

Polymer Functionalization as a Powerful Tool to Improve Scaffold Performances

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To address the increasing need for improved tissue substitutes, tissue engineering seeks to create synthetic, three-dimensional scaffolds made from polymeric materials, incorporating cells and growth factors to induce new tissue formation. Materials science, in conjunction with biotechnology, can satisfy these needs by developing artificial, synthetic substitutes and organ implants. Here, scaffold ability to promote cell growth and differentiation is a key point and, in this framework, orthogonal chemistry has led the field of biomaterial science into a new area of selective, versatile and biocompatible nature. In particular, the possibility to modify and functionalize scaffolds with compounds that are able to improve mechanical properties or cell viability and improve their differentiation in a tailorable manner opens new opportunities for researchers. In this review, we seek to emphasize the recent endeavors of exploiting this versatile chemistry toward the development of new cell culture scaffolds.

Introduction

TISSUE OR ORGAN transplantation is severely limited by the problems of donor shortage and immune rejection by receiving patients. Developments and recent achievements in tissue engineering allow the transplantation of cells from a patient's own tissue to regenerate damaged tissues or organs, avoiding adverse immune responses.^{1,2} Several recent developments point toward the combined use of cells together with specific drugs to enhance regenerative therapeutic effects.^{3,4} In this framework, three-dimensional (3D) biomaterial-made scaffolds were first developed as a temporary substrate to grow cells in an organized fashion, before performing the transplantation of such combined structures.⁵ Direct injection of *in vitro*-cultured cells is one attractive alternative.⁶ However, concerns emerge while trials are performed: the injected cells very often leave the zone of injection, as they are not confined by any support; thereby, they easily get into the circulation migrating all over the body toward a rather uncertain fate. Hence, joining materials science together with biotechnology and engineering is considered to be a promising strategy for developing 3D scaffolds that could help tissue replacement.^{7,8} In particular, recent advances in materials science have provided an abundance of innovations, underlining the leading role of polymers in this field.^{9,10} To design substitutes with improved biocompatibility, tissue engineers seek to create synthetic, three dimensional (3D) scaffolds made from

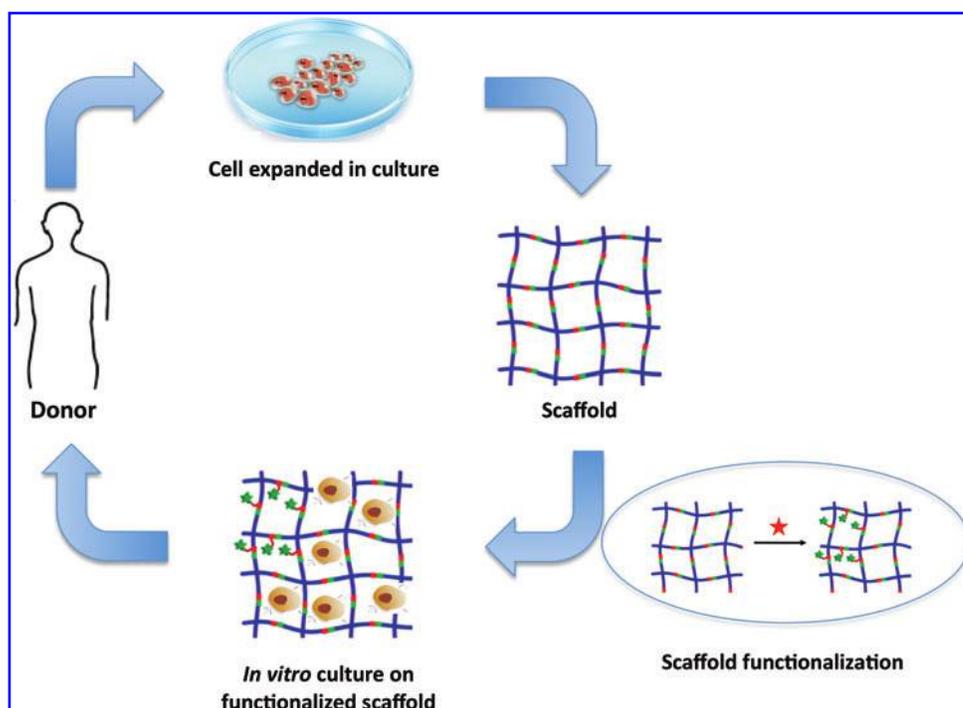
polymeric materials incorporating cells or growth factors to induce the growth of novel tissue.^{11,12} As a result, strong attention is paid to synthetic and natural polymers as they can be used to fabricate 3D scaffolds and also to develop injectable systems for tissue engineering.³ On one hand, synthetic polymers can be tuned in terms of composition, rate of degradation, mechanical and chemical properties. On the other hand, naturally derived polymers provide structures extremely similar to living tissues such as stimulating a specific cellular response, which sometimes supersedes the advantages of synthetic polymers.¹² Moreover, owing to their similarity with the extracellular matrix (ECM), natural polymers present higher biocompatibility *in vitro* and *in vivo*, but the possibility to induce immune reactions in humans is their main drawback.

