only when the pores are highly interconnected. One of the authors (Agrawal) has previously shown that scaffolds can possess different permeabilities while maintaining similar porosity.[1,2] Thus, permeability should be treated as an independent scaffold design parameter. A high permeability can translate into superior diffusion within the scaffold, which would facilitate the inflow of nutrients and the disposal of

degradation products and metabolic waste. Permeability is also affected by fluid-material interactions and thus influences the viscoelastic response of a scaffold. This in turn affects the fluid pumping movement of the scaffold.[48] This is of importance in the design of scaffolds for articular cartilage repair, where mechanotransduction and cell apoptosis may be affected by hydrostatic pressure and flow-induced shear.

The porosity and permeability of a scaffold are obviously related to the physical and mechanical properties of a scaffold. For example, better mechanical properties may be obtained for a scaffold if it is made more solid and less porous. Less intuitive is the fact that porosity and permeability can also have a significant impact on the chemical behavior of the scaffold, especially its degradation characteristics. For example, as stated earlier it has been shown that low porosity and permeability PLG scaffolds degrade faster.[2,6] Also, such scaffolds exhibit a lower decrease in their mass, molecular weight, and mechanical properties under dynamic fluid flow conditions compared to static conditions.[2] This phenomenon has been attributed to the inhibition of autocatalytic degradation due to better diffusion or forced fluid flow.

Li *et al*.[51] found that porosity and pore size alone were inappropriate to describe the accessibility of inner voids in macroporous scaffolds. They advocated the use of the permeability/porosity ratio, which is an indicator of the percolative efficiency per unit porous volume of a scaffold, where permeability can be termed as the conductance normalized by size of sample and fluid viscosity. A positive correlation could exist between porosity and permeability provided there was good interconnection between the pores. They found that permeability could represent a combination of five important scaffold parameters: porosity, pore size and distribution, interconnectivity, fenestration size and distribution and pore orientation.

Nutrient Mixing

In vitro nutrient availability for cells on a scaffold depends on the mixing technique used in the bioreactor. Freed and colleagues have published extensively on the effects of mixing in bioreactors on the growth of tissue-engineered cartilage. They found mixed culture conditions to yield implants with thicker and higher cell density as compared to static culture conditions.[30] This was attributed to reduction of diffusional constraints and elimination of external concentration gradients. Also, mixed seeding conditions were found to yield higher, more spatially uniform implant cell densities.[27] In one study, they grew chondrocytes on polyglycolic scaffolds under static and mixed conditions.[110] Mixed cultures exhibited 70% more cells, 60% more sulfated glycosaminoglycan (GAG), and 125% more total collagen compared to their statically cultured counterparts. Moreover, static conditions resulted in cells having a rounded morphology where as mixing yielded layers of elongated cells and collagen. Vunjak-Novakovic *et al*.[112] seeded PGA scaffolds with chondrocytes in well-mixed spinner flasks, where mixing promoted the formation of cell aggregates that enhanced the kinetics of cell attachment while simultaneously providing spatially uniform cell distribution and high yield. In a follow-up study they investigated the effects of mixing intensity on cartilage growth *in vitro*.[35] Static conditions were compared to nine different turbulent mixing conditions, and it was found that the absence of mixing had a greater impact than the different types of mixing. The authors reported an increase in GAG formation under mixing conditions but lower GAG retention. The retention rate decreased with an increase in mixing intensity. The media for mixed conditions contained equilibrium levels of O_2 and CO_2 while the static conditions exhibited depleted amounts of these gases. The positive effect of oxygen control has also been reported in another study.[17] However, low oxygen tension has been found to favor chondrogenesis.[47,68] Still other studies have demonstrated that cell growth rates under static culture conditions are adversely affected by decreased diffusion. Possible reasons for this decrease could be increased cell mass and/or pore occlusion resulting from cartilage regeneration.[29,111] Nutrient mixing, shown to be beneficial for tissue growth, can have several simultaneous effects including altered polymer degradation,[2] improved chemotransport, mechanotransduction effects on cells and cytokine secretion. It is not clear which of these effects are dominant or even if they work in concert or have mutually negating effects.

