

POLYMERS AS MATERIALS FOR TISSUE ENGINEERING SCAFFOLDS

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1.1 THE REQUIREMENTS IMPOSED BY APPLICATION ON MATERIAL STRUCTURES INTENDED AS TISSUE ENGINEERING SCAFFOLDS

The discovery of the multipotent nature of different kinds of stem cells opened new horizons for therapeutics for surgery and for medicine in general. Current treatments for diseased or injured tissues or organs range from transplantation from allogenic or xenogenic donors or reconstruction by transfer of autologous tissues to the use of nonbiological either implanted or extracorporeal devices. None of these strategies is free of inconveniencies (shortage and effectiveness of donors, clinical complications, need of immunosuppressive drugs, tumors formation, etc.). The hope to understand stem cells in their behavior up to the point of directing their differentiation toward desired lineages is at the base of the different new therapeutical strategies encompassed by regenerative medicine.

This process of cell differentiation is triggered and governed by a multiplicity of factors and stimuli, which cells receive from an immediate environment made from

other interacting cells and from the extracellular matrix (ECM). In cases of severe loss or degeneration of tissue, the sites of intended regeneration have lost their basic structures, and thus new grafted cells, even if having the right properties *in vitro*, fail to regenerate functional tissue *in vivo*. At this point, synthetic tridimensional structures, so-called scaffolds, may be of help, by providing grafted cells with a *niche* and adequate mechanical and chemical *stimuli*. As an example, cardiac tissue regeneration in cases of myocardial infarction and subsequent ventricular remodeling has been recently addressed by cell transplantation and cell sheet engineering, with a variety of cell populations and supply methods [1, 2]. Common difficulties found include lack of functional integration and a low survival of the grafted cells. These shortcomings could be overcome by means of biomaterials into which the cells would be encapsulated [3, 4].

Generally speaking, scaffolds must assist the regeneration process, performing as an artificial cellular environment during some stages of the tissue regeneration [5, 6]. Either *in vitro* or *in vivo*, they must replace as best as possible some of the functions of the ECM: they must (i) contribute to the structural and mechanical integrity of the diseased tissue, (ii) serve as a means of transport of nutrients and wastes and facilitate vascularization, (iii) act as a spatial guide for cell spreading and organization, and (iv) transduce mechanical or biochemical stimuli, and eventually transport, store, and deliver active molecules that effect the expression of the phenotype. Besides these functions, in defining the requirements on materials intended as scaffolds, two other sets of factors must be taken into account: those deriving from the specificity of the application (*in vitro* or *in vivo*, temporal or permanent, etc.) and those related to processability and manufacture (sizes and shapes of the implants, sterilization procedures).

Function, specific application and processability considerations thus define a number of requisite properties of mechanical, physicochemical, biological, and structural nature. From the mechanical side, strength (resistance to failure) and stiffness (characterized by shear, tensile, or compressive moduli) are the most important properties to be addressed. Modulus values as different as those of brain and bone determine a wide interval of magnitude, and mechanotransduction of signals to the cells depends significantly on this property, especially on the surface moduli. The most important physicochemical properties of scaffold materials are their degradable or stable nature, their permeability and diffusivity to fluids and gases, and their hydrophilic or hydrophobic nature. Material surfaces possess also specific biologically relevant properties: their chemical functionalities may be directly involved in the surface nucleation of compounds such as bone hydroxyapatite, or they may adsorb ECM proteins in different conformations, thus affecting cell adhesion, spreading, and proliferation. Lastly, microstructural properties of the materials, such as their pore volume fraction, pore connectivity and geometry (shape, dimensions of the pores, regularity) are critical for the scaffold's final performance. The scaffold's ability to host cells in required numbers, to allow vascularization throughout it, or to guide and organize spatially cell growth in specific ways, depends crucially on these properties.

Our ways to meet these mechanical, physicochemical, biological, and structural requirements is through bulk and surface chemistry for the first three, and through

different porogenic techniques for the fourth. A material with a given overall chemical composition may be, furthermore, in very different physical states: it may be a random or a block copolymer, it may be an interpenetrated network, it may be semi-crystalline or amorphous, vitreous or rubbery under physiological conditions. These possibilities are afforded by polymerization chemistry and/or subsequent processing or treatment, and make polymers such unique materials for tissue engineering applications.

The intended end uses of the scaffolds are widely different. Scaffolds may be implanted empty (acellular) when they are expected to be invaded and colonized by the cells in a short period of time; otherwise, it is necessary to seed the scaffolds with the appropriate cells before implantation or even to preculture them *in vitro* within the scaffold [7]. A bioreactor can be helpful in this stage to recreate *in vitro* some dynamic conditions and the mechanical and/or chemical stimuli that the cells would receive *in vivo*. Scaffolds may be chemically modified in order to direct cell anchorage or differentiation, through addition of proteins, peptides, growth factors (GFs), hormones, enzymes, or other regulators of the cell behavior [8–11]. Several methods for the controlled release of factors from scaffolds have been developed [12–14].

Polymer materials are especially suited to interface with cells. Being formed by long chain molecules, they share some basic properties with biological macromolecules. At the most fundamental level, both kinds of molecules deform with the inertial mechanism of conformational change, which gives rise to molecular dynamics with characteristic relaxation phenomena at long time scales. Moreover, both biological and synthetic macromolecules are able to exhibit structure at a subnanometer, molecular level (the local arrangement of different chemical monomers) and at a supramolecular, nano- to micrometer level: phase-separated domains, crystalline domains. And the more complex multimolecular associations leading to the macroscopic network structure of the ECM can be mimicked to some extent by the porous architecture of the polymer scaffolds. This represents a third level of structure, with typical dimensions ranging from tens to hundreds of microns.

1.2 COMPOSITION AND FUNCTION

1.2.1 General Considerations

1.2.1.1 The Influence of Surface Chemistry

The fate of an implant is determined by the host tissue reaction to it, and this is mainly a matter of surface interactions, chemical and topological [15, 16]. Cells react to events in their environment as a consequence of signaling processes transduced by cell membrane receptors. These are large molecules that bind to or react with chemical functionalities of the environmental molecules in specific ways, triggering a number of subsequent cellular processes [17]. The usual foreign body reaction to implanted synthetic material consists in a number of processes ending in the isolation of the implant, through its encapsulation in high-density fibrotic tissue. This circumstance may in some applications imply the failure of the implant as it makes

impossible a functional continuous integration of the grafted cells in the site of regeneration. The first stages in the encapsulation process involve the adsorption of ECM proteins onto the foreign surface, and the interaction of cell–membrane receptors with them [18]. The conformation of the adsorbed proteins thus may play an important role in the fate of an implanted scaffold. Since the cell–material interaction is always mediated by the ECM proteins adsorbed on the material's surface, the chemical and topological properties of the surface responsible for the adsorbed conformation of the proteins will always be determinant for the biological performance of a scaffold [19]. Cell adhesion and cell spreading, especially at early stages of the process, will depend on those properties.

The features of surface chemistry having the greatest influence in this respect are the hydrophilic–hydrophobic balance of functionalities, the surface charges, their spatial distribution on the surface, and the surface stiffness.

The more or less hydrophilic character of a surface is determined by the presence in its composition of hydrophilic functionalities (such as $-\text{OH}$, $-\text{COOH}$, $-\text{NH}_2$, $-\text{SH}$, polar groups, or bound ions) and by their mobility (large in the rubber state, very impeded in the glassy state). ECM proteins adsorb poorly onto highly hydrophilic surfaces, and consequently the cells adhere with difficulty, especially at the earliest times of contact. Adsorption sites of this kind of surfaces are preferentially occupied by water molecules, in a labile dynamic equilibrium that is difficult competing protein adsorption. In this situation, proteins adsorb, if at all, in small amounts and with a typically globular conformation, which minimizes the area of their interface with the material and thus the free energy. Correspondingly, cells attach, if at all, in small amounts and with a rounded shape, with a poorly developed cytoskeleton, frequently preferring cell-to-cell associations over cell–surface contacts. By contrast, on more hydrophobic surfaces ECM proteins tend to adsorb in larger amounts and with more extended conformations, now energetically preferred since the protein–material interaction destroys the water–surface bond, decreasing the free energy. This causes cell-binding sequences in the adsorbed proteins to be more accessible and, as a consequence, cells attach to hydrophobic surfaces more, and with more extended shapes, numerous processes, and larger focal adhesions and a developed cytoskeleton.

The state of affairs just described for hydrophilic and for hydrophobic surfaces applies, as a general rule, to the early stages of the cell–material interaction process. With time, different situations may arise as a consequence of new processes competing with those just described, such as the progressive build-up of protein–protein interactions (fibrillogenesis) [20] and the cell remodeling of the ECM. The early interactions, however, may be critical for the success of the implant since it is they who determine cell invasion, neovascularization, and the foreign-body response.

The spatial distribution of the surface functionalities is also important for the processes just mentioned. Since proteins have both hydrophilic and hydrophobic domains, the shapes they acquire upon adsorption may depend on the presence on the material surface of alternately distributed hydrophilic and hydrophobic domains at a scale that matches the separation of those domains in the proteins. Block copolymers, interpenetrating polymer networks, blends, or nanocomposite organic–organic or organic–inorganic materials are systems whose phase distribution can be tailored

at the nanometer scale relevant for the protein–surface, and thus for cell–surface, interaction. The issue of surface topography is also controllable to some degree by factors such as the sizes of the phase-separated nanodomains in heterogeneous material surfaces. These may include crystallites of different sizes, alternating with amorphous domains in semicrystalline polymers, or nanophases of different chemical and mechanical properties in block copolymers, polymer blends, or interpenetrated polymer networks. Protein adsorption, and hence cell early adhesion on the material, is very sensitive to these purely physical features of a surface; nano- and microroughness of the surface topography favors protein adsorption, acting as nucleation points for the adsorption process by diminishing the interfacial surface tension.

A different example of the import of surface chemistry is represented by the so-called “bioactivity” of surfaces. This term, which arguably should be referring to a wider class of phenomena, has come to be identified with the ability of certain synthetic materials to nucleate the growth of hydroxyapatite crystals at a rate relevant for physiological interaction [21]. This is of great importance in bone tissue engineering, where a hydroxyapatite layer grown on the surfaces of a scaffold may integrate, it is hoped, continuously with the surrounding bone tissue and avoid the formation of the aforementioned fibrous capsule, which, especially in the case of bone, would have disastrous effects on the vascularization of the implant and on its load-bearing capacity. Bioactivity thus understood is a process triggered by surface groups that may enter into exchange reactions, in aqueous medium (blood or physiological fluids *in vivo*, simulated body fluid (SBF) *in vitro*), to bind calcium ions, which in their turn start the process of apatite crystallization in the presence of phosphate ions. Usually hybrid organic–inorganic polymer composites exhibit this kind of bioactivity. In them, typically, a silica-based phase included in the polymer matrix exhibits silanol groups, $-\text{SiOH}$, at the surface, which, upon dissociation, $-\text{SiO}^-$, act as nucleation sites for the calcium ions acting as precursors for hydroxyapatite nucleation and growth.

Surface chemistry is important also from the mechanical point of view. Mechanotransduction, cell motility (migration), or the extension of cell processes (e.g., neurites) on the surface are all very sensitive to its stiffness.