Despite some good results in the last years, the attention of research is moving to orthogonal selective chemistry applied to polymer functionalization as a powerful tool able to increase scaffold performances.^{13–15} The (re)discovery of many highly efficient and orthogonal chemistries, combined with the development of various functional group-tolerant living/controlled polymerization techniques, has enormously expanded the scope of postpolymerization modification and resulted in an enormous increase in the use of this approach to synthesize functional polymers.^{16,17} Indeed, the modification of polymers could help tissue engineering in two ways: on the one hand improving material properties such as elasticity and degradation profiles and, on the other hand,

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FIG. 1. Tissue engineering approach: the smart combination of cells and functional materials to replace damaged or missing parts of living tissues. Adapted with permission from Azagarsamy *et al.*⁵ Copyright 2013 American Chemical Society. Color images available online at www.liebertpub.com/tea



enriching the cell environment to be closer to the natural situation of the tissue to be regenerated.^{18,19} In particular, recent studies underlined the possibility to build time-related degradable scaffolds,²⁰ hybrid (organic/inorganic) scaffolds,²¹ and materials able to increase cell viability and differentiation.²²

Hence, looking at the developments in this field, the aim of this review is to highlight the significant advances and breakthroughs and to provide the reader with a flavor of what has been accomplished and all the possibilities that are yet to be explored. Considering the high amount of publications of the last years, this approach promises to revolutionize the classic tissue engineering circle.^{6,7} Figure 1 presents the tissue engineering circle redrawn, adding scaffold functionalization before cell seeding.

Material Performances

Functionalization in a scaffold design must be able to create porous structures to attain the desired mechanical function and mass transport (i.e., permeability and diffusion) properties, and to produce these structures within arbitrary and complex 3D anatomical shapes. The physical aspects of a scaffold design, as with polymer choice, depend largely on the final application. The scaffold is meant to provide the appropriate chemical, physical, and mechanical properties required for cell survival and tissue formation. Essentially, the polymeric scaffold is designed to define the cellular micro-environment (cell niche) required for optimal function. In particular, several modifications could be considered to improve scaffold native performances, adding advantages typical for other classes of materials.^{23–26} This is the case of functionalization to create hybrid (organic/inorganic) scaffolds.²⁷ In bone tissue engineering, hydroxyapatite and calcium phosphates present high osteoconductivity, but low

mechanical properties.²⁸ In this direction, several research groups functionalized synthetic scaffolds (made of polylactic acid or polycaprolactone) with hydroxyapatite and calcium phosphate.^{29–35}

In the same direction, Armentano *et al.*²¹ investigated the role of carbon nanotube incorporation on poly-L-lactic acid-based scaffolds: mechanical properties together with cell adhesion demonstrated an enhancement related to the introduction of inorganic nanotubes in organic compounds. The aim of this approach is to guarantee the advantages typical for polymers as biocompatibility, high design possibility, injectability, and degradability together with properties typical for inorganic compounds such as osteoconductivity.^{36,37} Hence, magnetic nanoparticles showed a high possibility to address targeting therapies and imaging that could be linked with polymer chains^{38,39} improving their performances.