Goldstein *et al*.[32] studied different types of osteoblastic cell culturing techniques to determine which one provided optimal diffusion of nutrients into 3D osteoconductive scaffolds and outflow of metabolites thereof. The static cultures were found to be inferior to the culturing schemes that convect media (spinner flask, rotary vessel and perfusion flow system) due to the rates of diffusion being inadequate to satisfy the requirements of cells not only resulting in the suppression of their growth but also promoting preferential growth on the surface due to chemotaxis of cells from within. Burg *et al*.[15] compared three proliferation environments: static, dynamic and perfusion and found the perfusion bioreactor to be the best. Sittinger *et al*.[99] cultured tissue constructs for growing cartilage in perfusion chambers to provide for good nutrient diffusion.

Flow through scaffolds, though primarily aimed at increasing nutrient supply, could have a secondary effect on mechanotransduction. Cells growing within scaffolds are subjected to shear, compressive and/or tensile forces depending on their orientation with respect to flow direction. Saini *et al*.[90] cultured chondrocytes on PLA scaffolds under static and hydrodynamic loading conditions. Like other groups, they too found the statically grown constructs to support matrix production only superficially where as hydrodynamic loading resulted in greater abundance of GAG

and collagen in the scaffold interior. The authors used a concentric cylindrical bioreactor with a stationary bob serving as an anchor for the scaffolds surrounded by a rotating cup whose speed determined the amount of hydrodynamic loading. Although high rotation rates increased collagen content, they simultaneously decreased construct GAG composition thereby indicating that the shear forces generated by hydrodynamic loading had effects beyond increasing nutrient transport, namely altering matrix synthesis and composition.

Gomes *et al.*[34] studied the effects of static and flow perfusion culturing conditions on growth and differentiation of rat bone marrow stromal cells seeded on two types of starch-based scaffolds. They found a significant increase in calcium deposition, which is a marker of osteoblast maturation, on both types of scaffolds cultured under flow perfusion conditions. They attributed the enhanced mineralization brought about by flow perfusion to possible subjection of cells to fluid shear induced mechanical stimulation. They also found the scaffolds cultured under static conditions to limit most of the cells to the outer surface resulting from nonuniform cell distribution compared to flow perfusion, which improved their distribution.

Sikavitsas and colleagues[95] cultured rat marrow stromal cells (MSCs) on PLG scaffolds prepared by solvent casting/particulate leaching, under static conditions as well as in two types of bioreactors (spinner flask and rotating wall vessel). Spinner flask systems comprised scaffolds attached to needles descending from the lid of the flask. A magnetic stirrer minimized nutrient transport limitations by generating convective forces that continuously mixed the media around the scaffolds. The rotating wall vessel bioreactor consisted of two concentric cylinders: an inner stationary cylinder through which gaseous exchange took place and an outer cylinder that rotated at a predetermined rate. The free falling scaffolds, contained in the annular space, were balanced by the forces of gravity and centrifugation, establishing microgravity-like culture conditions. However, in all three cases nutrient transport to the interior of the scaffold was diffusion limited. The possible reasons given for this lack of diffusion in bioreactors were insufficient mixing at the surface of the scaffolds to deliver nutrients to the interior in case of the spinner flasks, and collisions of the scaffolds with the reactor's walls disturbing the settling of cells and possibly traumatizing them, in case of the rotating wall vessel.

The same research group explored using a flow perfusion bioreactor to overcome diffusional limitations and also to simultaneously subject the cells to mechanical stress.[7] Their perfusion bioreactor utilized a pump to continuously perfuse media through the scaffold, thereby not only eliminating external limitations to diffusion but also providing adequate diffusion within the scaffold. Titanium fiber mesh scaffolds seeded with MSCs were subjected to static and flow perfusion conditions and the latter were found to enhance early proliferation, differentiation and mineralized matrix production.[24] An increase in mineralized matrix production and calcium content with increasing flow rate was observed.[8] On perfusing fluid with different viscosities at a constant flow rate to increase the level of mechanical stimulation while keeping conditions for diffusion of nutrients and waste constant, they found the deposition of mineralized matrix to increase with increasing viscosity.[96]

Malaviya *et al.*[61] on subjecting monolayers of chondrocytes to shear stress in a parallel plate flow system found the anchorage-dependent cells to overgrow the monolayer and increase their number drastically in spite of adapting a rounded morphology. They attributed this behavior to the hydrodynamic environment created by the shear forces, which may have caused the chondrocytes to increase the secretion of cytokines, like TGF-β1, that regulate cell proliferation and matrix synthesis.

