Chapter 5
Nanofibers and Microfibers for Osteochondral Tissue Engineering

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Abstract The use of fibers into scaffolds is a way to mimic natural tissues, in which fibrils are embedded in a matrix. The use of fibers can improve the mechanical properties of the scaffolds and may act as structural support for cell growth. Also, as the morphology of fibrous scaffolds is similar to the natural extracellular matrix, cells cultured on these scaffolds tend to maintain their phenotypic shape. Different materials and techniques can be used to produce micrfibers- and nanofibers for scaffolds manufacturing; cells, in general, adhere and proliferate very well on PCL, chitosan, silk fibroin, and other nanofibers. One of the most important techniques to produce microfibers/nanofibers is electrospinning. Nanofibrous scaffolds are receiving increasing attention in bone tissue engineering, because they are able to offer a favorable microenvironment for cell attachment and growth. Different polymers can be electrospun, i.e., polyester, polyurethane, PLA, PCL, collagen, and silk. Other materials such as bioglass fibers, nanocellulose, and even carbon fiber and fabrics have been used to help increase bioactivity, mechanical properties of the scaffold, and cell proliferation. A compilation of mechanical properties and most common biological tests performed on fibrous scaffolds is included in this chapter.

Highlights

- The use of microfibers and nanofibers allows for tailoring the scaffold properties.
- Electrospinning is one of the most important techniques nowadays to produce fibrous scaffolds.
- Microfibers and nanofibers use in scaffolds is a promising field to improve the behavior of scaffolds in osteochondral applications.

Keywords Microfibers · Nanofibers · Fibrous scaffolds · Electrospinning

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Abbreviations

BDG	Butylene diglycolate
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
BMSCs	Bone marrow mesenchymal stem cells
BTDG	Butylene thiodiglycolate
CPP	Calcium pyrophosphate
CPP	Casein phosphopeptide
GAG	Glycosaminoglycan
HA	Hydroxyapatite
hESC	Human embryonic stem cells
hMSCs	Human mesenchymal stem cells
PA	Polyamide
PCL	Polycaprolactone
PDLA	Poly D,L-lactic acid
PEEK	Poly(ether-ether-ketone)
PEG	Poly(ethylene glycol)
PEO	Poly(ethylene oxide)
PET	Polyethylene terephthalate
PGA	Poly glycolic acid
PLA	Polylactic acid
PLGA	Poly(lactic-co-glycolic acid)
PLLA	Poly L-lactic acid
PVA	Polyvinyl alcohol
PVA-MA	Poly(vinyl alcohol)-methacrylate
PVP	Polyvinylpyrrolidone
rhBMP	Recombinant human morphogenetic protein
SBF	Simulated body fluid
TCP	Tricalcium phosphate
TFG-β1	Transforming growth factor-β1
TIPS	Thermally induced phase separation

5.1 Introduction

The use of fibers into scaffolds is a way to mimic natural tissues, in which fibrils are embedded in a matrix. Their use can also improve the mechanical properties of the scaffolds and may act as structural support for cell growth. Furthermore, due to their large surface, microfibers and nanofibers can be functionalized by the addition of antibiotics, peptides, RNA or other substances in order to increase their bioactivity or prevent infections, among other possibilities. There are different materials used as fibers within the tissue engineering field, depending on the intended objective, manufacturing process and scaffold material. The materials used as matrix also show a wide range of possibilities, from natural polymers (gelatin or collagen) to

bioglass or even carbon fibers. Electrospinning appears to be the most used technique in literature for microfiber and nanofiber production, although novel techniques are also being employed widely.

In the last years, fibers have been produced in a gradually increased materials range, from synthetic polymers (PCL, PLA, polyester, polyurethanes, etc.) to natural ones (silk, fibroin, chitosan, cellulose, etc.), from metals (titanium alloys) to ceramic materials (bioglass or calcium phosphates, even carbon fibers have been used for reinforcement of hyaluronic acid matrices. The main advantage in introducing microfibers or nanofibers within osteochondral tissue engineering is the possibility of tailoring the properties of scaffolds; porosity, pore size, mechanical properties, resilience, flexibility, bioactivity, and hydrophilicity constitute just a short list of potential adaptations. What is also of high interest is the combination of different materials to obtain a wider range of properties, both from the biological and mechanical sides.

As a summary, this is a very promising field, which has suffered a huge development in the last years, although further investigations on materials and manufacturing techniques need still to be performed. The ability of fibrous scaffolds to mimic extracellular matrix makes them definitely suitable for osteochondral applications.

5.2 Types of Fibers

5.2.1 Synthetic Polymeric Fibers

Different materials have been used to obtain microfibrous scaffolds by electrospinning, as this process is able to produce polymeric fibers from a molten or dissolved polymer at the micrometric and nanometric scale [1]. The benefit of using electrospinning in tissue engineering is that electrospun scaffolds show a similar morphology to the fibrous components of the natural extracellular matrix (ECM) [2], and so cells cultured on them tend to maintain their phenotypic shape [3]. Even though, electrospinning is not yet so widely implemented due to its slow production.

Electrospinning has been used as an efficient processing method to manufacture nanofibrous structures, enhancing cell proliferation and osteogenic differentiation [4]. Moreover, the small scale pores of electrospun nanofibrous scaffolds prevent cell migration, guiding tissue regeneration along the surface of the nanofibrous membrane [5], while porous hierarchical structures enable cell penetration, increasing the surface area for cell adhesion [6]. Furthermore, nanofibers, due to their vast surface, can be functionalized with drugs, antibiotics, bioactive peptides, proteins, RNA, and DNA [7].

Electrospun synthetic polymeric fibers have been widely explored for tissue engineering applications. Biodegradable materials like polylactic acid (PLA) or polycaprolactone (PCL) have suitable mechanical properties for the regeneration of cartilage and bone tissues and they degrade into nontoxic products. The use of polymeric micro – and nano – fibers allows obtaining wide wide range of proper-ties, as summarized in Tables 5.1 and 5.2 (also showing fibrous scaffolds in non – polymeric materials).