Polymeric scaffolds and hydrogels are widely used for controlled drug delivery purposes with the aim to avoid under- and overdosing, while maintaining the drug level within a desired range for a long period.⁸ Despite the good results obtained in many applications, several problems are present due to the fact that drug release from 3D matrices is mostly driven by a pure diffusion mechanism, where free solutes very rapidly escape to the surrounding solution. In this direction, researchers are aiming to develop scaffolds that are able to delay release rates or allow multiple release kinetics, for example, different factors. Release rates of several compounds could be delayed using a cleavable spacer (i.e., binding peptide) that links peptides^{40–43} or vitamins⁴⁴ with the polymeric chains of the scaffold. The possibility to deliver different drugs from a single device could be also realized by multilayer hydrogels⁴⁵ and by the use of scaffolds linked with liposomes, where drugs could be loaded either within the matrix or within liposomes.^{46,47} Moreover, polymer functionalization could be used to improve

mechanical properties: the most common approach is to combine synthetic and natural polymers to consider the advantages of both these systems.¹² On one hand, synthetic polymers can be tuned in terms of composition, rate of degradation, mechanical and chemical properties. On the other hand, naturally derived polymers provide compositional uniqueness, such as stimulating a specific cellular response, which sometimes overrides the advantages of synthetic polymers. In this direction, Chen *et al.*⁴⁸ and Su *et al.*⁴⁹ modified polyester scaffolds with collagen. This particular family of scaffolds (polyesters) presents problems due to the low hydrophilicity that does not allow high cell ad-

hesion and proliferation.⁵⁰ To overcome these problems, these scaffolds could be functionalized in different ways⁵¹ using polydioxanone,⁵² polylysine,⁵³ polyphosphazene,⁵⁴ heparin,⁵⁵ or inducing hydrophilicity by plasma treatment.⁵⁶

From a material point of view, scaffold functionalization could also improve thermal resistance⁵⁷ and mechanical properties to adapt materials to different medical needs^{58,59} together with the possibility to allow high material processability.⁶⁰ Here, a detailed discussion should be reserved to degradability, one of the most important characteristics of biomaterials. In particular, biodegradable materials should not evoke a sustained inflammatory or toxic response upon