The issue of surface chemistry is to some extent independent from bulk composition of the material: materials can be subjected to surface treatments, and functionalized in the desired ways; a surface layer with properties significantly different from the bulk can thus be achieved. Furthermore, surface properties are interfacial properties; this means that even in the case of nontreated surfaces, the outermost layer of a material may possess effective properties different from the bulk, due to the fact that interfacial interactions suffered during manufacture (e.g., contact with mould surfaces) or *in situ* (e.g., the hydrophobic interaction in the presence of water) have altered the conformation of the functionalities at the surface and thus affected their availability.

1.2.1.2 The Influence of Bulk Chemistry

Scaffolds, besides hosting cells, must in most applications withstand certain mechanical stresses. The overall mechanical stiffness of an implant determines its manipulability and its success in stressed environments. Stiffness depends on the

cohesive energy density of the material, and is thus a function of the bulk chemical composition. On it depend also other important properties, most singularly diffusivity and permeability to water and to small molecular weight species, the hydrogel character, the biostability, or biodegradability of the material, and, in the latter case, the degradation mechanism. If degradation takes place following a hydrolytic route, the material will degrade when in contact with water. If its bulk chemistry is hydrophobic, the process will start at the material's surface, and proceed gradually towards its interior. In this case, degradation erodes progressively thicker outer shells of the piece, but an inner nucleus remains unaffected for a time, which can preserve some of the mechanical properties of the piece. By contrast, in the case of hydrophilic chemistries bulk swelling occurs, which allows the onset of hydrolysis at all points of the piece from the start. Hydrogels and hydrophilic polymers will thus degrade more rapidly than hydrophobic polymers, and their bulk properties will start reflecting degradation at a faster rate.

1.2.2 Some Families of Polymers for Tissue Engineering Scaffolds

According to their biological stability polymers may be classified as biodegradable or biostable, and these characteristics condition their choice for applications. From the point of view of final use, biodegradable polymers offer the significant advantage of disappearing from the body in due time. The problems associated with long-term extraneous implants thus disappear. However, certain applications require permanent implants: corneal prostheses, cerebral stimulation devices, dental implants, cardiac restraint devices, and many others. In all these cases, one faces the impossibility of complete regeneration and the need to preserve function, or the necessity to preserve the long-term stability of an implant. Contrary to what may be a first thought on the matter, research on biostable synthetic polymers possesses a substantive interest of its own.

Biodegradable polymers. If, under specific *in vivo* conditions, a polymer undergoes chemical reactions that decompose it into nontoxic products that can be completely removed or metabolized by the human body, the material is regarded as biodegradable. Specifically, when a biomaterial is implanted in the human body, an inflammatory response to the foreign body occurs. This process is the result of the action of different cell types such as leukocytes and macrophages. Through oxidative reactions caused by reactive species secreted by the cells (H_2O_2 , NO , O_2^-), the polymer chains may suffer scission and hydrolytic degradation (chain scission through water-labile groups of the polymer structure, see Fig. 1.1). Depending on the hydrophilicity of the material, these degradation processes advance in a front-like manner, from the outside to the interior of the material (in hydrophobic polymers) or take place more rapidly, in a more homogeneous way in the bulk of the material (in more hydrophilic polymers). Hydrolytic degradation can be catalyzed by enzymes or by the fluids with high content of acidic or basic compounds in the body. The degradation process results in the loss of physical, chemical, and mechanical properties of the material. The kinetics of biodegradation is a matter of chemistry, but also of shape, size, and topology of an implant. In general, large *specific* surface areas (i.e., porosity,

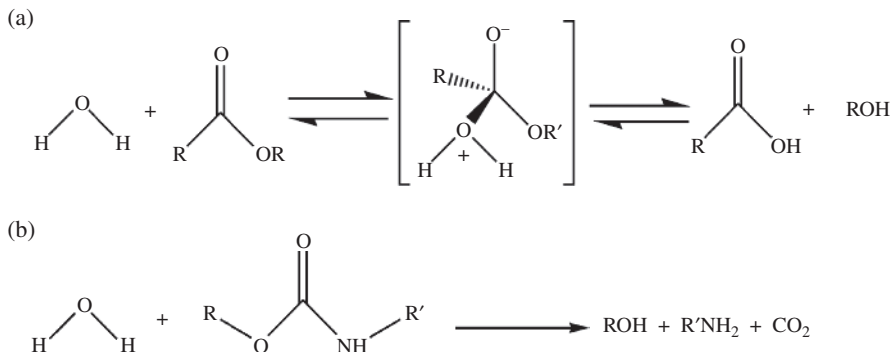


FIGURE 1.1 Hydrolytic scission mechanisms of polyesters (a) and polyurethanes (b).

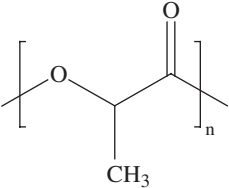
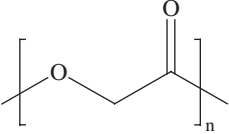
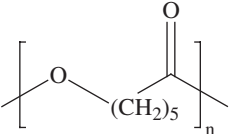
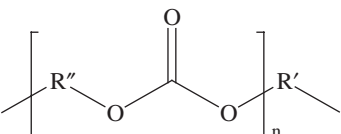
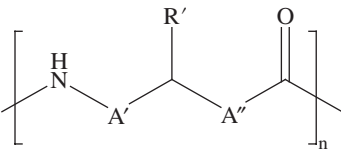
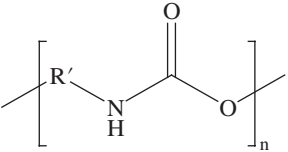
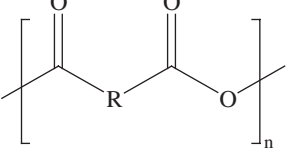
roughness, etc.) will result in rapid degradation, whereas smooth surfaces degrade with more difficulty; large implants with smooth surfaces tend more easily to be encapsulated, what may retard or even prevent completely their degradation, their chemistry notwithstanding. Natural polymers are treated in another chapter of this book; here we mention only some synthetic polymers. A list of structural units of some of these polymers is given in Table 1.1.

The family of α -hydroxy acids ($-\text{ORCO}-$) has been widely studied and used since the 1930s. The main methods to obtain these materials is the synthesis by polycondensation reaction using diols, diacids, and derivatives or through the ring opening polymerization (ROP) of cyclic di-esters. Poly(lactic acid), PLA, poly(glycolic acid), PGA, poly(ϵ -caprolactone), PCL, and their copolymers are the most extensively studied [22]. These polymers (except PGA) have hydrophobic character, an advantage in regard to the cell–material attachment. However, the lack of wettability properties may restrict the diffusion of grow factors, nutrients, and wastes along the structures made from such materials, and require specific seeding techniques. Polyesters are semicrystalline polymers. The hydrolytic degradation (see Fig. 1.1) in them starts in the amorphous phase, and the lower molecular weight chain fragments caused by hydrolysis may recrystallize, giving rise to a transient increase in the degree of crystallinity. Addition of hydrophilic functional groups to their chemical structure through esterification reactions increases the wettability of these polymers and thus also their degradation kinetics [23].

Poly lactides (L-PLA, D,L-PLA and copolymers) give lactic acid as the main degradation product after hydrolysis of the water-labile bonds. The lactic acid is metabolized by the human body through Krebs cycle, and excreted as carbon dioxide and water. In the case of polylactones, for example PCL, the degradation process occurs in two stages, the hydrolytic degradation due to the scission of esters links and the diffusion of low molecular weight polymers to the body. Fragments of PCL obtained after hydrolysis are digested by giant cell and phagocytes and excreted through urine and feces.

Degradation rates and mechanical properties of these polymers can be improved through copolymerization of different units, functionalization, blending, cross-linking,

TABLE 1.1 Structural units of some biodegradable polymers

Biodegradable polymers	Structural unit
	Poly(lactic acid)
	Poly(glycolic acid)
	Poly(caprolactone)
	Poly(carbonate ester)
	Poly(ester amides)
	Polyurethanes
	Polyanhydrides

modifying the crystallinity, etc. In Ref. [24] poly (L-lactide), PLLA, macromer was copolymerized with hydroxyethyl acrylate in order to enhance the wettability of the copolymer materials. Blends of polyesters have been used in bone tissue engineering as polymer/bioceramic composites with hydroxyapatite and carbon nanotubes (CNT) [25] as inorganic phase. Poly(ester carbonates), obtained by ROP of polyesters with trimethyl carbonate [26] or its derivatives, have products of

degradation less acidic than poly(ester amides) [27], which are obtained from a condensation reaction of amino acids, aliphatic dicarboxylic acid and diols, and have good thermal and mechanical properties, and cell attachment properties.

Polyurethanes ($-R-NH-COO-$) are versatile materials, which have good mechanical properties and biocompatibility, and can be biodegradable or biostable. Polyurethanes obtained through the reaction of di-isocyanates with polyester diols [28] are biodegradable and have been used in cardiac tissue engineering [29] and artificial blood vessel [30] among other applications. In this case, the bulk hydrolytic degradation mechanism is prevalent through the ester group susceptible to hydrolysis. Besides, the formation of new functional groups such as carboxylic acid can catalyze the degradation process.

Polyanhydrides ($-CO-R-COO-$) are obtained by different synthesis routes such as ROP, interfacial condensation, melt condensation, etc. Polyanhydrides are classified in aliphatic, aromatic, and unsaturated, according to the nature of R group located between the carboxylate functional groups. From the applications point of view, polyanhydrides are biocompatible, biodegradable, and nontoxic. These biomaterials have been widely used for short-term drug delivery due to their fast degradation rates. This property can be tailored in view of their final applications, for example, through intercalation of hydrophobic backbone between the water-labile anhydride bonds that hinder the water diffusion into the polymer matrix. Poly(glycerol sebacate), PGS [31], is one of the most important materials in this family, obtained from the equimolar polycondensation reaction between glycerol and sebacic acid. Due to its good chemical and mechanical properties, PGS has been widely used in cartilage [32], nerve [33], and cardiac tissue regeneration [34].

Biostable synthetic polymers. Nondegradable synthetic polymers have always played a fundamental role in the development of materials for biomedical purposes. Multiple applications in orthopedics, dental implants, suture materials, fixation devices, catheters, and, more recently, cements and adhesives rely on the chemical and mechanical properties that can be achieved with synthetic stable compounds. Poly(ethylene glycol), PEG, and poly(ethylene oxide), PEO, are very common industrial polymers, with many biomedical applications [35, 36]. PEO or PEG are obtained from ethylene oxide or ethylene glycol monomers and depending on the catalyst type, the mechanism of polymerization can be cationic or anionic. PEG is a polymer with hydrophilic features and resistant to protein adsorption; therefore, cell adhesion and spreading are quite poor on it. Fortunately, PEG is a versatile material and its functionalization with peptides or proteins can modulate the specific cellular response.