Table 5.1 Mechanical properties under compressive tests for fibrous scaffolds

Scaffold materials	Method of fabrication	Mechanical property	Value	Ref.
PLA nanofibers/ alginate-hyaluronic acid hydrogel	Electrospinning and aminolysis, esterification and cross-linking reactions	Young's modulus for a 1:1 hydrogel to fibers weight ratio	5.40 ± 0.90 kPa	[54]
PLLA nanofibers/ collagen	Freeze-drying and electrospinning	Young's modulus (week 12 after surgery)	~ 0.57 MPa	[5]
PLLA microfibrous sheets treated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/ gelatin–nanoHA	Electrospinning and freeze-drying	Compressive strength analysis (wet state) of a six PLLA layered scaffold	~ 6.0 MPa	[2]
P(LLA-CL) and collagen type I yarn mesh/hyaluronate/TCP	Electrospinning and freeze-drying	Compressive strength of the yarn-collagen type I/ hyaluronate hybrid scaffold	~ 0.25 MPa	[20]
PCL microfibrous discs/ PLGA	Thermally induced phase separation and electrospinning	Compressive modulus	125 ± 22 kPa for 90% porosity 75 ± 25 kPa for 95% porosity	[4]
		Increase in the elastic modulus between the first and last cycles of the test (%)	149 ± 45 for 90% porosity 135 ± 35 for 95% porosity	
		Increase in the strain at peak during fatigue (%)	204 ± 72 for 90% porosity 152 ± 15 for 95% porosity	
Oriented PCL fibrous membrane/collagen type I and hyaluronic acid/ TCP	Electrospinning and freeze-drying	Compressive modulus of the chondral phase	0.205 ± 0.029 MPa	[73]
		Compressive modulus of cylindrical TCP specimens	216.04 ± 48.08 MPa	
PVA nanofibers/ hyaluronate/type I collagen/fibrin	Sol-gel processing	Young's modulus at 20% strain of the scaffold enriched with liposomes, basic fibroblast growth factor and insulin	~ 2.0 MPa	[24]

(continued)

Table 5.1 (continued)

Scaffold materials	Method of fabrication	Mechanical property	Value	Ref.
Multiphasic calcium phosphate fibers/ chitosan	Freeze-drying	Compressive yield strength for a 1:1 chitosan to fibers weight ratio	~ 420 kPa	[59]
		Elastic modulus for a 1:1 chitosan to fibers weight ratio	~ 3.87 MPa	
Collagen fibers/ hydroxyapatite	Freeze-drying	Young's modulus of a 50HA–50COL scaffold	~ 7 kPa	[72]
Fibrous collagen/PEG hydrogels	Lyophilization and photopolymerization processes	Tangent modulus at 15–20% strain	~ 400 kPa	[38]
Collagen-PCL nanofibers/PCL-coated 45S5 bioactive glass	Foam replication process and electrospinning	Compressive strength of PCL dip-coated 45S5 BG scaffolds	0.24 ± 0.06 MPa	[7]
Collagen fibrils/alginate/ hyaluronic acid	Sol-gel processing	Compressive stress at 30% strain	~ 65 kPa	[11]
Alginate/hydroxyapatite/ bacterial nanocellulose			~ 80 kPa	
Knitted silk-collagen sponge with hESC-MSCs	Knitting technique and freeze-drying	Young's modulus	34.91 ± 5.08 MPa	[17]
Silk fibers/regenerated fibroin	Freeze-drying	Ultimate compressive strength for scaffolds seeded with autologous chondrocytes after 9 months	0.258 ± 0.158 MPa	[14]
		Young's modulus for scaffolds seeded with autologous chondrocytes after 9 months	2.661 ± 1.79 MPa	
Silk fibroin yarns/ polyethylene terephthalate	Knitting technique	Elastic modulus	41.9 ± 17.1 kPa	[53]

(continued)

Table 5.1 (continued)

Scaffold materials	Method of fabrication	Mechanical property	Value	Ref.
Pullulan/cellulose acetate	Electrospinning, cross-linking and freeze-drying	Young's modulus of a P50/CA50 scaffold	4.13 ± 0.68 MPa	[50]
		Compressive strength of a P50/CA50 scaffold	0.43 ± 0.01 MPa	
		Strain of a P50/CA50 scaffold (%)	27.64 ± 2.89	

Table 5.2 Mechanical properties under tensile and flexural tests for fibrous scaffolds

Scaffold materials	Method of fabrication	Test	Mechanical property	Value	Ref.
P(LLA-CL) and collagen type I yarn mesh/hyaluronate/TCP	Electrospinning and freeze-drying	Tensile	Tensile strength of the yarn-collagen type I/hyaluronate hybrid scaffold	3.43 ± 0.15 MPa	[20]
PCL microfibrous discs/PLGA	Thermally induced phase separation (TIPS) and electrospinning	Tensile	Elastic modulus	~ 7 MPa for 90% porosity ~ 5 MPa for 95% porosity	[4]
			Ultimate stress	~ 1.6 MPa for 90% porosity ~ 1.1 MPa for 95% porosity	
			Ultimate strain	400% for 90% porosity 250% for 95% porosity	
			Increase in the elastic modulus between the first and last cycles of the test	~ 120%	
			Increase in the strain at peak during fatigue	~ 220%	

(continued)

Table 5.2 (continued)