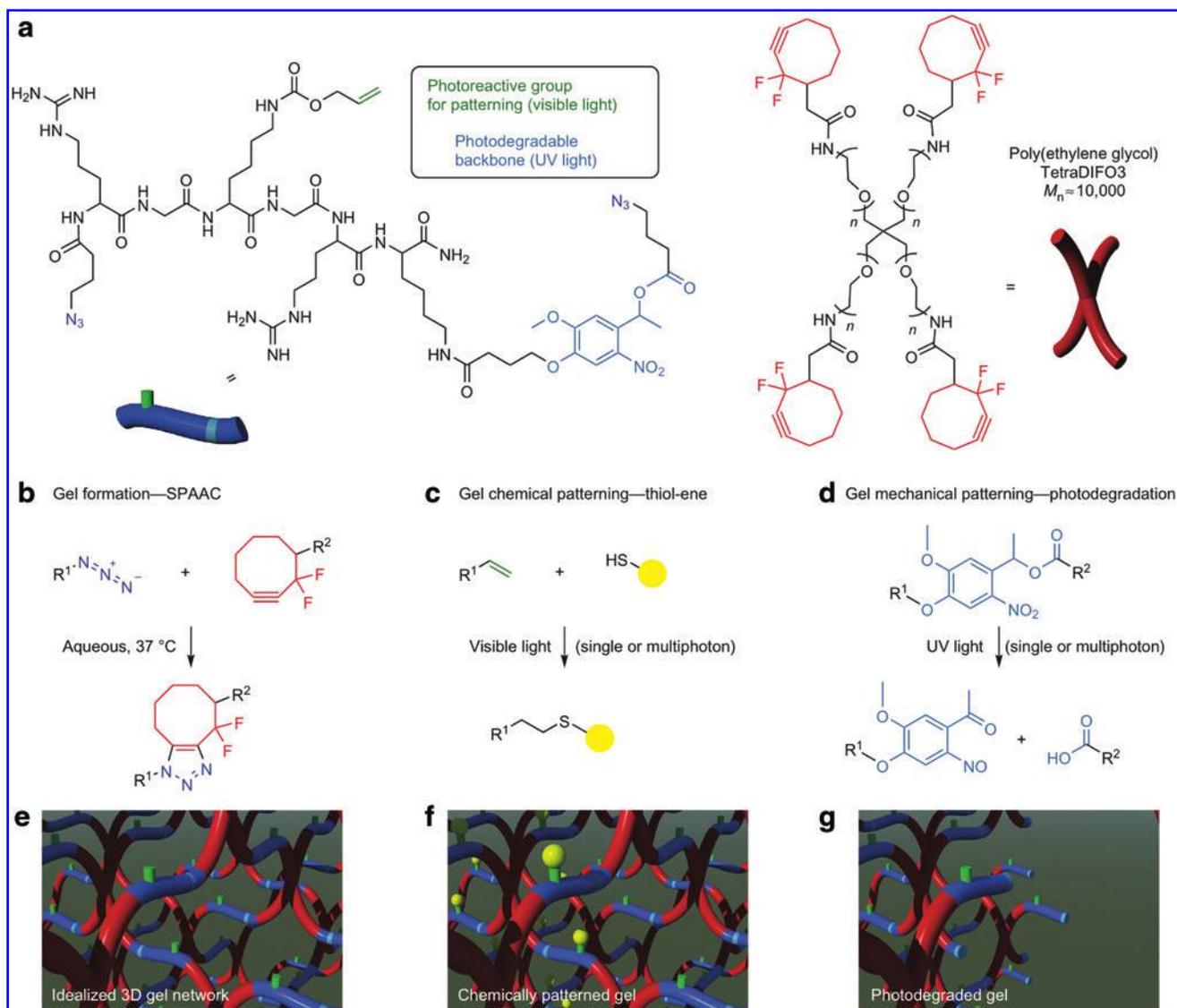


FIG. 2. Synthesis, photocoupling, and photodegradation for tuning chemical and physical properties of click-based hydrogels. **(a, b)** Click-functionalized macromolecular precursors (PEG-tetraDIFO3 and bis(azide)-functionalized polypeptides) form a three-dimensional ideal hydrogel structure **(a)** by means of a step-growth polymerization mechanism through the SPAAC reaction **(b)**. **(c)** In the presence of visible light ($\lambda = 490\text{--}650$ or 860 nm), thiol-containing biomolecules are covalently affixed to pendant vinyl functionalities throughout the hydrogel network through the thiol-ene reaction. **(d)** A nitrobenzyl ether moiety within the backbone of the polymer network undergoes photocleavage in the presence of single or multiphoton ultraviolet light ($\lambda = 365$ or 740 nm) that results in photodegradation of the network. Schematics of the formed SPAAC-based idealized gel **(e)**, the network after thiol-ene functionalization **(f)** and the material after photodegradation **(g)**. (Adapted from DeForest *et al.*²⁰ [with permission from the Nature Publishing Group]). Color images available online at www.liebertpub.com/tea

implantation in the body together with having an acceptable shelf life. The degradation time of the material should match the healing or regeneration process and the degradation products should be nontoxic, and able to get metabolized and cleared from the body.⁶¹ Chien *et al.*⁶² added disulfide-containing crosslinkers to form a biodegradable carboxy-betaine hydrogel, which can be self-degraded as cells grow or degraded in an accelerating way by means of adding a cysteine-contained medium to NIH 3T3 cells.

Another approach was the one developed by the Anseth group,^{20,63} where step-growth networks are formed rapidly through a copper-free, azide-alkyne click chemistry between tetrafunctional poly(ethylene glycol) molecules and difunctionalized synthetic polypeptides. A sequential photochemically activated thiol-ene chemistry allows subsequent functionalization of the network through reaction with pendant alkene moieties on the peptide (Fig. 2). Because the thiol-ene reaction is light driven, the degree of modification is directly related to the dosage of light delivered to the system. Functional groups incorporated into this class of materials are able to produce networks that are capable of degrading in the presence of light, allowing the effects of physical material cues on cell function to be probed.