Thus, nutrient mixing has been shown to be beneficial and it follows that scaffolds should be designed to facilitate nutrient mixing with appropriate porosity and permeability.

Modeling of Diffusion in Tissue Engineering Scaffolds

With diffusion in tissue engineering scaffolds increasingly receiving importance, some research groups have attempted to model the process. For example, Botchwey *et al.*[13] developed a one-dimensional model to study the efficiency of glucose diffusion to osteoblast-like cells in PLG scaffolds under static and dynamic culture conditions. They assumed that the scaffold possessed cylindrical channels with a fixed cell density consuming the same amount of glucose in the absence of flow. They utilized the equation developed by Petrov *et al.*[76] to formulate the homeostatic diffusion problem within lacunar–canalicular systems in conjunction with suitable boundary conditions to obtain an analytical solution for concentration of glucose (C) as a function of depth (x) within the scaffold:

$$C(x) = C_{\mathrm{o}} - \frac{Nr}{2lDn\sigma}(Lx - x^2)$$

where, C_o, glucose concentration at the exterior boundary of the scaffold (g m^{-3}); N, total number of cells within the scaffold; r, single osteoblast rate of glucose consumption (g s^{-1}); L, thickness of scaffold (m); l, length of cylindrical pores within scaffold (m); D, Diffusivity of glucose (m^2 s^{-1}); n, total number of cylindrical pores; σ, average cross-sectional area of pores (m^2).

For the dynamic case they assumed that neither would the geometry of the scaffold change nor would the scaffold degrade over the period they considered for modeling purposes. Also, the scaffold cell density was once again held constant and glucose consumption was assumed to be uniform as a function of depth within the scaffold. They utilized equations[14] for calculating permeability in a cylindrical scaffold having cylindrical channels in conjunction with the drag force imparted on a cylinder and Darcy's law

to derive the velocity of fluid flow through the scaffold (V):

$$V = \frac{-K}{\eta}\frac{\Delta P}{L}$$

where, K, permeability (m^2); η, fluid viscosity (kg m^{-1} s^{-1}); $\Delta P/L$, pressure gradient (N m^{-3}).

Volume of fluid within pores of the scaffold (ΔM), quantity of glucose consumed by this volume (ΔQ) and average residence time of fluid within this volume (T) were used to relate V to change in nutrient concentration (ΔC) within the scaffold, resulting in the following equation:

$$\Delta C = \frac{N}{n}\frac{\Delta x}{l}\frac{r}{\sigma V}$$

where, Δx, incremental depth within scaffold (m).

Based on their model they concluded that passive glucose diffusion was unable to maintain minimum glucose concentration beyond a few hundred microns within modeled constructs. Also, the maximum depth of modeled glucose penetration was found to increase linearly with internal perfusion rate and pore-size. The internal fluid flow rate, in turn, was influenced by pore volume, pore diameter and pore tortuosity of the scaffold. Their calculations indicated that static and some incorrectly designed dynamic culturing environments led to areas of nutrient concentration inadequate to sustain cell viability. They suggested a need to balance flow-induced nutrient flux with the effects of fluid shear stress, which could interfere with attachment and growth of cells on the exterior during culturing.