Scaffold materials	Method of fabrication	Test	Mechanical property	Value	Ref.
Oriented PCL fibrous membrane/ collagen type I and hyaluronic acid /TCP	Electrospinning and freeze-drying	Tensile	Tensile strength for PCL fibrous membranes	4.07 ± 0.37 MPa	[73]
			Tensile modulus for PCL fibrous membranes	36.14 ± 3.58 MPa	
Poly(butylene succinate) mesh	Electrospinning	Tensile	Elastic modulus of polymeric films	~ 500 MPa	[13]
Cellulose acetate nanofibers/ polyethylene terephthalate	XanoMatrix™ (commercial product)	Tensile	Modulus of elasticity	~ 0.509 GPa	[45]
70S bioactive glass/silk fibroin	Electrospinning	Tensile	Young's modulus	27.48 ± 3.96 MPa	[52]
			Elongation at break (%)	8.52 ± 1.43	
Hydroxyapatite nanofibers/ cellulose	Electrospinning	Tensile	Tensile strength of 5% nano-HA scaffold	~ 70.6 MPa	[44]
			Elastic modulus of 5% nano-HA scaffold	~ 3.12 GPa	
			Elongation at break of 5% nano-HA scaffold	~ 5.56%	
Collagen-PVA nanofibers/ collagen sponge	Freeze-drying and electrospinning	Tensile	Young's modulus	~ 0.25 MPa	[12]
			Ultimate tensile strength	~ 0.07 MPa	

(continued)

Table 5.2 (continued)

Scaffold materials	Method of fabrication	Test	Mechanical property	Value	Ref.
Collagen-PCL nanofibers/ PCL-coated 45S5 bioactive glass	Foam replication process and electrospinning	Tensile	Young's modulus of collagen-PCL fibrous meshes	23 ± 10 MPa	[7]
Gelatin mesh	Electrospinning	Tensile	Tensile modulus	426 ± 39 MPa	[62]
Collagen mesh				262 ± 18 MPa	
Elastin mesh				184 ± 98 MPa	
Tropoelastin mesh				~ 289 MPa	
Pullulan/cellulose acetate	Electrospinning, cross-linking and freeze-drying	Tensile	Young's modulus of a P50/CA50 scaffold	1.54 ± 0.13 MPa	[50]
			Ultimate tensile strength of a P50/CA50 scaffold	0.11 ± 0.02 MPa	
			Strain of a P50/CA50 scaffold (%)	33.93 ± 2.18	
Titanium fibers/13–93 bioactive glass	Freeform extrusion fabrication	Flexural	Flexural strength of scaffolds made with 0.4 vol% Ti fibers	14.9 ± 1.3 MPa	[70, 71]
			Modulus of elasticity of scaffolds made with 0.4 vol% Ti fibers	15.2 ± 4.1 GPa	
			Fracture toughness of scaffolds	0.79 ± 0.07 MPa·m1/2	

5.2.1.1 Polylactic Acid (PLA)

The use of polymers such as PLA in the fibrous form offers structural support to the cells and is more similar to gelatin or collagen naturally present in terms of resilience, fracture toughness, elasticity and flexibility [4].

Biodegradable microfibrous PLLA/PVA sheets were incorporated into a gelatin–nanoHA matrix, achieving better cellular migration towards the center of the scaffold [4] and reducing the brittleness of the gelatin–nanoHA scaffolds.

The combination of collagen and electrospun PLLA nanofibers has been reported to synergistically promote osteochondral regeneration [5]. These tests have shown a more important osteogenic differentiation in cells seeded on collagen/PLLA scaffolds than in pure collagen ones, also leading to better cartilage formation and, in consequence, to better functional repairing of the osteochondral defects. This can be explained by the lower mechanical properties of collagen sponge to the subchondral bone, thus providing lower mechanical support for cartilage formation [5].

Apart from collagen, other natural polymers have been explored in combination with PLA fibers. For example, Mohabatpour et al. [8] proposed a hydrogel consisting of alginate-graft-hyaluronate. The presence of the nanofibers improved the mechanical properties of the hydrogel alone: the compressive modulus increased around 81%.

PDLLA nanofibers have also been used to coat bioglass scaffolds [9], with a decreased HA mineralization by increased the PDLLA thickness, thus ensuring a strong bond between the glass substrate and the PDLLA nanofibers and a smooth transition of the HA content; in vitro studies with chondrocyte cells shown good cell attachment and proliferation, leading to cell migration into the fibrous network. Hydroxyapatite and PLLA electrospun scaffolds have also been reported, showing differentiation of hMSCs, achieving chondrocyte-like phenotype with generation of a proteoglycan based matrix [10]. Copolymers derived from PLA, such as PLG (poly-D,L-lactide-co-glycolide) have been also explored for the treatment of osteochondral defects. For example, Toyokawa et al. [11] tested this type of material on the femoral condyles of rabbits.

5.2.1.2 Polycaprolactone (PCL)

Polycaprolactone is a biocompatible aliphatic polyester widely used in tissue engineering. Vaquette and Cooper-White [1] have combined electrospinning of PCL with a thermally induced phase separation (TIPS), also using PLGA. With this combination, they have been able to produce scaffolds made of electrospun membranes achieving better mechanical properties than scaffolds made by TIPS at shorter times and with no limits in the scaffold thickness. PLGA/PCL electrospun fibrous scaffolds also showed that rat bone marrow cells were infiltrated into the scaffold; GAG assays showed an abundant cartilage matrix after in vitro chondrogenic priming, leading to new bone formation in in vivo analysis [12].

Alginate hydrogels have been also combined with polycaprolactone fibrous matrices [13–15]. For example, the scaffolds proposed by Kook et al. [13] consisted of a nanofiber PCL matrix with infiltrated hydrogel and a second compartment of pure alginate hydrogel. The matrix was treated with oxygen plasma to improve the affinity with the alginate hydrogel. This structure allowed the coculture of different types of cells within the scaffold.

Bioactive glass scaffolds have been covered with PCL to enhance mechanical properties and collagen/PCL were electrospun over the coated scaffold [16]. This structure is justified by the high bioactivity of the PCL-coated bioglass scaffold, acting as support in the bone side, while the microfibers are intended for the cartilage side. Results from in vitro SBF tests show that HA crystals have grown along the surface of the collagen-PCL fibers, confirming their viability for osteochondral tissue engineering.