Acrylates and methacrylates [$-CH_2-(CXCOOR)-$, with $X=H$ (acrylates) or $X=CH_3$ (methacrylates)] are among the oldest polymer families with well-established applications in the medical industry [37]. Poly(2-hydroxyethyl methacrylate) (PHEMA, $R=CH_2CH_2OH$, $X=CH_3$) is an FDA-approved and biocompatible material amply used since decades in contact lenses, breast and orthopedic prostheses and for drug delivery systems. Its hydrophilic character is due to the lateral hydroxyl group present in the homopolymer. Surface modifications of PHEMA materials with proteins and peptides, and chemical modifications with other reagents like ϵ -caprolactone

and L-lactide have been made [38] in order to enhance cell attachment and spreading on PHEMA. PHEMA has been produced also as scaffolds with different morphologies [39] and is a main component of the promising family of electroconductive hydrogels for medical uses [40]. Another interesting polymer in this family is poly(ethyl acrylate), PEA ($R=CH_2CH_3$, $X=H$), obtained through the radical polymerization of ethyl acrylate monomer using a variety of initiators and cross-linker agents [41]. Substrates and scaffolds from EA and EA copolymers have very good cell attachment properties [42–46]. Poly(methyl methacrylate), PMMA ($R=CH_2CH_3$, $X=CH_3$), is the main acrylic component in orthopedic applications. It is a biocompatible, nondegradable and hydrophobic material. PMMA has been used as bone cement [47], in cranial reconstruction [48], as prosthetic material in dental applications, in permanent intraocular implants and as drug delivery device, among many other uses.

Monomers with ionizable functional groups, such as acrylic and methacrylic acid ($R=H$) and acrylamides [$-CH_2-(CXCONRR')$ –, with $X=H$ (acrylamides) or $X=CH_3$ (methacrylamides)] have also found interest. Materials obtained from derivatives of these monomers with different functional groups are able to respond to a variety of specific changes in the surrounding medium, such as pH, temperature, ionic strength, etc. This is the case of poly(*N*-isopropylacrylamide), PNiPAAm, homopolymer or its copolymers, which experience a phase transition when heated above 32°C owing to their lower critical solution temperature (LCST) [49, 50]. This property is used to modulate the hydrophilic character and the release profile of substances, or to detach and harvest cell sheets after culture on these substrates [51].

1.2.3 Composite Scaffold Matrices

Composite polymer matrices are multiphasic materials, in which two or more polymers are blended, or in the form of interpenetrated networks, or one polymer is mixed with a finely dispersed filler phase. In some applications, such as in mineralized tissues, polymers display too poor mechanical properties and are consequently in need of some reinforcement. This can be obtained combining polymers with bioactive glasses [52], hydroxyapatite (HAp) [53], calcium orthophosphates [54], or pure silica [55], in the form of particles or fibers to obtain bioactive composites, which aim to simulate the composition of natural bone, where the inorganic phase HAp is dispersed in a collagen matrix. Porous scaffolds of hybrid composites mimicking natural bone or dentin mineralized ECM have been proposed to serve as a support, strengthen and guide new tissue in-growth and regeneration [56–59]. Still another approach is the concept of nanohybrid matrices, including an inorganic silica phase polymerized in a sol–gel process simultaneously with the organic monomer. These nanohybrids include those of poly(2-hydroxyethyl methacrylate) (PHEMA) polymerized with tetramethoxysilane (TMOS) as silica precursor [60], or polycaprolactone (PCL) [61], poly(hydroxyethyl acrylate) (PHEA) [62], and P(EMA-co-HEA) [63, 64] polymerized with tetraethoxysilane (TEOS) as silica precursor. In Ref. [65], the mechanical reinforcement and the bioactivity of P(EMA-co-HEA)/SiO₂ nanohybrids was seen to depend on the structure of the silica network formed, on its continuity

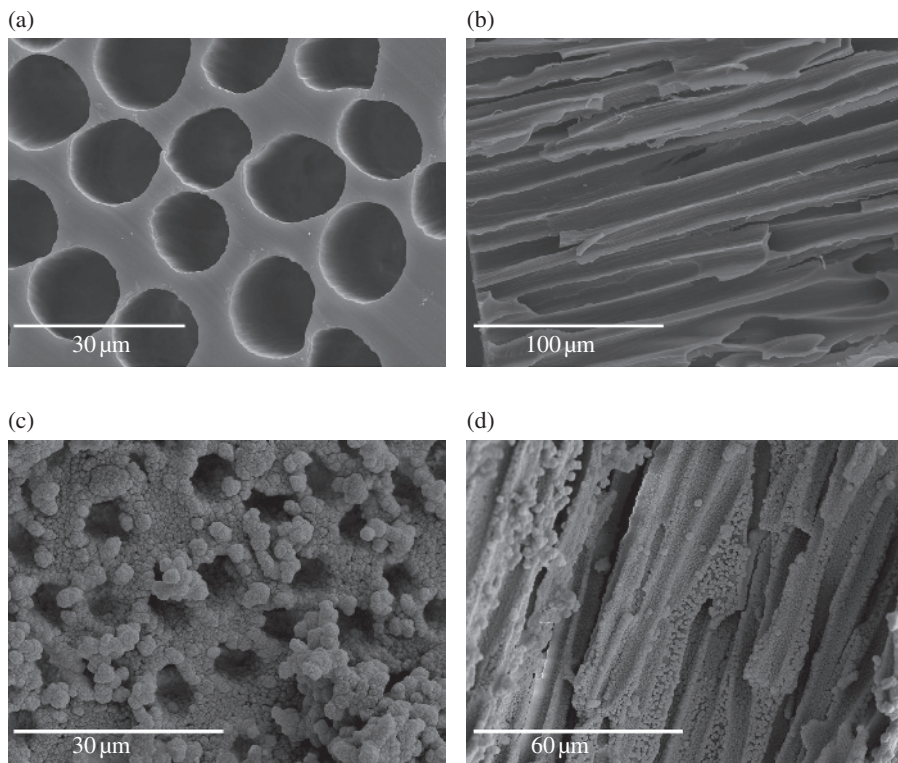


FIGURE 1.2 SEM images of nanohybrid scaffolds of P(EMA-co-HEA) 70:30 wt% copolymer containing a 15 wt% of silica interpenetrated network obtained by simultaneous sol-gel polymerization, with aligned 12 μm -diameter tubular pores (a and b). After immersion in simulated body fluid for 14 days the tubular and outer surfaces are coated by hydroxyapatite showing its typical cauliflower aggregates (c and d). The silica inorganic network acts as a nucleating agent for the crystallization of hydroxyapatite. Images (a, c) correspond to a cross section normal to the cylinder axis, and (b, d) to longitudinal sections.

and on the number of silanol groups available initially at the surface and those formed later by dissolution of the silica network. Channeled scaffolds with this composition mimic the dentin ECM (Fig. 1.2) and were able to guide odontoblasts processes into the tubules and improved their integration providing stimuli for cell invasion and differentiation when implanted *in vivo* [66, 67].

Nanocomposite matrices are obtained also incorporating CNTs into polymeric materials [68–70]. CNTs are tubular molecules made either of one sheet (single-walled, SWCNT) or more (concentric cylinders or multi-walled, MWCNT), their highly regular geometry conferring them salient mechanical and electrical properties. The key issue in the fabrication of these nanocomposites is a good interaction between the tubes and the polymeric matrix. When this happens, a dispersion of very small amounts of CNTs (<1% wt) can lead to significant improvement of the strength, the electrical conductivity, and the ability to nucleate HAp on the surface. Additionally,

CNTs can be functionalized for the delivery of drugs. These nanocomposites in the form of scaffolds could find application in bone [71] and interestingly in neuroregeneration [68], where the polymer guides the elongation of neurons while CNTs are expected to stimulate cell growth and improve their synaptic connectivity. However, CNTs are not biodegradable, and debate is still open as to their biocompatibility and toxicity [72].

1.3 STRUCTURE AND FUNCTION

1.3.1 General Considerations

Polymer materials can be conformed into different kinds of structures in order to address the widely differing applications: microparticles, microfilaments, membranes, or three-dimensional scaffolds. The choice depends on the main intended function: cell carrier for localized supply, protective cell niche, growth-guiding structure, etc. Three-dimensionality, though not always needed, is nonetheless the defining property of the type of synthetic structures usually designated as *scaffolds*. And this for a number of reasons.

The earliest experiences of cell cultures for regenerative purposes were developed under standard culture conditions, employing culture wells that may be assimilated to two-dimensional surfaces. Cells such as chondrocytes, but also others, were seen to de-differentiate in those conditions after some passages. An awareness grew out of those experiences of the importance of environmental influences beyond the purely biochemical stimuli that can be supplied in a two-dimensional culture plate; spatially arranged stimuli, such as stress transduction and cell-to-cell contacts, were recognized to play a major role in cell differentiation. Thus emerged the idea of the scaffold, as a means to provide cells with an actual three-dimensional environment that could better simulate the physiological (or even developmental) entourage of the cells [73].

The most important features of the three-dimensional inner structure of a scaffold are the overall porosity (apparent density) and the sizes, shapes, and geometrical arrangement of the different kinds of pores. These features may be produced with different porogenic techniques (see later text). The applications dictate the type of pore architecture: thus, tubular pores may be of interest in nerve or dentin regeneration, while a more isotropical pore structure may be in place in the cases of bone and cartilage regeneration. In all cases, this architecture has an influence on nutrient and metabolite diffusion, stress transfer, cell–cell contact, cell spreading, and on the organization of the ECM secreted by the cells. A critical circumstance that must be borne in mind is that the viability of the seeded cells, or of the cells invading the scaffold, requires their proximity to capillaries, their source of nutrients; although some cell types, such as chondrocytes, are viable at millimeter distances from capillaries [74], most cells do not survive at a distance greater than 200 μm from a capillary [75, 76]. Depending on the size of the scaffold, this may imply the need that it be rapidly vascularized when implanted. This neoangiogenesis within the scaffold needs the

fast migration of endothelial cells through the scaffold, which imposes lower bounds on the sizes of the pores and of the pore–pore interconnections, the bottleneck of the cells' migrating path.

Does a scaffold really represent a three-dimensional niche for the cells? This question has been sometimes legitimately raised. After all, it is said, if cells adhere onto the scaffold's pore *surfaces*, they continue to “feel” a two-dimensional substrate. While this is true, cells nonetheless experience a truly three-dimensional environment, if only because the pores' surfaces are *curved* with curvature radii of cellular scale, to which the cytoplasm adapts. This curvature facilitates cell-to-cell contacts coming from different directions, and also the truly three-dimensional disposition of the secreted ECM, which fills the pore cavities. Adhered cells are often seen sprouting processes in different space directions, touching the pore surface at different angles. In the course of time, moreover, different cell structures, such as capillaries, may develop within the pores. This in itself is a truly three-dimensional event, implying the reorganization of the ECM and the original cell arrangement within the pores. It may be concluded that, though not a “natural” environment, scaffolds constitute nonetheless a niche able to provide three-dimensional cues to seeded or invader cells.

1.3.2 Structuring Polymer Matrices

Different applications require different material structures: cell encapsulation may be best accomplished within microvesicles; a gel may be the best solution to inject cells; cell sproutings, such as axons or odontoblastic processes in dentin, can be directed with tubules or filaments; porous scaffolds may be the solution in cases where cell supply to bulk defects is needed, etc.