Malda *et al.*[62] measured oxygen concentrations as a function of depth within osteochondral explants and spinner flask-seeded cylindrical scaffolds, made of segmented block copolymers of alternating polyethylene glycol terephthalate and polybutylene terephthalate, using a glass microelectrode system. The microelectrode, having a tip diameter of 5 μm, was used in combination with a micromanipulator to determine oxygen profiles with a spatial resolution of 10 μm. The samples were confined so as to allow diffusion only from the top. The electrode was lowered into the constructs up to a depth of 2500 μm and oxygen concentration was recorded at 100 μm intervals during the ascent of the electrode. The electrode was lowered at three locations on the surface of each cylindrical scaffold, including the center and the edge. At all three locations the decrease in oxygen concentration with depth followed the same trend. The increasing rapidity of this decrease with depth was attributed to greater cellularity and low diffusivity of oxygen in the constructs. Up to 27 days, oxygen tension towards the periphery of the scaffold was found to decrease faster and this was speculated to be due to higher cell concentrations in the region. However, by day 41, all three locations had almost identical profiles, owing to the chondrocyte distribution becoming more homogeneous.

They developed a mathematical model to quite accurately predict the oxygen profiles within the explants and constructs, using the following mass balance:

$$\frac{\partial C_{O2}(x,r,t)}{\partial t} = D\left(\frac{\partial^2 C_{O2}(x,r,t)}{\partial x^2} + \frac{\partial^2 C_{O2}(x,r,t)}{\partial r^2} + \frac{2}{r}\frac{\partial C_{O2}(x,r,t)}{\partial r}\right) - r_o(x,r,t)$$

with the following boundary conditions:

$$x = Z, \quad \frac{\partial C(Z,r)}{\partial x} = 0,$$
$$x = 0, \quad C(0,r) = C_{\text{medium}}(= 21\%),$$
$$r = R, \quad \frac{\partial C(x,R)}{\partial r} = 0,$$

where, C_{O2}, local oxygen concentration (mol m^{-3}); x, distance within scaffold (m); r, radius (m); t, time (s); D, diffusion coefficient of oxygen in construct (m^2 s^{-1}); Z, thickness in the x direction (m); R, radius of construct (m); r_o, oxygen consumption rate by chondrocytes (mol m^{-3} s^{-1}), given by:

$$r_o(x,r,t) = C_{\text{cell}}(x,r,t)\frac{Q_{\max}C_{O2}(x,r,t)}{K_m + C_{O2}(x,r,t)}$$

where C_{cell}, cell density (cells m^{-3}); $Q_{\max}$, maximal oxygen consumption rate (mol $cell^{-1}s^{-1}$); K_m, oxygen concentration at half maximal oxygen consumption (mol m^{-3}).

These equations could not be solved analytically owing to their complexity and had to be solved numerically by dividing the construct into layers in the transverse and radial directions. This, however, gave them the ability to alter the value of r_o from one layer to the next, which can change dramatically as a function of cell distribution, especially as a function of depth, within the scaffold. The model was found to be very sensitive to changes in C_{cell}, $Q_{\max}$ and D. Using ranges of C_{cell}, D and K_m available from literature, the model was fitted to the measured gradients in native cartilage by varying $Q_{\max}$. However, for the constructs, the value of r_o obtained after measuring C_{cell}, estimating D and K_m and varying $Q_{\max}$ was found to be lower than the range found for chondrocytes in native cartilage.

CONCLUSION

As new technologies for fabricating and culturing tissue engineering constructs are developed, it is imperative that the issue of adequate diffusion receive attention. In the absence of blood supply (as in the initial periods of tissue regeneration) a lack of proper diffusion will result in decreased nutrient inflow and a slower metabolic and degradation waste outflow. This may translate into cell migration to the periphery due to chemotaxis or eventual cell necrosis. We propose that in the case of vascularized tissues, a central goal of tissue engineering scaffolds should be to provide an architecture that ensures adequate nutrient supply prior to the establishment of blood supply. This aim will involve the

simultaneous optimization of several factors related to scaffold architecture, including porosity, pore size, permeability, channel tortuosity, and degradation properties. These need to be addressed along with choice of appropriate materials, which may influence cellular response, and manufacturing processes that will provide adequate mechanical properties for the production of functional scaffolds for musculoskeletal tissue engineering applications.

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