5.2.1.3 Other Synthetic Fibers

Anisotropic scaffolds have been obtained using 45S5 bioactive glass foam as substrate, gelatin as adhesive and short polyamide (PA) fibers, placed on the top surface of the scaffold by electroflocking [17]. This technique allows tailoring the surface porosity of the scaffold by varying the flocking time. After submersing these scaffolds on SBF for 21 days, the surface was entirely covered by HA, thus meaning that mineralization also occurs over the PA fibrils.

Another application of fibrous scaffolds is related to the tailoring of scaffolds properties, not only referred to mechanical ones but also to degradation rates. Chen and collaborators [18] have fabricated electrospun scaffolds from a block poly(butylene succinate)-based copolyesters containing either butylene thiodiglycolate (BTDG) or butylene diglycolate (BDG) sequences. The molecular architecture of the polyesters (and the heteroatom they contained, O or S) made it possible to change the mechanical properties and the hydrolysis rate of produced scaffolds. As a conclusion, they have demonstrated that copolyesters containing thioether links were more favorable for chondrogenesis, while those with ether linkages enhanced scaffold mineralization.

5.2.1.4 Fibers Including Additives

To improve the bioactivity of the synthetic fibers, several additives have been proposed, especially natural polymers and biological substances. For example, PVA has been electrospun with liposomes, bFGF, and insulin to obtain nanofibrous scaffolds [7], which, even without been cell seeded prior to implantation, showed enhanced osteochondral regeneration towards hyaline cartilage and/or fibrocartilage.

The incorporation of nanoapatitic particles to a PLGA-based nanofibrous scaffolds [19] significantly improved the tissue response of a subcutaneous implanta-

tion, thus demonstrating that the electrospun fibrous scaffolds made of PLGA/PCL at 3/1 rates with up to 30% of nanoapatitic particles allow controlling the in vivo adverse reactions of PLGA materials, leading to optimized clinical application of these materials in biomedical devices. Liu has proved that fibrous scaffolds made of electrospun hydroxyapatite/chitosan fibers show higher proliferation of BMSCs than the membranous compound [20], meaning fibrous scaffolds provide superior ability of bone reconstruction. Similarly, PLGA/PCL scaffold combined with electrospun PCL, hyaluronic acid and chondroitin sulfate nanofibers, also demonstrated that this combination stimulates the different regions of osteochondral tissue regeneration: collagen type II and aggrecan expression in the cartilage region and BMP-2 in the bone area [21]. Oriented poly(L-lactic acid)-copoly(ε-caprolactone) P(LLA-CL)/collagen type I(Col-I) nanofiber mesh made by electrospinning over a collagen I/hyaluronate sponge was fabricated to enhance the mechanical properties of the scaffold, also getting better infiltration [22]. These yarns were also produced over a TCP porous structure, obtaining improved repairing times and good compressive modulus.

Fibrous scaffolds made of PVA-MA and chondroitin sulfate–MA were obtained by electrospinning, obtaining fiber dimensions on the nanoscale for application to articular cartilage repair [23]. The low density of obtained nanofiber scaffolds allows immediate cell infiltration and optimal tissue repair, as shown in the in vitro tests. Furthermore, scaffolds containing chondroitin sulfate nanofibers lead to an increase in the deposition of type II collagen, specific to hyaline cartilage, enhancing the endogenous repair process without exogenous cells. Table 5.3 shows a summary of most usual in vitro tests in fibrous scaffolds and measured parameters.

Electrospinning has also been applied for the production of biphasic nanofiber scaffolds made of poly(lactide co-caprolactone, PLCL) and its mineralized form (obtained after activation in a NaOH solution, and then dipped alternatively in a $CaCl_2$ and Na_2HPO_4 solutions) [24]. In vitro studies shown that PLCL favored ECM secretion of cartilage, while mineralized PLCL favored bone secretion; in vivo tests in small animal model (nude mice) revealed that new cartilage and bone tissues were formed in the implanted area. This polymers combination was also used by Cui [25], but impregnating the scaffold into a chitosan-AHP solution, although reported results were similar to those without chitosan. In this case, as scaffolds did not incorporate cells neither bioactive compounds, only bone was formed.

Nanofibers in scaffolds also allow encapsulating active principles. Drugs can be encapsulated in electrospun fibers [26] to achieve a controlled release of the actives during the scaffold degradation; several authors have reported the release of various compounds, such as TFG-β1 from PCL microfibers and nanofibers, BMP-2 from PEG/PCL core/shell nanofibers, and fenbufen from PLGA [26]. Fibrous scaffolds have also been applied to the control of fibrous capsule formation, which leads to tissue fibrosis [27]. Small interfering RNA (siRNA) has been used to virtually make disappear any gene of interest; Rujitanaroj and team have used this approach to modulate fibrous capsule formation by RNAi is collagen type I. siRNA–poly(caprolactone-co-ethylethylene phosphate) nanofibers have been investigated for this purpose [27], leading to a significant decrease in fibrous capsule thickness;

Table 5.3 Most common biological assays for fibrous scaffolds

Scaffold materials	Method of fabrication	Evaluation	Cell culture/animal model	Biological assay	Ref.
PLLA nanofibers/collagen	Freeze-drying and electrospinning	In vitro	Rabbit bone marrow mesenchymal stem cells	Methylthiazolyl-diphenyl-tetrazolium-bromide (MTT)	[5]
				Von Kossa staining	
			Human bone marrow mesenchymal stem cells	RNA isolation and semiquantitative reverse transcription polymerase chain reaction	
		In vivo	Adult male New Zealand white rabbits	Hematoxylin and Eosin (H&E) and Safranin O staining	
				μ-Ct	
Poly(D,L-Lactide) nanofibers/45S5 bioglass derived foams	Foam replica technique and electrospinning	In vitro	ATDC5 chondrocyte cells	Alamar Blue	[39]
Mineralized poly(lactide-co-caprolactone) nanofibers	Electrospinning	In vitro	Primary rat articular chondrocytes and bone marrow-derived mesenchymal stem cells	Safranin O and Alcian Blue staining	[74]
				Alkaline phosphatase activity	
				Gene expression	
		In vivo	Male athymic mice	H&E, Safranin O, and Alizarin Red S staining	
				Immunostaining for type II collagen	
PCL microfibrous discs/ PLGA	Thermally induced phase separation and electrospinning	In vitro	3T3 fibroblasts	Alamar Blue	[4]