Biological Performances

The scaffold, by definition, is a temporary supporting structure for growing cells and tissues. It is also called synthetic ECM and plays a critical role in supporting cells. These cells then undergo proliferation, migration, and differentiation in three dimensions, which lead to the formation of a specific tissue with appropriate functions as would be found in the human body.¹ Cells primarily interact with scaffolds through antigens on their surface and chemical groups (ligands) on the material surface.⁶⁴

Cell attachment is strongly affected by the biophysical nature of the ligand. Hence, the ability to design biomaterial substrates with known and quantifiable densities of adhesion molecules makes it possible to manipulate cell behavior by the correct preparation of the substrate. Recent literature supports the idea that density^{65–67} of a ligand is a fundamental scaffold requirement. Ligand density is mainly influenced by the specific surface area of pores to which cells can adhere. Pores thus need to be of an optimal size to allow cells to migrate into the structure, but do not need to have contact. The cells become eventually bound to the ligands within the scaffold, but the ligands need to be small enough

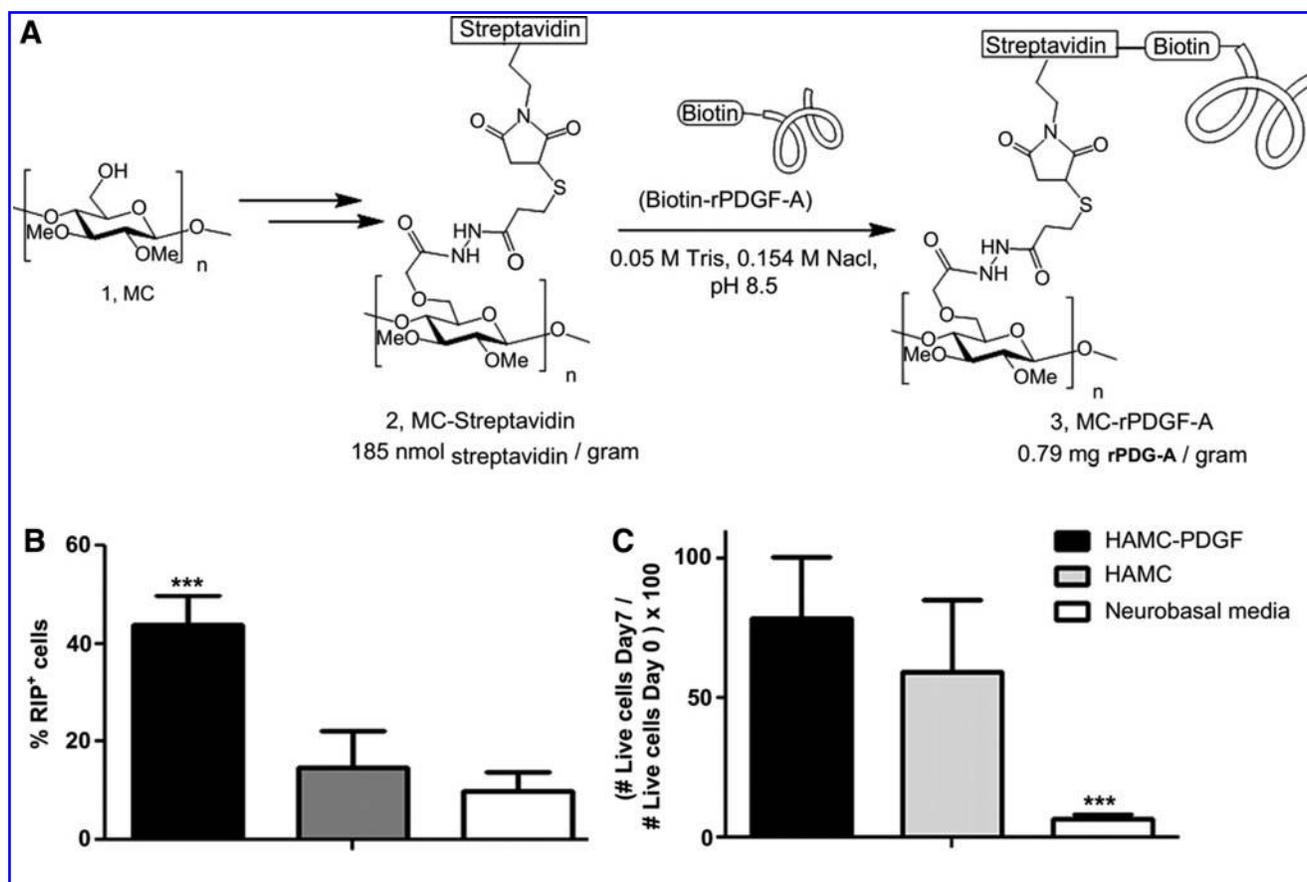


FIG. 3. (A) Chemical conjugation of biotin-rPDGF-A to MC. MC (1) was chemically modified to covalently conjugate maleimide streptavidin (B, C) *in vitro* characterization of neural stem cells (SCs) in various culture conditions. Cells were premixed into the hyaluronic acid–methylcellulose (HAMC) hydrogel or in neurobasal media and then plated at 4×10^4 cells/well into an eight-well chamber slide for 7 days. (B) HAMC-platelet-derived growth factor (PDGF) promotes oligodendrocyte differentiation and shows a higher percentage of RIP β cells compared to cells cultured in HAMC alone (gray) or in neurobasal media alone (white). (C) Cell viability of SCs, as determined by live/dead assay. Viability of SCs cultured in neurobasal media was significantly lower than cells cultured in HAMC-PDGF or HAMC alone. *** $p < 0.001$. (Adopted from Mothe *et al.*²² [with permission from Elsevier]).

to establish a sufficiently high-specific surface, leading to a minimal ligand density to allow efficient binding of a critical number of cells to the scaffold. Therefore, for any scaffold, a critical range of pore sizes exist, which may vary depending on the cell type used and tissue being engineered. Moreover, porous interconnectivity is equally important in the same sense for cells to be able to penetrate the scaffold. To improve cell attachment to the scaffold, other parameters also should be taken into account like stiffness,⁶⁸ cell phenotype,⁶⁹ and nature of ligand.