1.3.2.1 Polymer Gels

Hydrogels retain large amounts of water and their soft and rubbery consistence resembles that of living tissues. They are made either from synthetic or natural polymers and are the preferred materials whenever mechanical strength is not a stringent requirement. Hydrogels are highly hydrated polymer networks, not soluble in water as a result of a physical or chemical cross-linking through covalent bonds, physical cross-links, hydrogen bonds, strong van der Waals interactions, or crystallite associations, and even, frequently, a combination of them. Poly(2-hydroxyethyl methacrylate) and poly(ethylene oxide) are probably the most widely used synthetic gels in the pharmaceutical industry. Acrylic precursor monomers are converted into gels by radical polymerization reaction through the double bonds in presence of an initiator and a cross-linker molecule. Obtention methods of gels from linear polymers (polyvinyl alcohol, polyethylene glycol, chitosan, alginate, etc.) are different. For example, ionotropic gels are based on polyelectrolyte complexes like chitosan-hyaluronic acid or alginate-polylysine. These gels are formed by ion-exchange reactions giving rise to stable intermolecular ionic bonds. In addition, linear polymers can be also chemically modified in order to graft double bonds or other functional groups like thiol groups for the subsequent polymerization reaction. In the last decades, the macroporous hydrogels called cryogels have attracted attention for biotechnological and biomedical

applications [77]. Cryogels are formed in partially frozen solutions of monomeric or polymeric precursors, when ice crystals perform as porogens. Specifically, the polymer gel can form during the cryogenic treatment steps, including (i) the freezing of the initial system, (ii) during the storage of the system at frozen state, or (iii) during the thawing stage. Therefore, cryogels can be of many chemical types: noncovalent, ionic or covalent. The macroporous gels formed by cryotropic gelation procedures reveal a sponge-like morphology, in contrast to the gel morphology obtained in non-frozen systems. Freeze–thaw methods have been used to make hydrogels with highly elasticity.

Among hydrogels, the injectable ones deserve a special consideration, for they permit a noninvasive simple administration. Injectable gels are materials in the *sol* state with a viscosity sufficiently low to be injected, and able to gel *in situ* either by physical association or chemical cross-linking [78]. The physical association is commonly mediated inversely by temperature as occurs with polyethylene glycol/polyester block copolymers such as poly(L,D-lactic-*co*-glycolic) acid-polyethylene glycol-poly(L,D-lactic-*co*-glycolic) acid (PLGA-PEG-PLGA), which forms some micelles at low temperature that do not percolate but at higher temperatures, when the hydrophobicity of the PLGA segment increases sufficiently [78, 79]. On the another hand, examples of natural polymers able to be cross-linked covalently are collagen by means of carbodiimide, glutaraldehyde, or genipin as cross-linkers [80], or hyaluronic acid employing divinyl sulfone, glutaraldehyde, or carbodiimide among others [81].

Another group of interesting injectable gels are the self-assembling polypeptides (SAPs). They are short repetition units of amino acids, injectable in aqueous solution, which form a nanofibrous gel responding to pH changes or to increase in the saline concentration when being injected *in vivo*, or when culture medium is added *in vitro*. As charges become partially neutralized, hydrophobic packing followed by β -sheet parallel ordering occurs, leading to nanofibers that at concentrations sufficiently high are able to percolate in a continuous network. This self-assembling process to form ordered structures is mediated by van der Waals forces, hydrogen bonds, or electrostatic forces between the complementary groups of the peptides. The shape of the nanofibrils depends on the character of the peptides.

Ionic peptides of the RAD16 (R: arginine, A: alanine, D: aspartic acid) family have from 8 to 32 amino acids (multiple of the RADA sequence), with alternate hydrophobic (A) and hydrophilic lateral groups (at one side and another of the chain), and the polar groups in their turn alternate positive (R) and negative (D) charges. They form dimers by hydrophobic packing when these charges are sufficiently neutralized, and the dimers formed order parallelly through interactions between complementary units, leading to fibrils of 5 nm width [82, 83]. Among them, the most employed is RAD16-I, which has been found to promote the adhesion of endothelial cells, their proliferation, and formation of capillaries [83–85], or support neuronal growth and promote the formation of synapses [86, 87]. Conversely, amphiphilic peptides, with a bulky hydrophilic head and a hydrophobic tail, self-assemble in cylinder nanofibers of 6–8 nm in diameter [88].

The interest of these injectable gels lies in that they (i) can be administered by injection in the *sol* state and physically adapt to the available space, which permits

noninvasive implantation *in vivo*; (ii) are biodegradable, soft, and absorb large amounts of water, which permits the diffusion of nutrients or cellular waste substances; (iii) can be loaded with drugs or GFs to be delivered in a controlled pattern, and additionally, some of them (iv) resemble the native ECM in terms of composition and gelly structure, so they can be employed to encapsulate cells in a friendly environment, and (v) allow the incorporation of recognizable peptidic sequences of interest (Arg-Gly-Asp (RGD), Ile-Lys-Val-Ala-Val (IKVAV), etc.). Furthermore, SAPs are synthetic in nature, which avoids some drawbacks of molecules of natural origin. The shortcomings of injectable gels as biomaterials falls on their poor mechanical properties, which may difficult their manipulation and their form stability once implanted in stressed or mobile environments.

1.3.2.2 Microparticles as Scaffolds

Microparticle-based scaffold designs have gained increasing interest over the past few years due to the feasibility of combining controlled release functions into a three-dimensional niche, in pursuit of better control of neo-tissue development [89]. This use is very flexible since it is possible to tailor the physical, chemical, and mechanical properties of the microparticles in order to better adapt to the *in vivo* microenvironment. Polyester-based microparticles, including polylactic and polycaprolactone, are frequently used alone or combined with polysaccharide and protein-based microparticles such as gelatin, chitosan, alginate, hyaluronic acid, etc. Polyester microparticles can be prepared by the oil-in-water emulsion and solvent evaporation technique, while polysaccharide-derived microparticles are commonly prepared by the coacervation method, or by water-in-oil emulsion and subsequent cross-linking reaction. Microparticles can be injected as a loose three-dimensional scaffold into variously shaped defects for bone repair and even in other tissues. García et al. [90, 91] studied the use of biodegradable chitosan and gelatin microparticles as an injectable carrier for cell transplantation and demonstrated that microparticle size and seeding procedure are key parameters in order to avoid the de-differentiation of human chondrocytes to chondroblasts and the subsequent formation of fibrocartilage. Such systems are highly dynamic in nature, and microparticles are free to move during cell growth, allowing the natural expansion of the tissue. Microparticle-based scaffolds may provide more versatile applications than preshaped scaffolds because minimally invasive strategies for the scaffold transplantation can be used. Laurencin et al. [92] reported a microsphere-based approach to create a porous interconnected scaffold using a sintering process for bone tissue regeneration. Sintered microsphere scaffolds offer several benefits, which include the ease of fabrication, control over morphology and physicochemical characteristics by altering the size or changing the interior morphology of the microspheres, and versatility of controlling the release kinetics of encapsulated bioactive compounds.

1.3.2.3 Microfilament Scaffolds

Some tissues or parts of tissues possess a very anisotropic organization. This is the case of nervous tracts, or of tendons and ligaments, for example, where cells and their ECM are almost parallelly aligned as bundles of axons or of collagen fibers,

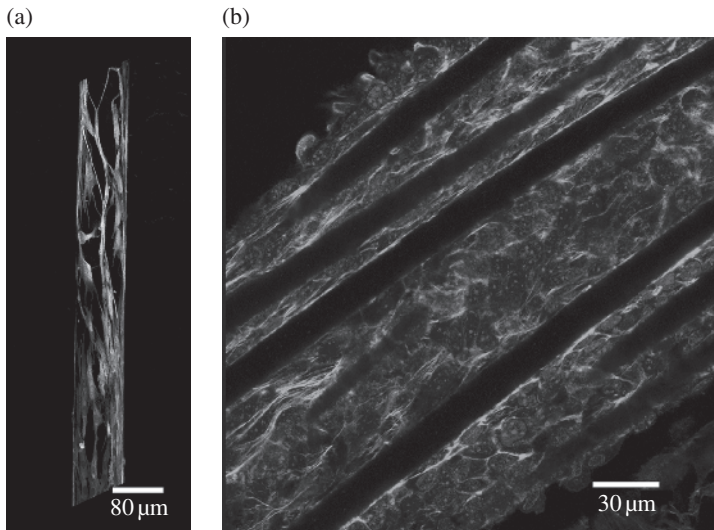


FIGURE 1.3 Confocal laser scanning microscope images of cells cultured on microfilament scaffolds. (a) Rat olfactory ensheathing cells (OECs) on a PCL filament after a 4-days culture, stained for immunocytochemical analysis for nuclei (DAPI, in blue) and for the glial cell markers S100 (red) and p75 (green). (b) Mouse fibroblasts cultured for 14 days on a PLA multifilament bundle. Cell nuclei in blue (DAPI stain) and actin cytoskeleton (phalloidin, in green). (See insert for color representation of the figure.)

respectively. Regeneration of these structures can be attempted with the help of filamentous scaffolds. Monofilaments of biodegradable polyesters, such as PLA or PCL, can be obtained by extrusion from the melt and subsequent drawing until the desired fiber diameter is obtained. In Ref. [93], neural glial cells were grown on PCL filaments of 60–80 μm of diameter, and their migration on this kind of scaffold was studied (see Fig. 1.3a). In Ref. [94], cells were cultivated on braids made from PLA microfibrils, in a multicomponent construct intended as a regenerative tendon prosthesis (see Fig. 1.3b). Apart from polyesters, silk is another filamentous material being promisingly studied as scaffold, due to its extraordinary mechanical strength and its biocompatibility [95–97].

1.3.2.4 Electrospun Membranes

The mats produced by electrospinning mimic to some extent some features of the ECM fibrous components closer than conventional scaffolds. In recent years, there has been a substantial interest in exploiting this technology to produce fibers with diameters of nanometer size from a variety of natural and synthetic polymers for tissue engineering. The process versatility and the high-specific surface area of these nanofiber meshes may facilitate their use as local drug-delivery systems [98]. Common electrospun nanofiber mats are characterized by a random orientation of the fibrils. Aligned oriented fibers can also be obtained with this technique by special collector devices. A major concern in electrospun scaffolds is the tendency to

accumulate densely packed fibers, resulting in a porosity of too small effective dimensions, which hinders cellular infiltration inside the scaffolds and eventually compromises tissue regeneration.

More complex membranes can be obtained with the same basic principle: two or more polymer solutions can be mixed previously and loaded in a single syringe to give composite nanofibers [99], nanofibers with a core material and a coating of another material if a coaxial syringe is loaded with different polymer solutions [100], cross-linked nanofibers if the cross-linker solution is loaded in a secondary syringe connected to the primary one by a three-way adapter previous to the needle [101, 102], those composed of several kinds of fibers if different polymer solutions are electrospun simultaneously on a moving collector, or multiple layers if the solutions are electrospun sequentially [103, 104], and even combinations of these techniques leading to bilayer membranes one of which is cross-linked [102].