Polyvinyl alcohol-methacrylate/chondroitin sulfate	Electrospinning and UV light cross-linking	In vitro	Goat bone marrow-derived mesenchymal stem cells	DNA quantification	[10]
				Sulfated glycosaminoglycan quantification	
				Total collagen quantification	
				Immunohistochemical staining for type II collagen	
				Gene expression	
		In vivo	Rat osteochondral defects	Safranin O staining for proteoglycans	
				Immunohistochemical staining for collagen deposition	
Carboxymethyl cellulose nanofibers/hydroxyapatite	Electrospinning	In vitro	Mouse osteoblastic MC3T3-E1 cells	Methylthiazolyl-diphenyl-tetrazolium-bromide (MTT)	[49]
70S bioactive glass/silk fibroin	Electrospinning	In vitro	MG63 osteoblasts and primary porcine chondrocytes	Alamar Blue	[52]
				Alkaline phosphatase activity	
				Sirius Red dye based colorimetric assay for total collagen estimation	
				1,9-Dimethylmethylene Blue (DMMB) assay for sulfated glycosaminoglycan estimation	
				Gene expression	
			Murine macrophage cells	Immune response using an ELISA kit	
Multiphasic calcium phosphate fibers/chitosan	Freeze-drying	In vitro	Immersion in simulated body fluid	FE-SEM/EDX to evaluate the formation of a calcium phosphate-rich layer	[59]

(continued)

Table 5.3 (continued)

Scaffold materials	Method of fabrication	Evaluation	Cell culture/animal model	Biological assay	Ref.
3D nonwoven silica gel fabric	Electrospinning	In vitro	Mouse calvaria-derived preosteoblastic MC3T3-E1 cells	MTT	[64]
				Alkaline phosphatase activity	
				Total RNA extraction and reverse transcription-polymerase chain reaction	
		In vivo	New Zealand white male rabbits	Osteoconductivity test	
Collagen-PCL nanofibers/ PCL-coated 45S5 bioactive glass	Foam replication process and electrospinning	In vitro	Immersion in simulated body fluid	SEM and XRD to evaluate the formation of hydroxyapatite	[7]
Silk fibroin yarns/ polyethylene terephthalate	Knitting technique	In vitro	Human adipose-derived stem cells	DNA quantification	[53]
				Alkaline phosphatase activity	
				Alizarin Red staining	
				Immunodetection of bone-specific proteins	
				RNA isolation and real-time reverse transcriptase-polymerase chain reaction	
		In ovo	White fertilized chicken eggs	Chick chorioallantoic membrane	
				Analysis of blood vessel convergence	
				Hematoxylin and Eosin staining	
				Immunohistochemical detection	
		In vivo	CD-1 mice	H&E staining	
Gelatin mesh	Electrospinning	In vitro	Human embryonic palatal mesenchymal cells	Alamar Blue	[62]
Collagen mesh					
Elastin mesh					
Tropoelastin mesh					

Carbon nonwoven fabrics/ hyaluronic acid	Thermal treatment and carbonization	In vitro	Human lung adenocarcinoma A549 cells	Trypan Blue staining	[1]
				Cellular cytokine production to assess inflammatory potential	
			Human osteoblast-like MG-63 cells	Cell-material interaction evaluated with epifluorescence microscopy	
		In vivo	Knee cartilage of rabbits	Masson-Goldner method for histological analysis	
Titanium fibers/13–93 bioactive glass	Freeform extrusion fabrication	In vitro	Immersion in simulated body fluid	SEM and XRD to evaluate the formation of hydroxyapatite	[70, 71]

the in vitro silencing of collagen I was sustained for at least 4 weeks, in contrast to conventional bolus delivery of siRNA. In this research, scaffolds were obtained by electrospinning PCL and PCLEEP nanofibers, in which siRNA was encapsulated together with cell penetrating peptides.

5.2.2 Cellulosic and Cellulosic Derivative Fibers

Cellulose fibers are mainly found to be used in scaffolds for the osseous part, as fibers can act as reinforcement, improving the scaffold stiffness. Bacterial nanocellulose has also been investigated as scaffold for cartilage tissue engineering [24, 28]. Regarding the treatment of osteochondral defects, Iamaguti et al. [29] used cellulose membranes in experimental trochleopasty in dogs. They found that this type of implant could support the migration of chondrogenic cells.

Cellulose fibers have been also tested in combination with other biocompatible materials, such as alginate [11], hydroxyapatite [30, 31] or gelatin [31, 32]. Channel-like pores can be obtained when using nanocellulose in an alginate based scaffold. The use of bacterial nanocellulose fibers lead to an increase in the stiffness of alginate scaffolds under compression tests; this is also observed for the introduction of collagen fibers. No toxic effects have been found for scaffolds containing cellulose, as cell culturing is not influence by the presence of nanocellulose in alginate scaffolds [24]. Chenghong et al. [30] obtained an electrospun scaffold of nanofibrous cellulose and nanohydroxyapatite. The addition of the hydroxyapatite strengthens the matrix: a content of 5% of hydroxyapatite is able to provide a scaffold with a Young's modulus of 3.12 GPa. Moreover, the presence of nanohydroxyapatite also implies an improvement on the bioaffinity of the hybrid scaffolds compared to pure cellulose ones.