⁷⁰ Scaffolds synthesized from natural extracellular materials (e.g., collagen) naturally possess these ligands in the form of Arg-Gly-Asp (RGD) binding sequences, whereas scaffolds made from synthetic materials may require deliberate incorporation of these ligands through functionalization.^{12,43,71} If these ligands are not present in the polymers themselves, they must be incorporated to be able to have the cells attached. In this framework, research developed a large amount of strategies for scaffold functionalization.⁵ Of note, differences should be considered between two-dimensional (2D) scaffolds, where cells are cultured onto the matrix (superficial functionalization) and 3D hydrogels, where cells are included within a 3D polymeric network (bulk functionalization). In the first case, researchers provide different possibilities to improve cell adhesion onto polyester scaffolds, mainly improving their hydrophilicity.⁷² In particular, polycaprolactone and poly-L-lactic acid-based scaffolds were modified with gelatin,⁷³ RGD peptides,⁷⁴⁻⁷⁶ and collagen I.⁷⁷ Moreover, Lopez-Perez *et al.*⁷⁸ developed the possibility to functionalize the surface of polycaprolactone scaffolds with surface grafting of sulfonic or phosphonic groups through plasma-induced polymerization of vinyl sulfone: the presence of these anionic functional groups can modulate cell adhesion mediated through the adsorbed proteins present in the culture medium. Functionalization of implantable scaffolds (2D) is important to improve their performances, but the topic will become even more fundamental if we consider 3D injectable composites of cells with PEG or polysaccharides.^{79,80} Indeed, material adhesion properties should be improved functionalizing them with RGD,⁸¹⁻⁸⁴ cysteine,⁸⁵ collagen-like proteins,⁸⁶ heparin,^{87,88} laminin,⁸⁹ lysine,⁹⁰ and streptavidin.⁹¹

In several cell therapies, materials should not only guarantee high cell-material adhesion and viability after having been implanted, but also sufficient cell orientation to improve medical success.^{92,93} This is, for instance, the case in nerve regeneration, where modified substrates often provide better environments for neurite outgrowth. Multiwalled carbon nanotube-coated electrospun poly (l-lactic acid-co-caprolactone) nanofibers improved the neurite outgrowth of rat dorsal root ganglia neurons and focal adhesion kinase expression of PC-12 cells.⁹⁴ The high ability of carbon nanotubes to orient cell culture was also studied by Luo *et al.*,⁹⁵ where electrospun cellulose acetate nanofibers were assembled with positively charged chitosan and negatively charged multiwalled carbon nanotubes through a layer-by-layer self-assembly technique.