1.3.2.5 Porous Scaffolds

Sponges and foams are the most common three-dimensional porous scaffolds. Traditional techniques used for producing these kinds of scaffolds from natural and synthetic polymers include freeze drying/gelation/extraction, particle leaching, phase separation, template sintering, solvent casting, gas foaming, and even the combination of more than two techniques in order to control the porous architecture, pore size distribution, pore volume fraction (overall porosity), and pore interconnectivity of the three-dimensional resulting structures. The combination of particle leaching method with other techniques has the advantage of increasing pore interconnectivity and at the same time controls the shape and pore size through the size and geometry of the porogen particles. Porous scaffolds produced by template sintering and particle leaching techniques result in highly porous interconnected materials with very regular and controlled pore size structure [66, 105–107] (see Figs. 1.2 and 1.4). Similar features can also be obtained by thermally induced phase separation (TIPS), which, however, yield less regular porous structures. This technique exploits the onset of immiscibility as a function of temperature while quenching a homogeneous polymer solution. The phase separation can take place as solid–liquid or liquid–liquid demixing, resulting in polymer-rich and polymer-poor phases [108]. By adjusting cooling rate, freezing temperature, polymer concentration, and using different solvents, varied pore morphologies can be generated. TIPS has been used to produce microporous membranes for medical applications such as hemodialysis, drug carriers for controlled release, filtration membranes, and others. For cell invasion or seeding, the porous material resulting from TIPS may be not adequate due to small pore size. Therefore, many researchers have attempted to combine the TIPS with the particle leaching technique in order to obtain pore sizes of 100 μm or higher. In Ref. [109], a fabrication method is employed that yields micro and macropores, based on the combination of both the freeze gelation and the particle leaching techniques. The resulting scaffolds have an interconnected pore structure with smaller and larger pores, the first ones generated during the freeze gelation process and the larger pores generated by the porogen particles.

New methodologies have been developed to reduce or to eliminate the use of organic solvents and avoid the thermal treatments at high temperatures of the

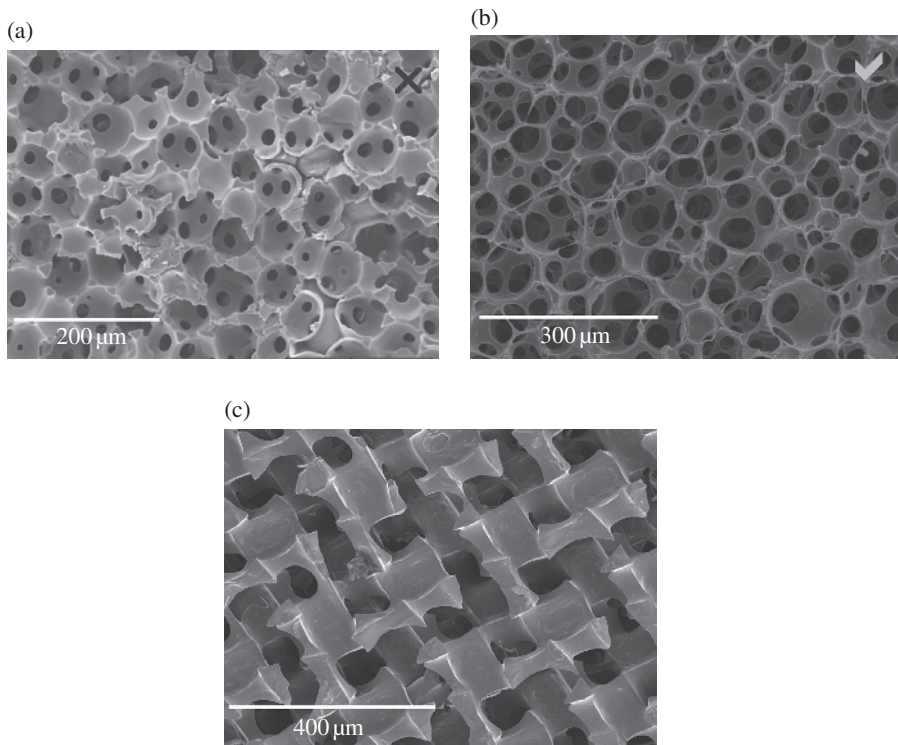


FIGURE 1.4 SEM images of PEA scaffolds (sections) obtained by the template leaching technique, with different porogen templates: sintered microspheres (a and b), and sintered fabrics (c). If the sintering of the template is not properly controlled the interconnections of the pores may be too small for cell invasion, giving as a result a “bad” scaffold (image a). Proper sintering, through temperature and pressure programs, leads to “good” scaffolds, with highly regular pore structures and well developed interconnecting pores (images b and c).

traditional manufacture protocols of porous scaffolds. Supercritical fluid and solid free-form fabrication (SFF) technologies are interesting and potential alternatives for the preparation of advanced materials, and for the processing of biopolymers and bioactive compounds. Murphy and coworkers first showed the feasibility of manufacturing porous scaffolds using CO_2 , as porogen, and the encapsulation of GFs during scaffold fabrication [110]. Advanced supercritical processes have been developed in order to improve the poor connectivity obtained. Positive results reported for materials produced with this technique include the controlled release of proteins, the promotion of bone formation *in vitro* and *in vivo* and the induction of angiogenesis *in vitro*. SFF techniques have brought a new dimension to the field of tissue engineering. SFF scaffold manufacturing methods provide excellent control over scaffold internal pore interconnectivity and external shape and geometry. SFF techniques can be integrated with imaging techniques to produce scaffolds that are customized in size and shape allowing tissue engineering to be tailored for specific applications.

These techniques include selective laser sintering (SLS), stereolithography (STL), fused deposition modeling (FDM), and direct 3D printing (3DP). Biomaterials/cell/GF hybrid structures obtained by multi-head deposition have developed and successfully tested [111].

The main features of the fabrication techniques for porous polymeric scaffolds are listed in Table 1.2.

1.3.2.6 Surface Mineralization

In mineralized-tissue applications such as bone or dentin, avoiding the formation of a fibrous capsule between the synthetic material and the surrounding tissue is a crucial question. Since the discovery of 45S5 Bioglass® by Hench in 1971 [112], various kinds of ceramics such as $\text{Na}_2\text{O}-\text{CaO}-\text{SiO}_2-\text{P}_2\text{O}_5$ glasses, sintered hydroxyapatite (HAp) and glass-ceramics containing apatite or wollastonite, are known to bond to living bone without the formation of fibrous tissue [113, 114]. When implanted *in vivo*, these bone-bonding materials form on their surface a layer of bone-like HAp similar to the bone apatite. Briefly, two simultaneous chemical changes are involved in the apatite deposition mechanism on bioactive glasses [115, 116]: (1) preferential diffusion-controlled extraction of Na^+ and/or Ca^{2+} ions out of the glass by exchange with protons from the solution, which increases the ionic activity product of the apatite in the medium, accelerating its precipitation, and facilitates the hydrolysis of silica, and (2) hydrolysis and silica network dissolution, increasing the number of $\equiv\text{Si}-\text{OH}$ groups that provide favorable sites for nucleation of the apatite, for their negative charge that enhances electrostatic interaction with the positively charged Ca^{2+} ions in the fluid. In 1991, Kokubo [117] developed a simple biomimetic test to reproduce the formation of an apatite layer *ex vivo* and thereby predict the *in vivo* bioactivity of a biomaterial [114, 115, 118, 119]. An acellular protein-free SBF with ion concentrations, pH, and temperature nearly equal to those of the human blood plasma, is employed as the medium for apatite nucleation. It was found that silanol group formation was the key factor to induce the apatite nucleation and layer deposition on the surface of these materials, even with Ca and P absent from the composition, that is, biomineralization could be induced by different functional groups acting as effective sites for heterogeneous nucleation of apatite, if they are able to develop negative charge at the physiological pH: silanol, phosphate, carboxy, hydroxy and amine groups [118, 120, 121]. This test, or its variants [122], has been employed to premineralize *in vitro* the surface of different kinds of materials containing these groups, for example in [65, 123] poly(ethyl methacrylate-co-hydroxyethyl acrylate) P(EMA-co-HEA) nanohybrids with SiO_2 (see Fig. 1.2c and d).

Additionally, other surface modification techniques have been proposed with the aim of enhancing the bone-bonding ability of *a priori* bioinert substrates, or of increasing the number of surface apatite nucleating sites of less bioactive ones, or of activating the surface of bioactive materials to shorten the HAp coating process. These treatments are based on (i) the precalcification of the surfaces, immersing the samples in SBF in the presence of a plate of $\text{CaO}-\text{SiO}_2$ -based glass or similar as a source of nucleating ions of apatite [124, 125], (ii) the impregnation of the surfaces with alternative nucleating agents such as sodium silicate [126] or silane-coupling

TABLE 1.2 Summary of main scaffold fabrication techniques

Fabrication techniques		Comments	
		Advantages	Limitations
Conventional methods	Freeze gelation/drying	Highly porous structures	Difficult to prevent porogen agglomeration
	Freeze extraction	High pore interconnectivity	Limited reproducibility
	Particle leaching		Random pore size and porosity
	Template sintering	Sorted pore size distribution	Residual porogen, incomplete removal of harmful solvents
		No thickness limitations Crystallinity can be tailored	Limited ranged of pore sizes Limited to two-dimensional films up to 2 mm of thickness
Solvent casting/ particle leaching Gas foaming/salt leaching		Large range of pore sizes Water soluble porogen	Lack of mechanical strength Residual porogen
		Low-temperature process Solvent-free	Lack of interconnectivity Formation of nonporous skin
		Interconnected foamed structures	
			Polymers
			CHT, PCL, HA
			PCL, CLMA, PHEA, CLMA-PEA, PEA-PHEA
			PLGA, PCL, PU, PEG, PVA
			PLLA, PLGA

Advanced methods	Supercritical fluid	Easy process High porosity High porosity High surface area to volume ratio	Use of toxic organic solvents Lack of mechanical strength High processing temperatures Limited material range	CHT, Starch, PLLA, PMMA
	3DP			PCL and derivatives PVA
	FDM	High degree of reproducibility Solvent free Use of powdered polymeric materials	High processing temperatures Limited to small pore sizes Risks of degradation, cross-linking and oxidation	PCL, PLLA, PLGA
	SLS	Rapid fabrication of hybrid scaffolds High resolution Use various biomaterials at the same time High degree of reproducibility Solvent free		PE, PPF/DEF copolymers
MHDS				HA/PCL

agent and titania or calcium silicate solutions [127, 128], or (iii) the introduction of hydrophilic polar groups effective for the apatite nucleation in the surfaces to confer them bioactivity [129–131]. More sophisticated postprocessing treatments combine the modification of the surfaces with functional groups effective for the apatite nucleation, and the rapid precalcification [132, 133]; in the latter, poly(ϵ -caprolactone) scaffolds can be treated with aqueous NaOH to introduce carboxylate groups onto the surface, and next soaked alternatively in CaCl_2 and $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ solutions. In Ref. [134] the possibility of shortening of the HAp coating process on the surface of P(EMA-co-HEA)/ SiO_2 nanohybrids by this pretreatment before immersion in SBF was investigated.

1.4 PROPERTIES OF SCAFFOLDS RELEVANT FOR TISSUE ENGINEERING APPLICATIONS

Some of the properties of polymer structures important for their use as scaffolds have already been mentioned, such as their apparent density, mechanical stiffness, and degradability. Here we review them and other, and introduce the quantities and measurements that are useful for their characterization.