Besides, derivatives from cellulose have been also proposed as suitable materials to be used in osteochondral regeneration. For example, XanoMatrix™ is a hybrid material of polyethylene terephthalate and cellulose acetate that was studied by Bhardwaj and Webster [33]. These authors report suitable adhesion and proliferation of chondrocytes for in vitro testing. Furthermore, the cells aligned along the fibers resembling the structure of the natural cartilage. The strategy proposed by Atila et al. [34] was the electrospinning of pullulan and cellulose acetate and their subsequent cross-linking with trisodium trimetaphosphate. The cross-linking is a useful tool to maintain the characteristics of the scaffolds after soaking the samples in PBS because the pullulan component is not dissolved.

Hydroxyapatite coated carboxymethylcellulose nanofiber mat was analyzed by Yamaguchi et al. [35]. This nonwoven mat has potential applications for bone tissue regeneration, owing to its ability to support the growth of osteoblastic cells as shown by the authors.

5.2.3 Mineral Fibers

The use of ceramic fibers in scaffolds is mainly justified by the mechanical properties achieved, as these fibers act as reinforcement of hydrogels or polymer matrices. Calcium phosphate salts, like hydroxyapatite, have been used for this purpose. In the last years, other compounds such as bioactive glasses and silicate based ceramics have been investigated [39]. Also, bioactive mesoporous particles have been found to shown hemostatic properties, and so healing materials also tend to be in the form of fibers [36].

Fibers from different materials have been used in calcium phosphate cements to increase the similarity in mechanical properties to the natural bone, mainly in terms of toughness, ductility and fatigue resistance. Chitosan, PA, PCL, PLLA, PGA, carbon and glass fibers have been used to this purpose [40, 41]. The addition of fibers with higher resorption rate than the calcium phosphate matrix would allow creating macropores, thus favoring cell colonization and angiogenesis.

5.2.3.1 Glass Fibers

Bioglass nanofibers can be produced in several ways [36, 37]. Concentrating a laser on a bioglass monolith nanofibers can be produced [36]. These fibers, due to their small diameter and their bioactivity, are rapidly dissolved in SBF, leading to hydroxycarbonate apatite tubes. Electrospinning technique has also been recently used to produce nanofibrous scaffolds of bioactive glass [37–39]. Due to their high surface area, bioactive glass nanofibers degrade quickly, converting to HA. The bioactivity of these glass nanofibers is maintained over a larger SiO_2 compositional range when compared to melt-derived glasses. Electrospinning can take place from organic or inorganic solutions, being after heated to 600–700 °C to decompose any residual group; fibers prepared in this way exhibit a diameter in the micro to submicron range and are commercially available. Because of their rapid degradation rate, they have a huge potential in the regeneration of non-loaded bone and in the healing of soft tissue.

Submicron 45S5 bioglass fibers (with and without copper) were used in gelatin/collagen scaffolds at a 70/30 ratio (30% of submicron bioglass fibers) [39]. Those scaffolds doped with copper have provided better behavior in terms of cell proliferation and distribution, demonstrating that copper-doped bioglass fibers are non-cytotoxic and that their surface is ideal for osteoblast attachment, growth, viability, and bone regeneration.

In some cases, small amounts of polymer (polyvinyl butyral, PEO or PVA) were firstly introduced to the sol to obtain optimal viscosity for the process; a burning stage was later needed to decompose the polymer and obtain the glass fibers. Hybrid scaffolds (silica and PCL, PLGA, or PLLA) have been successfully electrospun.

Bioactive glass particles have demonstrated to be useful in bone defects regeneration, although approved compositions are not suitable for making fibers. Scaffolds with 50% porosity made from these materials, with 75 μm thick, were completely degraded in 6 months after implantation in rabbit tibia.

5.2.3.2 Calcium Phosphate Fibers

Calcium phosphate compounds are widely used in bone regeneration because of their osteoconductive properties [42, 43]. Zhang and collaborators [3] developed a woven-bone-like beta-tricalcium phosphate (β-TCP)/collagen scaffolds by sol-gel electrospinning, preparing pure β-TCP fibers with dimensions close to mineralized collagen fibrils in woven bone. They have observed that osteoblasts showed 3D morphologies and multicellular layers, shortening to time to produce new bone.

Polycrystalline CaP fibers can be obtained by electrospinning an aqueous solution of $CaCl_2$ and H_3PO_4, using PEO as spinning aid [44]. Fibers from 10 to 25 μm of diameter were obtained after pyrolysis and sintering to remove the polymer. The so prepared fibers show no cytotoxicity under in vitro tests.

Multiphasic calcium phosphate fibers (HA, β-TCP and CPP) have been used as reinforcement of chitosan matrices, finding an increase in compressive properties, pore size and density and a decrease of porosity and swelling ratio [45]. Calcium phosphate was formed on the scaffold surface after immersion of the scaffolds in a PBS solution, demonstrating their in vitro bioactivity. Fibers were obtained by dissolving $Ca(NO_3)_2 \cdot 4H_2O$ and $(NH_4)_2HPO_4$ in distillated water at pH 3 and with small amounts of urea; the precipitated formed was treated with ethanol and subjected to 800 °C for 2 h. Chitosan scaffolds were obtained by freeze-drying of a chitosan solution containing up to 50% of fibers. Urea has demonstrated to modify the structure of precipitated calcium phosphate fibers [46], depending on the urea concentration and reaction time. Low concentration of urea leads to the production of whisker-like monetite/HA fibers, while higher concentrations tend to produce a combination of whisker-like fibers and spherulites, made of HA and octacalcium phosphate. Reaction times of 10 days allow producing HA monophasic whiskers.