Another key challenge in tissue engineering is overcoming cell death in the scaffold interior due to the limited diffusion of oxygen and nutrients therein. Odedra *et al.*⁹⁶ hypothesized and demonstrated that immobilizing a gradient of a growth/survival factor from the periphery to the center of a porous

TABLE 1. PRINCIPAL FUNCTIONALIZATION STRATEGIES RELATED TO MATERIAL PROPERTIES

Material properties	Functionalization
Adhesiveness to cells	Carbon nanotubes ^{94,95}
	Collagen ^{48,49,73,77,86}
	Glucagon-like peptide ⁸⁵
	Growth factor ^{79,96,110}
	Heparin ^{55,87,88}
	Laminin ⁸⁹
	Mussel adhesive proteins ^{102,103}
	Polydioxanone ⁵²
	Polylysine ^{53,90}
	Polyphosphazene ⁵⁴
	Resveratrol ¹⁰⁶
	RGD peptide ^{74-76,81-84,97-101}
	Silanol group ¹⁰⁷
Degradability	Sulfonic group ⁷⁸
	Streptavidin ²²
Imaging capacity	Disulfide crosslinkers ⁶²
	RGD peptide ^{11,20,63}
Osteoconductivity	Magnetic nanoparticles ^{38,39}
	Calcium phosphate ³³⁻³⁵
Protein/drug retention and release	Carbon nanotubes ²¹
	Hydroxyapatite ²⁹⁻³²
	Chemical linkers ⁴⁰⁻⁴⁴
	Layer polymers ⁴⁵
	Liposomes ⁴⁶

scaffold would guide endothelial cells into the interior of the scaffold, thus overcoming a necrotic core.

Moreover, functionalized scaffolds, once being cultured with stem cells (SCs), are able to create an environment favorable for their differentiation. In this sense, RGD peptides were used for human mesenchymal SCs,^{11,20,97,98} retinal^{99,100} and neural SCs¹⁰¹ and mussel adhesive peptides for pre-osteoblast cells¹⁰² and human adipose tissue-derived SCs.¹⁰³ Furthermore, streptavidin modification is able to induce neural SC^{22,104,105} differentiation: Mothe *et al.*²² encapsulated neural SCs either in unmodified (hyaluronic acid-methylcellulose [HAMC]) and in functionalized (HAMC-platelet derived growth factor/[PDGF], Fig. 3A) hydrogels for 7 days. HAMC-PDGF (black) significantly increased the percentage of oligodendrocytes and live cells if compared to cells cultured in unmodified HAMC (gray) or in neurobasal media (white) alone (Fig. 3B, C). In bone tissue engineering, polycaprolactone-based scaffolds, were functionalized with resveratrol,¹⁰⁶ silanol, and growth factors¹⁰⁷⁻¹⁰⁹ and when implanted *in vivo* and cultured with adipose-derived SCs, are able to promote the expression of neovascularization mediators, which may help overcome the nutrition deficit in the inner part of the scaffold.¹¹⁰ Diffusion of nutrients is able up to distances of maximum 200 μm .

In summary, in Table 1, the principal functionalization strategies are related to the material properties they are aiming to improve.

Conclusions and Future Directions

The creation of complex tissues and organs is the ultimate goal of tissue engineering. The main aim of this review is to provide information about the methods used for preparing functional scaffolds. Following this approach, modified polymer scaffolds could improve their performances in terms

of mechanical properties, degradation kinetics, cell adhesion, and differentiation with respect to the unmodified ones. Despite recent advances in the development of functional biomaterials, several challenges still remain, including the dynamic cellular microenvironment in the design of scaffolds. Novel scaffolds that are able to allow simultaneous spatial and temporal resolution, being also cell responsive, promise to be a great challenge for the future. In particular, the possibility to modulate different scaffold areas with different properties is the key point that should be addressed. Following this strategy, different cell types can grow together in a unique scaffold creating mutual networks and thereby providing a more biomimetic environment. Engineered polymers can be used as a platform to better mimic the cell niche, allowing for multiple stimuli and many cell types to be explored individually or in combination. Regarding *in vivo* applications, innovative biomaterials should overcome the current problems of cell survival and integration after implantation or injection.

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Disclosure Statement

No competing financial interests exist.

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