1.4.1 Porous Architecture

Basic features of the porous architecture relevant for the performance of scaffolds include the pore volume fraction, the distribution of pore sizes, the specific surface area and the geometric layout of the pores. In the case of polymers that swell significantly, care must be taken that these properties be evaluated when swollen, their relevant state for the applications.

The pore volume fraction (π) is defined by

$$\pi = \frac{V_{\text{pores}}}{V_{\text{pores}} + V_{\text{pol}}} \quad (1.1)$$

where V_{pores} and V_{pol} are, respectively, the volume occupied by pores and by the bulk polymer in a scaffold. This quantity is related to the apparent density by

$$\pi = 1 - \frac{d_{\text{scf}}}{d_{\text{pol}}} \quad (1.2)$$

where d_{pol} and d_{scf} are, respectively, the density of the bulk polymer and the apparent density of the scaffold (the ratio of its mass and its apparent volume). The pore volume fraction can be determined by gravimetric methods, or by intrusion porosimetry. Important quantities for the clinical application, such as the total number of seeded cells in a scaffold and the mass of material implanted for this purpose, are determined by the pore volume fraction of a scaffold.

The distribution of pore sizes is the relationship that gives the fraction of pores having a typical linear dimension (their “diameter”) comprised within an interval. This is an interesting quantity for characterizing the heterogeneity of the set of pores. It can be determined by image analysis, from scanning electron microscopy (SEM) images, or from micro-computerized tomography (μ CT), or by intrusion porosimetry. In this latter case, pressure differences needed to intrude a liquid (usually mercury) into the scaffold are converted to sizes by making some assumptions (e.g., Washburn’s equation), which involve the choice of a geometrical shape for the pores (cylinders, for example). Since this geometry does not necessarily correspond to the actual case under study, the pore distribution profiles obtained with this technique must be cautiously interpreted: they are representative of the distribution of a linear dimension taken as typical, but that linear dimension is not necessarily the “pore diameter.” The same caution applies to the interpretation of data for the specific surface area of a scaffold if determined by intrusion or adsorption porosimetry. This quantity relates the total inner area of the pores’ surfaces to the mass of the scaffold. It can also be determined by image analysis and computerized microtomography. These techniques, besides SEM, are adequate for a general characterization of the geometric features of the pore architecture [135]. *Delesse’s principle* can also be employed to estimate pore volume fraction in the case of random structures (isotropic, random porosity). This principle states that, in a random bi-phasic structure, the volume fraction of one phase is equal to the mean area fraction of the phase in cross sections taken at random. Thus, the pore volume fraction can be estimated by the area pore fraction in a set of cross sections of a sample.

1.4.2 Solid State Properties: Glass Transition, Crystallinity

The single most important property of a polymer is its glass transition temperature, T_g . It separates the temperature domains where the polymer behaves as a glass (below T_g) or as a soft material (a rubber, if cross-linked, above T_g) [136]. Glassy polymers are brittle and stiff (moduli of the order of 10^9 Pa), rubbery polymers are ductile and soft (moduli of the order of 10^6 Pa). In the rubber state polymer chains have enough energy to change conformation; the water-containing phase of a polymer hydrogel is always in the rubbery state, since the small-sized water molecules act as a plasticizer of the polymer chains, increasing the conformational mobility. Free volume and all the properties that depend monotonously on it (density, permeability, diffusivity) are greater in the rubbery state than in the glassy state. Not only state properties (such as density) experience pronounced changes across T_g , but also transport properties, such as the diffusivity to gases and liquids. Temperature changes suffered by the material (they may be due to changes during storage or transport, or during sterilization) may alter the structure of the scaffold if they take it through its glass transition. If, at use temperature, the material is in the glassy state, its structure may evolve even in the absence of temperature changes, that is, isothermally. This is due to the fact that glasses are in a metastable state due to their formation history; they continuously evolve towards a relaxed, equilibrium state, but with a kinetics so slow, that the relaxation process may last years. These changes are subtle, but noticeable as

densification and changes in mechanical properties. The glass transition temperature may be determined with the help differential scanning calorimetry (DSC).

The degree of crystallinity of a polymer is defined by the mass fraction of the material in crystalline form. It can be determined with the help of DSC. Also with DSC one determines the melting T_m and the crystallization T_c temperatures of the material that undergoes the phase transition. Because the nucleation and grow of the crystallites implies the creation of an interface, the free energy barrier must be compensated by undercooling, and T_c is always lower than T_m . The relevance of these parameters lies in that they establish an upper bound for the temperatures that the scaffold could withstand without destruction (by melting) of its structure. This may be important in choosing the sterilization procedure.

1.4.3 Mechanical and Structural Properties

The mechanical properties that can be of interest in the case of scaffolds depend on the application to which it is destined, and may include tensile, compressive and bending moduli [137]. These moduli can be time-dependent quantities, if the material, in the conditions of use, behaves as a viscoelastic solid. For elastic materials, tensile or compression stress–strain assays will deliver a “Young modulus” E as a way of characterizing the material’s mechanical properties in the small deformation, linear range of behavior; in the case of viscoelastic materials, these moduli can be obtained from frequency-dependent assays in dynamic-mechanical spectrometry assays, or from time-dependent creep or relaxation assays [138]. Further circumstances that must be taken into account in characterizing a scaffold’s mechanical behavior is whether it has isotropic properties, and whether it is a hydrogel. In the case of anisotropic materials, measurements on samples taken from different parts and orientations of the material will be needed; in the case of hydrogels, or, more generally, of materials absorbing a significant amount of water, tests must be performed immersed in liquid environment.

Since the bare output of all the mechanical tests is a relationship between measured force and deformation, it is necessary to define well the geometrical characteristics of the sample to obtain, from those quantities, the moduli E . If these geometrical characteristics (length, thickness, area, etc.) are the overall, or apparent, dimensions of the scaffold, apparent or overall moduli E_{scf} will be obtained from the measurements. These overall moduli characterize the sample as a whole, but, in the case of porous scaffolds, may be not representative of the local, true, mechanical properties of the material, E_{pol} . Both are relevant for scaffolds: single cells experience the local property E_{pol} , which influences biological mechanotransduction, and the load transfer dictated by overall mechanical equilibrium is dependent on E_{scf} . Scaffolds with an isotropic inner porous structure can confidently be treated as cellular materials [139]: lattices made of struts; for these, fairly universal expressions have been derived relating the scaffold’s and the material’s modulus [140]:

$$E_{scf} = b \cdot E_{pol} \cdot \left(\frac{d_{scf}}{d_{pol}} \right)^n = b \cdot E_{pol} \cdot (1 - \pi)^n \quad (1.3)$$

where b is a constant, π is the pore volume fraction, related to the densities d of the scaffold and of the material as in (2), and n has the value $n=2$ in most cases, when the deformation of the scaffold is dominated by the bending of its “struts”; the exponent n may have other values in different situations, and can be considered, as is the case of b , as an adjustable parameter to be determined experimentally.

In the case of polymer networks, the most important structural parameter is the molar concentration of chains, n_c/V (with n_c the mol number of chains, and V the volume), or, equivalently, on the molar concentration of cross-links, n_x/V , or the mean molecular weight between cross-links, $\bar{M}$. These quantities are mutually related:

$$n_x = \frac{2n_c}{\varphi}, \quad \frac{n_c}{V} = \frac{d_{\text{pol}}}{\bar{M}} \quad (1.4)$$

where φ is the functionality of the knot in the network. On the concentration of chains depend such properties as the swelling degree Q (see below, in 4.4) and the tensile modulus E in the rubber state [136, 141]:

$$E = 3 \left(\frac{n_c}{V} \right) RT \quad (1.5)$$

Here R is the gas constant, T is absolute temperature. These dependences are important in the case of hydrogels. The concentration of chains can be directly determined from stoichiometric considerations (if the composition of the polymer-precursors reactant mixture is known), or it can be experimentally determined through Equations (1.5 and 1.13), with the help of mechanical measurements (E) or swelling measurements (Q , see Section 1.4.4).

Apart from its interest in relation to the mechanical and swelling properties of a polymer network, the parameter n_c/V is important also for the diffusion of molecules through the polymer matrix. The diffusion coefficient of a species through a network depends on the mean distance between cross-links of the network, ξ , also called the *mesh size* of the network,

$$D = D(\xi) \quad (1.6)$$

If a is the molecular size of the diffusing species, too low values of ξ may completely impede diffusion ($a > \xi$), and even in the case $a < \xi$ the concentration of cross-links is a measure of the tortuosity of the diffusing path, thence $D = D(\xi)$. The mean distance between cross-links ξ can be estimated with the following considerations. If the number of repeat units in a chain between cross-links, ν , is not too low ($\nu > 10$), the mean squared end-to-end distance $\langle r^2 \rangle$ in an unperturbed chain is [141, 142],

$$\langle r^2 \rangle = C\nu \ell^2 \quad (1.7)$$

with ℓ the length of the repeat unit (for vinyl chains, $\ell = 2.8 \text{ \AA}$) and C the so-called *characteristic ratio* of the chain, a measure of its conformational rigidity that is

tabulated and known for most polymers. Now ξ may be identified with the square root of $\langle r^2 \rangle$:

$$\xi = \sqrt{\langle r^2 \rangle} = (Cv)^{1/2} \ell \quad (1.8)$$

Since $v = (\bar{M} / M_u)$, with M_u the molecular mass of the repeat unit, all the quantities considered above turn out to be related.

1.4.4 Swelling Properties

The most common measures of the water contained in a polymer phase are the *water content*, WC, the *swelling degree*, Q , and the polymer volume fraction in the phase, ϕ ; they are defined and related in the following way:

$$WC = \frac{m_w}{m_{pol}}, \quad Q = \frac{V_w + V_{pol}}{V_{pol}}, \quad \phi = \frac{V_{pol}}{V_w + V_{pol}} = Q^{-1} \quad (1.9)$$

where m_w and V_w are, respectively, the mass and volume of water in the phase, and m_{pol} and V_{pol} are the mass and volume of the dry polymer. The quantity WC is usually determined gravimetrically; Q can be determined either through the change of dimensions of the sample, or, indirectly, by gravimetry,

$$Q = \frac{WC \cdot v_w + v_{pol}}{v_{pol}} \quad (1.10)$$

where v_w and v_{pol} are the specific volumes of water and the polymer.