HA fibers can be prepared by treating a block of β-$Ca(PO_3)_2$ fibers with $Ca(OH)_2$ particles heating it at 1000 °C and then treating it with a HCl solution [47]. Also, hydrolysis of TCP in a water-aliphatic alcohol solution at 80 °C and growing the HA fibers in an agar gel system, using $Ca(NO_3)_2 \cdot 4H_2O$ and $(NH_4)_2HPO_4$ solution have been reported to be used for HA fibers obtaining. Wu and collaborators have obtained them by electrospinning a mixture of its precursors (a mixture of $Ca(NO_3)_2 \cdot 4H_2O$ and $(C_2H_5O)_3PO$ with a polymer additive), and then annealing the electrospun fibers (containing the polymer) at 600 °C for 1 h [48]. By this procedure, HA fibers about 25 μm were obtained. Other researchers have used P_2O_5 and $Ca(NO_3)_2 \cdot 4H_2O$ as precursors to also obtain submicron fibers by electrospinning, but in this case from a mixture of the gel formed from the mentioned salts with PVP in water and ethanol/water [47]; post-heating is also required to obtain the HA

fibers. Diameters of 567 ± 70 nm and 122 ± 32 nm for fibers were obtained starting with a PVP concentration and 50 and 100% in water, respectively. This is due to the higher conductivity and lower viscosity of the water solution, in comparison with the 50% ethanol/water one. Also, composition studies show that after sintering the fibers were made of carbonated hydroxyapatite, as the human bone.

Similarly, the use of PLGA dissolved in hexafluoroisopropanol has been used to produce electrospun fibers of nanohydroxyapatite [49]; thermal processing at 1100 °C is required to evaporate the polymer. These authors also propose the use of PVA or PVP as sacrifice polymers, indicating that polymers with low melting point, such as PCL, are not an option, due to their incapacity to maintain the fibrous structure during the thermal treatment stage. On the other hand, considerations about the low mechanical properties of the so obtained fibers are made in the paper, fact which would need to be solved prior to their use as scaffolds reinforcement.

5.2.3.3 Silica Fibers

Silica fibers, coming from natural sponges skeletons, with an average diameter of 10 μm, have also been used to produce composite scaffolds based on PEEK; to increase mechanical properties, both materials were pretreated by atomization and using citric acid [50]. The composite was prepared by compression molding at 350 °C. The use of silica fibers has led to an increase of over 50% in elastic modulus at flexural testing and of 26.7% in microhardness. High cytocompatibility of the composite was found, as the metabolic activity of fibroblasts was also increased.

Silica fibers have also been produced from tetraethyl orthosilicate, which is hydrolyzed and condensed by water, ethanol and HCl [51]; the solution produced is then electrospun to obtain a nonwoven mesh, which is thermally treated at 300 °C for 3 h. Wide diameter distribution of the fibers is found: from 0.7 to 6 μm. The produced mesh show good cellular behavior, allowing preosteoblastic differentiation and osteoconductivity.

5.2.4 Fibers from Animals: Collagen, Silk, and Fibrin

5.2.4.1 Collagen

Collagen is mainly used in scaffolds for chondral applications, as it is naturally found in cartilage regions. Collagen fibrils have been added to the chondral part of biphasic alginate-based hydrogels to improve their mechanical properties, as well as to act as a binding site for living cells [24]. As for cellulose fibers, an increase in the collagen amount of an alginate scaffold reduces the pores density, while the pores diameter is not affected. Collagen in the alginate scaffolds shows no cytotoxicity and does not affect the cells growth.

Collagen–PVA nanofibers have been electrospun onto a collagen sponge to make aligned and random composites [52]. Average diameters for the random nanofibers was 203 ± 74.91 nm and 301.05 ± 96.53 nm after glutaraldehyde cross-linking, while for aligned fibers diameters were significantly smaller: 94.82 ± 25.57 nm before and 198.20 ± 33.61 nm after cross-linking. The swelling ratio was higher for the random nanofibers composite, due to the capillary effect. The aligned composites scaffolds showed higher mechanical properties, making them more suitable for articular cartilage repair, while both scaffolds showed similar cell proliferation and secretion of cartilage II.

Multiphasic composite scaffold made of an upper collagen I fiber layer and a lower part made of PLA, β-TCP and HA, seeded with hMSCs, showed chondrogenic differentiation and a homogeneous cell distribution when cultured in a TGF-β1 medium. Cells were also surrounded by a proteoglycan and collagen type II; also a high deposition of GAGs was measured [53].

Fibrous collagen has also been used as reinforcement of PEG hydrogels [54], obtaining increased modulus and toughness, and decreasing lateral expansion under compressive loading.

In other medical fields, these materials are also showing promising results; for instance, PLLA meshes have been filled in with collagen fibers for the reconstruction of abdominal wall with good results [55].

5.2.4.2 Silk

Silk fibroin, extracted from silk fibers, shows low immunogenicity, cell affinity, tunable degradation rates [56], and impressive mechanical properties [57], which provide exceptional advantages over other polymers [58]. The use of embedded silk fibers into a regenerated silk matrix led to the obtaining of scaffolds with great mechanical properties and high porosity levels; it was also found that silk fibers boost the degradation rates, due to an increased number of immigrated cells into the silk matrix. If chondrocytes are seeded in the scaffold, results are better [58]. Similar studies also reported that transplantation of mesenchymal stem cells grown in a silk fibroin/hydroxyapatite scaffold can enhance tissue repairing [59]; also, scaffolds made of silk fibroin containing mesenchymal stem cells and chondroitin provided improved behavior [60].

Silk fibers have also been used by Chen and collaborators to produce a knitted structure in which openings collagen microsponges were placed [27]. Again, seeding the scaffold with hESC-MSCs in in vivo tests provided good tendon healing, with cells differentiation into the tenocyte-lineage morphology. Ribeiro et al. [61] also proposed silk-based biotextiles for bone regeneration. They produced a silk fibroin-PET fabric and they tested the osteogenic differentiation on its surface of human adipose-derived stem cells. The alkaline phosphatase activity quantification showed a higher differentiation on the silk fibroin-PET samples than on PET fabrics taken as reference.