If swelling is isotropic, then $Q = \Lambda^3$, with Λ the linear *stretch ratio* in any direction. If the hydrogel had a mesh size ξ (unperturbed end-to-end distance, see Eq. 1.8) in its preparation state Q^* , then in a swollen state of $(Q/Q^*) \neq 1$ it will have a mesh size ξ' (perturbed end-to-end distance) given by

$$\xi' = \left(\frac{Q}{Q^*} \right)^{1/3} \xi \quad (1.11)$$

The moduli of a rubbery xerogel, E_{xero} , and of its swollen hydrogel, E_{hgel} , in a state with Q are related as

$$E_{hgel} = E_{xero} Q^{-1/3} \quad (1.12)$$

The value of WC, when it corresponds to a state of equilibrium with the environment, is termed the *equilibrium water content*, EWC. The water content in a phase in equilibrium with its environment is dependent on the activity of water in the environment, a_w : $EWC = EWC(a_w)$, $Q = Q(a_w)$, $\phi = \phi(a_w)$, etc. These equilibria are governed by the thermodynamics of phase equilibria and by the expressions for the free energy

of polymer-water mixtures (theory of Flory–Huggins) and of elastic polymer networks; from them one gets a relationship between the swelling properties *in equilibrium* and the structural parameter (n_c/V) of the network [141, 142]:

$$\ln a_w = \ln(1 - \phi) + \phi + \chi_{wp} \phi^2 + v_w \frac{n_c}{V} \phi^{1/3} \quad (1.13)$$

where χ_{wp} is the water–polymer apparent *interaction parameter* (the *Flory–Huggins parameter*), v_w is now the molar volume of water, and the rest of symbols have already been introduced. The well-known Flory–Rehner equation is the particular case of Equation (1.13) for $a_w = 1$ (left-hand side of (1.13) equal to zero); it corresponds to swelling in pure water, a circumstance never met *in vivo*, where solutes are always present. Since changes in dimensions of samples depend on Q , this relationship is useful for controlling those changes through modification of the cross-linking density of the hydrogel network. The water–polymer interaction parameter can be estimated from correlations with other properties (such as the solubility parameter and the cohesive energy density, [143], or else it can be measured with the help of Equation (1.13) and model networks).

1.4.5 Degradation Properties

Degradation studies are important both for biodegradable and biostable polymers. *In vitro* studies, though they represent a condition very different from that met *in vivo* by the polymers, may nonetheless yield results that can serve to extrapolate, at least qualitatively, what is to happen to a material when implanted.

In vitro degradation studies are commonly performed in phosphate buffered saline (PBS) solution or in cell culture medium, with and without enzymes, at 37°C, to track the degradation time-profile of polymeric materials. As a result of degradation there is a loss of mass, changes in morphology and structural properties of the material (e.g., in crystallinity), a reduction in the mechanical properties, etc. The very same degradation products may act as catalysts of the degradation process (e.g., by increasing the acidity of the medium) [144, 145].

All these chemical and physical events can be employed to characterize the effects of degradation. They can be monitored by several methods such as mass variation through weight measurements; morphological changes by SEM; degradation products analysis by high performance liquid chromatography (HPLC) and gel permeation chromatography (GPC), changes in chemical structure and properties by infrared spectroscopy (FTIR) and DSC, changes in crystallinity by X-ray tests spectroscopy or DSC, and changes in the mechanical properties by stress-strain test and compression assays [146]. The use of more than one of these methods is always advisable since degradation may manifest itself in some property, but not (or less) in others. For example, mass loss may be in early stages insignificant if the oligomers produced by degradation have difficult diffusion through the bulk polymer; however, mechanical, swelling, or calorimetric properties will be sensitive to the changes.

1.4.6 Diffusion and Permeation

Water, solutes and gases are species that can diffuse through the scaffold and, in the case of hydrogels, even through the polymer matrix itself. The *diffusivity* of a species in a medium along some fixed direction x characterizes the relationship between the mass flow per unit area across a surface normal to the chosen direction, J , and the concentration gradient along that direction, $\partial c/\partial x$:

$$J = -D \frac{\partial c}{\partial x}; \quad (1.14)$$

D is the *diffusion coefficient* of the species in the medium. Together with the continuity equation, the aforementioned relationship gives *Fick's equation* for diffusion when D does not depend neither on x nor on c :

$$\frac{\partial c}{\partial t} = D \frac{\partial^2 c}{\partial x^2}. \quad (1.15)$$

This equation has known solutions for some geometries; for a slab of thickness ℓ it can be solved, and, since the concentration of the species c can be related to the mass m of the species in the medium, the solution of (1.15) can be expressed in terms of m [147]:

$$\frac{m(t) - m(0)}{m(\infty) - m(0)} \approx 4 \left(\frac{Dt}{\pi \ell^2} \right)^{1/2}, \quad (1.16)$$

thus permitting the determination of D through weighing in time of the mass $m(t)$ until equilibrium, $m(\infty)$, is reached. Equation (1.16) is valid for values of the left-hand side less than or equal to 0.6.

When the mass flow takes place across a pressure gradient along the direction x , the *permeability* gives the relationship between the mass flow J and the pressure gradient,

$$J = -P \frac{\partial p}{\partial x}; \quad (1.17)$$

P is the *permeability coefficient* of the medium to the species along x . From (1.14) and (1.17)

$$P = D \frac{\partial c}{\partial p} = D \cdot S, \quad (1.18)$$

where S is the *solubility* of the species in the medium (a constant in the domain of validity of Henry's law). Permeability properties of scaffolds are important in bioreactor culture conditions [148–150].

1.4.7 Surface Tension and Contact Angle

Adhesive and cohesive properties, capillarity and wettability are some of the important properties of biomaterials that depend on the area of the interface between the material and its environment. The surface tension, or *surface energy* of a material is a measure of the work that it costs, under specified circumstances (constant volume and temperature) to increase the area of its surface of contact *with a given environment*. Surface energy is thus always an interfacial property [151]. Wettability, for example, refers to the spreading of a liquid on a surface, that is, to the extent of their mutual contact surface. Since protein and cell adhesion on material surfaces are processes involving the creation or destruction of interfaces (protein–material, cell–material), these processes cost free energy and thus can be correlated with the surface energy of a material.

Basic methods for estimating the interfacial energy of a couple of media (M,N) involve the use of the Young–Dupré formula for the *contact angle* θ_{ML} between a liquid L and a solid M,

$$\sigma_{MN} = \sigma_{ML} + \sigma_{LN} \cdot \cos \theta_{ML} \quad (1.19)$$

together with some mathematical hypothesis on the dependence of σ_{ML} on σ_{MN} and σ_{LN} , and thus allows the calculation of σ_{ML} if the contact angle θ_{ML} of the interface between M and L is experimentally measured and the liquid interfacial tensions σ_{LN} are known; these are usually tabulated, or can be measured through the pendant drop method. In usual laboratory circumstances M is the material of interest, N is ambient air, and L is one of several liquids; if L is water, the contact angle θ_{MW} serves to characterize the wettability of the material surface. The calculation of σ_{MN} involves the measurement of contact angles θ_{ML_i} with a number of different liquids L_i , in order that the approximations implied by the hypothesis on σ_{ML} have little influence on the result [143, 151]. Correlations between the surface energy and other parameters (such as the compressibility, the cohesive energy density, or the solubility parameter) exist, and, in lack of better possibilities, σ can even be estimated with the help of group contribution methods (the *paracor* [143]).

1.4.8 Biological Properties

The biocompatibility of a material does not ensure the success of a scaffold, since a numerous set of different factors are determinant. The fate of an implant depends on such a huge number of factors present in the living environment, that no set of *in vitro* experiments will suffice to predict the biological performance of a material. However, passing certain *in vitro* tests represents a mandatory *minimum* for any candidate material, so as to reduce animal tests as much as possible.

1.4.8.1 Cytotoxicity

In vitro screening assays are used to assess the biocompatibility of a material and to evaluate the potential adverse biological effects it could produce in a specific cell culture, according to norm (ISO 10993-5: 2009, part 5). Quantitative and qualitative

analysis methods by indirect and/or direct cell contact have to be used with specific cell lines. After a period of exposure (typically 24–72 h) of the cells to the extracts in contact with the material, or to the material itself, cytotoxicity is evaluated by the loss of viable cells.

1.4.8.2 Pro-Inflammatory Response

Cell toxicity results in cell death, and thus in the reduction in the number of live cells revealed by the cytotoxicity assay. Absence of cytotoxicity, however, may be not sufficient as a filter for biomaterial selection, since cells may be alive, but in a stressed state that will unfavorably condition their fate. Inflammatory processes occur when a material is implanted, which trigger the release of cytokines (low molecular weight proteins) from the cells. A way to assess *in vitro* the stressed state of cells in a scaffold is to quantify the expression of these cytokines (TNF- α , IL-1, IL-6, ...) and to compare the data with the release when the cells are activated by inflammatory *stimuli*, such as lipopolysaccharide LPS, a bacterial pathogen that activates immune response. For example, activated endothelial cells express the adhesion molecule E-selectin, which is involved in inflammatory response; quantifying its expression can be employed for testing the pro-inflammatory response of biomaterials [152, 153]. For human use, endotoxin tests are strict, and represent a stringent requirement on biomaterials [154].

1.5 COMPOUND, MULTICOMPONENT CONSTRUCTS

The different scaffold structures discussed in Section 1.3.2 may be combined into compound constructs to meet specific objectives. Some examples follow.

1.5.1 Scaffold-Cum-Gel Constructs

Natural and synthetic hydrogels are cell-friendly environments: they constitute an ideal medium of transport for dissolved gases and species, and they are easy to remodel by the cells: thus, migration processes and neovascularization take place faster in such media. However, gels have low consistency and very poor mechanical properties. They can be included into the pores of three-dimensional scaffolds to render them more resistant and localizable. In Ref. [102], hyaluronic acid gel was incorporated into the pores of an elastomer scaffold of poly(ethyl acrylate). Depending on the preparation conditions, the scaffold can be filled to different degrees, ranging from a complete occupation of its pores to a sparse surface coating. The former situation may be preferred by cells such as chondrocytes, which exist in cartilage as relatively isolated cells in lacunae. To enhance nerve regeneration in [155, 156], silicone guiding tubes were filled with collagen, laminin and fibronectin, and in [157] cellulose channels have been filled with the RAD16-I self-assembling peptide gel. In Figure 1.5a the pores of poly(ethyl acrylate) channeled scaffolds have been filled with RAD16-I and the length gelled *in situ*. As coatings, fibrin gels have been used to enhance cardiomyocyte retention in chitosan scaffolds [158], or improve the

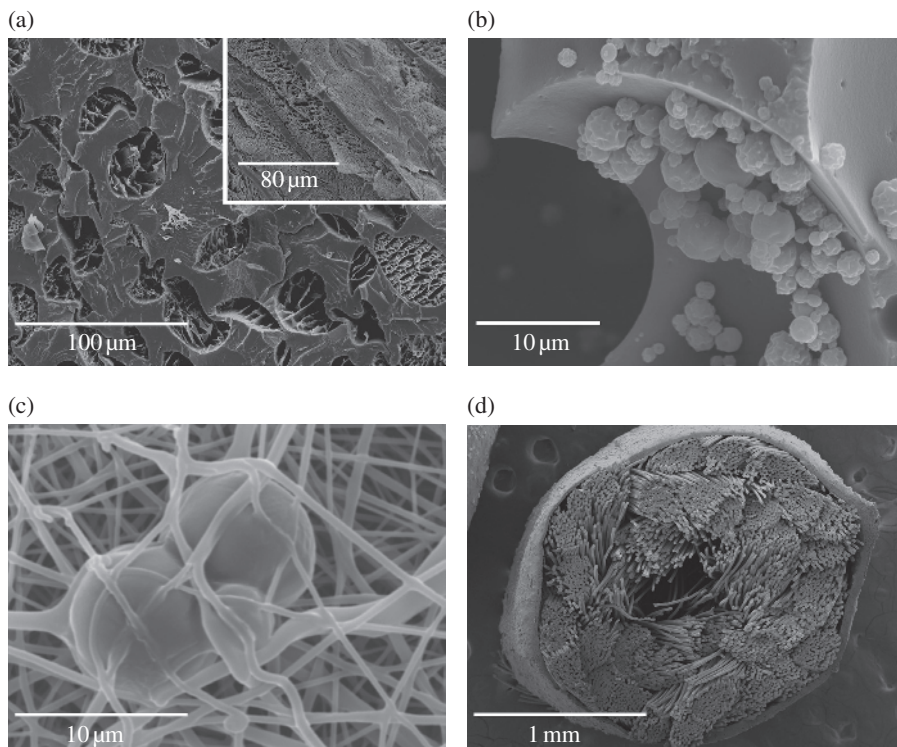


FIGURE 1.5 Different examples of compound scaffolds. (a) CryoSEM image (transversal section) of a P(EMA-co-HEA) 70:30 wt% copolymer scaffold with aligned tubular pores, filled with a 0.15% RAD16-I peptide solution and gelled *in situ* with PBS. The inset corresponds to a longitudinal fracture. The SAP hydrogel is a cell-friendly artificial ECM, but with very low mechanical properties. Its incorporation into the pores of an elastomer scaffold yields a construct combining the advantages of both materials. (b) SEM image of gelatin microparticles adsorbed on the cup surfaces of a PEA scaffold with spherical pores, and (c) hyaluronic acid microparticles embedded in PLLA microfibers of an electrospun mat. Angiogenic or chemotactic factors released from the particles lodged in these scaffolds may accelerate the vascularization of the constructs, or their colonization with cells and cell processes, such as axons. (d) SEM image of a section of a construct aimed to serve as a regenerative tendon prosthesis: a hollow PLA multifilament braid, loaded in its lumen with microparticles as cells carriers, is externally coated with an antiadherent hyaluronic acid (HA) electrospun mat. Each element of the construct serves a definite purpose: the braid supplies immediate mechanical strength (necessary to replace the tendon's load-bearing function); the stem cells supported on the microparticles must differentiate into tenocytes under the mechanical and environmental stimuli, producing collagen I and progressively regenerating the tendon structure. The outer HA coating is suited for intrasynovial tendons, in order to avoid undesired adherences to the tendon sheaths and nearby tissues; its microporous hydrogel nature allows for transport of nutrients, metabolites and signals, but is impervious to cells and nonadherent.

development of endothelial cells within polylactide (PLA) scaffolds [159, 160]. The same concept applies for gel-coatings of microfibers in mats, or wrapping bundles of microfilaments working as guides [161]. The gels employed in these constructs may additionally carry drugs or GFs to be released *in situ* as the gel layer swells or degrades. Thus, in [162] a fibrin gel loaded with glial cell-derived neurotrophic factor (GDNF) or nerve growth factor (NGF) was used within silicone tubes, and in [163] polysulfone tubes filled with agarose gel loaded with laminin and NGF were employed.

1.5.2 Scaffolds and Membranes Containing Microparticles

The success of cells transplanted within a scaffold may depend on the rapidity of the neovascularization of the implant. In order to enhance this process, vascular endothelial growth factor (VEGF)-releasing vehicles may be envisaged within the scaffold, such as the gels just considered, or also microparticles located on the inner surfaces of the scaffold. Or, axonal extension processes may be wanted within a structure. To aid and direct such processes it may be interesting to locate chemotactical stimuli within scaffolds. Figure 1.5b and c shows two possible such configurations, in which microparticles are either adsorbed on the scaffold's pores, or embedded in the microfibers of an electrospun mat. PLGA microparticles loaded with NGF and incorporated into a porous scaffold of the same material were considered in [164]; PLGA particles loaded with dexamethasone within alginate scaffolds were employed in [165]. The drug delivery profile will depend on the encapsulation method, the loading capacity of the material, its hydrophilicity and degradation kinetics depending on its composition, geometry, site of implantation, among others, and can be sequenced temporally combining microparticles of different sizes and/or compositions or more sophisticated systems, as in [166], where double-walled PLGA/PLA microparticles loaded with GDNF were incorporated into PCL tubes.

1.5.3 Other Multicomponent Scaffold Constructs

Patches intended as vehicles for cell supply to a tissue surface are best made of cell-adherent, hydrophobic polymers, such as are the polyesters. Because of this very same fact, however, these materials may induce unwanted fibrotic adherences to neighboring tissues. With this problematic in mind, in [102] a bi-layered membrane was devised, with cell-adherent properties on one face, and cell-repellent properties on the opposite face; the construct was obtained in a two-step electrospinning process, one of the sides of the membrane being formed by a PLA mat, the other being a hyaluronic acid mat.

The larger the dimensions demanded by application for a scaffold, the more likely it will be that the designed solution requires several components. In Ref. [94] a regenerative prosthesis for tendons was presented. It consists in braided fiber bundles made from a resorbable PLA polyester that are externally covered by an electrospun cross-linked hyaluronic acid coat (see Fig. 1.5d); the braid has a hollow nucleus, where microparticles carrying seeded adipose-tissue derived stem cells are hosted.

1.6 QUESTIONS ARISING FROM MANIPULATION AND FINAL USE

Scaffolds are destined for use, either in the laboratory for *in vitro* experiments, or to be implanted by a surgeon. Both in the practical work of the laboratory and in the clinical praxis scaffolds interface three groups of actors with very different know-hows: the material scientists that produce them, the biologists who seed them with cells and analyze the results, and the surgeons who implant them in living organisms. This raises a number of specific questions that have to be addressed, and that may condition the choices of materials, structures and other design parameters. Biologists usually know the best way to seed cells on a culture well and to mark them with specific antibodies, but seeding and marking a culture in a scaffold may be an entirely different problem. Surgeons are used to standard sterilization methods of their instruments, but these may in some cases be destructive for scaffolds... . Three partite dialogue is imperative.

1.6.1 Sterilization

Since there is no mandatory standard sterilization procedure, the choice of one will depend on the characteristics of the material, and of the final use. It must comply with the general requirements of being effective and of not altering the material's properties. Efficacy of the method chosen must be assessed according to what demanded by norm. Autoclave sterilization is one of the most widely employed methods in hospitals. Since it involves exposure to water vapor at temperatures of 115–125°C during 30 min, many polymers and structures made from them may suffer irreversible changes of properties. Autoclaving is not advisable as a sterilization method for polymers that undergo hydrolytic degradation, and for those with semicrystalline structure and melting temperatures falling below and near those temperature values. Coming next in popularity in hospitals is sterilization with gases, most frequently with ethylene oxide. The biocidal efficacy of this agent is based on the high reactivity of the double bonds of its molecule. This may be also a cause of chemical alteration of the polymer matrices. Moreover, sterilization with gases includes a process of adsorption, and a subsequent process of desorption of the sterilization agent, which lasts usually much longer than the first one. Since scaffolds have a high specific surface area and an intricate inner geometry, desorption may last quite long until it is completed to a safe degree. Both these circumstances are in detriment for ethylene oxide as a sterilization method for polymer scaffolds in most cases. Things being so, irradiation with a gamma-ray source may be the recommended method in most circumstances: it is highly effective as biocidal, and gamma rays are able to penetrate a structure in the bulk, thus ensuring that the inner pores in a scaffold get treated. Doses may range typically from 5 to 25 kGy; as before, the chosen dose must be assessed for its efficacy with specific tests according to norm. However, gamma irradiation may also cause permanent changes in the material [167]: energetic radiation may induce chain scission reactions and free radicals, which may lead to subsequent oxidation reactions; these possibilities must in any case be assessed. For nonhuman use, sanitization

processes of the scaffolds may suffice. These processes, to be distinguished strictly from sterilization, may consist in UV light irradiation, or repeated rinsing in ethanol/water 70:30 solution. It is important to bear in mind that sterilization processes do not eliminate endotoxins from the materials; since the sources of endotoxin contamination are ubiquitous, the sole way to guarantee endotoxin-free materials is to produce them under “GMP” (good manufacturing practice) conditions. This is mandatory for applications in humans.

1.6.2 Cell Seeding, Cell Culture, Analysis

The use of scaffolds as niches where cells are seeded poses new problems when compared with conventional cell cultures “in two dimensions,” that is, on the flat surface of culture wells [160, 168]. Cell seeding techniques and subsequent analysis methodologies must be modified or developed, taking into account the restricted accessibility of the inner porous structure of scaffolds. Pore size and pore connectivity, as well as the hydrophilicity of the material, determine the degree of difficulty in achieving a uniform cell seeding; the chemistry and mechanical properties of the matrix determine the difficulties that arise in cutting and fixing the samples for later analysis.

An effective cell seeding determines the fate of the scaffold-cell construct. Previous experience with 2D cultures is difficult to translate to the 3D context: cell densities per unit area now lose their meaning; drop seeding may now be ineffective. The number of cells to be incorporated into a scaffold must now be estimated with different criteria, taking into account the scaffold’s volume and surface area, which may be many times larger than its apparent, cross-sectional outer area. Depending on the hydrophilicity of the material, the cells can be simply deposited on top of it, or they may require injection into the structure with the help of a syringe or other means. This operation can be repeated more than once, and at different spots in the scaffold, depending on the size of the construct. The seeded construct can then be left to itself in the culture medium (“static seeding”), or it may be subjected to gentle shaking and other dynamical processes, such as centrifugation or perfusion [169] (“dynamic seeding”). A static seeding may be advisable if the material is a poor cell-adherent, but, generally, dynamic seeding improves the uniformity of the cell distribution within the scaffold [170].

After culture, the scaffold-*cum*-cells constructs must be processed for analysis. The concentration of the cell fixation media (paraformaldehyde or methanol) and the fixation times must be chosen depending on the characteristics of the material. Many cells can be lost during these processes, especially when the cells prefer cell-to-cell contact and are poorly adhered on the material’s surfaces; this is often the case of different types of stem cells, which grow making spheroids. Immunohistochemical staining of cultured scaffolds does also have its specific problems. Opacity and light refraction properties of the bulk material difficult the observation. Furthermore, some materials absorb the fluorochromes employed as secondary antibodies becoming autofluorescent, sometimes impeding a clear recognition of the cellular marking. This makes mandatory to cut the construct in slices of few microns with cryostat or microtome, and to reconstruct from them the overall picture.

1.6.3 In the Surgeon's Hands

A final set of considerations to be borne in mind for scaffold design refers to their final use in the operating room. Implantation of a scaffold in a living organism is a surgical act; as such, it implies the manipulation of the material with the surgeon's instruments and abilities, and possibly its suture to a tissue. This poses definite mechanical requirements on the material. Additionally, the translation of results obtained *in vitro* to clinical or preclinical practice must always consider the factor of *scale*: scaffold-*cum*-cells constructs that are viable in a culture well may fail in other circumstances simply because of rescaling their size. Typical dimensions employed *in vitro* can ensure nutrient inflow and metabolite outflow under culture conditions (static or dynamic), but, with other dimensions, the same scaffold may not be viable if the construct is not rapidly vascularized to ensure those flows *in vivo*.

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