Christakiran et al. [62] developed scaffolds consisting on an osteogenic matrix of 70S bioactive glass and a chondrogenic matrix of silk fibroin. They evaluated the suitability of these scaffolds for the treatment of osteochondral defects by culturing chondrocytes from pigs on the silk membrane and MG63 (osteosarcoma cell line) on the bioactive glass side. They tested two types of silk: non-mulberry and mulberry based ones. The authors concluded that the non-mulberry silk based membrane performs better both from the mechanical and the biological points of view.

5.2.4.3 Other Proteins: Fibrin, Elastin

PLGA sponges have also been used in combination with fibrin fiber, BMSC, plasmid DNA TGF-β1. After culturing for 4 weeks under in vitro conditions and implantation for 12 weeks, cartilage defects were completely repaired in rabbits, being the new cartilage well integrated with the surrounding tissue and subchondral bone. GAGs confirmed similar amount and distribution of collagen type II in the new cartilage and in the hyaline one [63, 64].

Apart from collagen and gelatin, α-elastin [65] has also been electrospun to obtain 0.6–3.6 μm width and from 1.4 to 7.4 μm for tropoelastin, depending on the electrospinning parameters (concentration of the solution and delivery rate). Elastin fibers have also found to be more brittle than the other three, although cell viability is higher for elastin, followed by collagen.

5.2.5 Carbon Fibers

Carbon fiber is a not biodegradable material that can be obtained both at the nano [66] and micro levels. This feature has attracted the interest of the researchers to include this material as scaffolding in tissue engineering [67]. For the treatment of osteochondral injures, carbon fibers are potentially interesting because they enable the restoration of damaged cartilage [68, 69]. Besides, Aouri et al. [66] demonstrated they are also an effective support for the delivery of recombinant human morphogenetic protein 2 (rhBMP-2). This characteristic was useful to promote bone regeneration. In fact, in this study, SEM observation of samples implanted in mice showed that the carbon fibers and the bone matrix were fully integrated.

Bencano et al. [69] carried out the in vivo assessment of using carbon fibers to treat osteochondral defects. They evaluated the histological progression of the osteochondral defects created on the articular surface of the patella of a population of rabbits and treated with carbon fiber implants. They found that a year after the treatment, the defects had been covered with hyaline cartilage tissue.

Carbon nonwoven fabrics have a higher surface area and an interconnected pore structure, providing increased surface area for cell attachment as well as convenient channels for nutrients transportation, diffusion of gases and cell migration [68].

However, even though carbon fibers are biocompatible they do not have enough biological activity to stimulate the cells proliferation. To overcome this limitation, different modifications have been proposed, such as coatings with hyaluronic acid [68]. This has proofed to provide good cellular attachment and viability and higher speed of tissue regeneration regarding the non-modified carbon nonwovens at in vitro and in vivo studies. Several authors have explored the possibility of obtaining carbon fibers doped with osteoinductive components by previously mixing this component with polyacrylonitrile, precursor of the carbon fibers [67, 70–72]. Following this strategy, Fraczek-Szczypta et al. [67] obtained carbon nonwovens with different ceramic nanoparticles (bioglass and wollastonite). The improvement of the bioactivity of the fabrics was evaluated by the assessment of the apatite forming ability of the material when immersed in SBF solution for 21 days. All the fibers tested promote the apatite precipitation. However, the apatite layer was more uniformly distributed on the nonwoven samples containing wollastonite. On the other hand, Zhang et al. [70] demonstrated that the presence of bioglass in a carbon nanofiber matrix accelerates the proliferation rate of BMSCs when compared to pure carbon nanofiber and, besides, it improves the differentiation ability of the cells.

Another approach is the utilization of composite materials. However, the main limitation for the manufacturing of composite materials containing carbon fibers is their poor dispersion and chemical inertness in the common matrix used for tissue engineering applications [73]. For example, Chlopek et al. [74] proposed a composite of carbon fibers ($d = 7$ μm) in a PGLA matrix. They followed the degradation profile of these implants and pure PGLA ones in vivo on a population of New Zealand rabbits for 48 weeks. In this study, they conclude that the presence of the carbon fibers accelerates bone regeneration and the overall process of resorption of the implant. On the other hand, Shi et al. [75] activated carbon fiber via a high temperature process and subsequent air plasma treatment. With these activated carbon fibers, a composite material with PLGA was obtained. This composite exhibited an improvement on the porosity when compared to the pure PLGA scaffolds.

5.2.6 Titanium Fibers

Thomas et al. [76, 77] have produced printed glass scaffolds reinforced with titanium fibers to increase the mechanical properties of the bioactive glass. They started from a composite paste made of bioactive glass and titanium microfibers (16 μm diameter, up to 0.4% in volume fraction) and extruded it at 0–90° orientation. The use of titanium fibers led to an increase in the fracture toughness of about 70%, with an increase of flexural strength near 40%. It has also been demonstrated that the introduction of titanium fibers do not affect bioactivity, as HA is precipitated after 2 weeks of immersion in SBF solution in the same extent and

morphology that in bioglass scaffolds. Biodegradation tests on these scaffolds have also been performed by these researchers [76, 77], showing that compressive strength in bioglass is reduced by 30% after 4 weeks in SBF, while this reduction is near 40% for titanium fiber/glass scaffolds (67 MPa and 88 MPa for glass and Ti/glass scaffolds, respectively, after 4 weeks test).

5.3 Conclusions

As we observe, the most important technique to obtain fibrous scaffolds is electrospinning. The combination of different materials allows obtaining a wide range of properties, both from the mechanical and biological points of view; what makes fibrous scaffolds especially interesting for osteochondral applications as they are able to mimic extracellular cartilage matrix.

Even if the background in this field is quite large, more effort is needed in order to continue evaluating other material alternatives and their combinations. Furthermore, the adhesion between materials in the scaffold needs to be studied in more detail. Also, other manufacturing techniques, less common, should be further investigated.

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