



## Review

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**Authors for correspondence:**  
 Marwa Tallawi  
 e-mail: [marwa.tallawi@ww.uni-erlangen.de](mailto:marwa.tallawi@ww.uni-erlangen.de)  
 Aldo R. Boccaccini  
 e-mail: [aldo.boccaccini@ww.uni-erlangen.de](mailto:aldo.boccaccini@ww.uni-erlangen.de)

# Strategies for the chemical and biological functionalization of scaffolds for cardiac tissue engineering: a review

Marwa Tallawi<sup>1</sup>, Elisabetta Rosellini<sup>2</sup>, Niccolotta Barbani<sup>2</sup>,  
 Maria Grazia Cascone<sup>2</sup>, Ranjana Rai<sup>1</sup>, Guillaume Saint-Pierre<sup>3</sup>  
 and Aldo R. Boccaccini<sup>1</sup>

<sup>1</sup>Institute of Biomaterials, Department of Materials Science and Engineering, University of Erlangen-Nuremberg, 91058 Erlangen, Germany

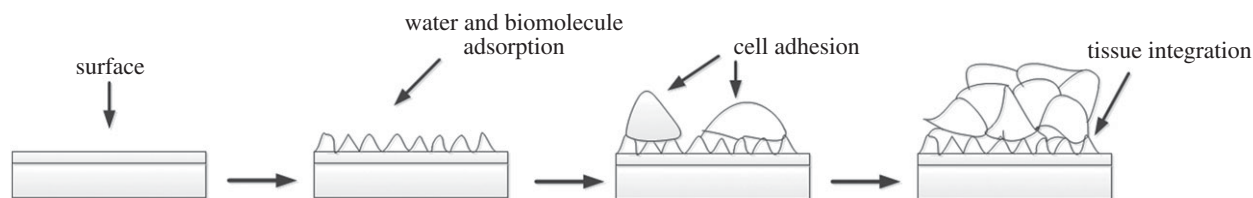
<sup>2</sup>Department of Civil and Industrial Engineering, University of Pisa, Largo Lucio Lazzarino, 56126 Pisa, Italy

<sup>3</sup>Inspiralia, Materials Laboratory, C/Faraday 7, Lab 3.02, Campus de Cantoblanco, Madrid 28049, Spain

The development of biomaterials for cardiac tissue engineering (CTE) is challenging, primarily owing to the requirement of achieving a surface with favourable characteristics that enhances cell attachment and maturation. The biomaterial surface plays a crucial role as it forms the interface between the scaffold (or cardiac patch) and the cells. In the field of CTE, synthetic polymers (polyglycerol sebacate, polyethylene glycol, polyglycolic acid, poly-L-lactide, polyvinyl alcohol, polycaprolactone, polyurethanes and poly(N-isopropylacrylamide)) have been proven to exhibit suitable biodegradable and mechanical properties. Despite the fact that they show the required biocompatible behaviour, most synthetic polymers exhibit poor cell attachment capability. These synthetic polymers are mostly hydrophobic and lack cell recognition sites, limiting their application. Therefore, biofunctionalization of these biomaterials to enhance cell attachment and cell material interaction is being widely investigated. There are numerous approaches for functionalizing a material, which can be classified as mechanical, physical, chemical and biological. In this review, recent studies reported in the literature to functionalize scaffolds in the context of CTE, are discussed. Surface, morphological, chemical and biological modifications are introduced and the results of novel promising strategies and techniques are discussed.

## 1. Introduction

Myocardial infarction (MI) or heart attack occurs as a consequence of abrupt blocking of one or more of the blood vessels supplying the heart (coronary arteries). In an adult heart, the damaged tissue does not repair spontaneously, and scar tissue is formed instead, as the mature contracting cardiac cells (cardiomyocytes) have limited capacity to proliferate [1]. When occlusions in the coronary arteries occur, a sudden decrease in nutrient and oxygen supply to the portion of the heart muscle supplied by the occluded arteries is observed. Therefore, if the flow of blood is not rapidly restored within the affected area of the heart muscle, permanent cell death occurs. The scar tissue replacing the heart muscle cannot conduct electrical or mechanical stimuli thus leading to a reduction in the pumping efficiency of the heart's main pumping chambers: the ventricles. Owing to the reduced cardiac output, several compensatory mechanisms are initiated which at first stabilize the damaged heart and maintain the cardiac output, such as: vasoconstriction, leading to an increase in blood pressure in order to return more blood to the heart; increase in heart rate; hypertrophy or enlargement of the heart muscles for greater pumping force and left ventricles (LVs) enlargement or dilation, so that more blood can fill into the heart. However, extra complications may affect the weakened heart leading to further deterioration of the cardiac function, ultimately reaching heart failure. Thus, the heart fails to pump enough blood around the body at the right



**Figure 1.** The interaction of cells with biomaterials is governed by the surface properties of the biomaterial.

**Table 1.** Summary of pros and cons of both natural and synthetic materials.

	advantages	disadvantages
natural	<ul style="list-style-type: none"> <li>—biological origin</li> <li>—biocompatibility</li> <li>—presence of adhesive sequences that facilitate cell adhesion and cell differentiation</li> </ul>	<ul style="list-style-type: none"> <li>—inadequate mechanical properties</li> <li>—rapid degradation</li> <li>—variable properties depending on extraction procedure</li> <li>—risk of contamination</li> <li>—high production costs</li> </ul>
synthetic	<ul style="list-style-type: none"> <li>—suitable mechanical properties</li> <li>—highly reproducible chemical and mechanical properties</li> <li>—low production costs</li> <li>—low immune response</li> </ul>	<ul style="list-style-type: none"> <li>—low biocompatibility</li> <li>—risk of biodegradation side effects</li> </ul>

pressure and as a result body functions fail. For example, kidneys functions fail causing retention of fluids and salts, therefore, fluid builds up in the arms, legs, ankles, feet, lungs and/or other organs and the body becomes congested [2]. The only therapeutic option available for patients with end-stage heart failure is to undergo heart transplantation or the use of mechanical ventricular assist devices (VADs) [3]. Unfortunately, many patients die while being on a waiting list, owing to the limited availability of organ donors and to the limitations of VADs, such as peri-operative and post-operative bleeding complications, thrombotic events and infection, occasional thrombus in the circuit, failure of the electrical motor and haemolysis owing to non-laminar blood flow. In addition, it has to be considered that not all patients have a body size that allows the device to be implanted into the chest cavity. Moreover, the majority of available devices are somewhat bulky, making normal patient movement difficult. Finally, the high costs must be taken into account.

Lately, replacement of the defective myocardial tissue with fetal or neonatal cardiomyocytes, skeletal myoblasts, embryonic stem cells, bone marrow-derived mesenchymal and haematopoietic stem cells and adipose-derived stem cells [4–7] has been proposed. Currently, the injection of cells in suspension, either into the circulating blood or directly into the myocardium, is the preferred method. However, the efficiency of these methods for cell delivery may be low owing to a significant loss of cells [8]. Therefore, alternative methods for cell delivery are being investigated to open new approaches for cardiac tissue engineering (CTE) [9]. The goal of CTE is thus to repair or replace damaged and injured heart tissue.

In general terms, a tissue engineering/regenerative medicine (TERM) approach consists of cell seeding on a scaffold, followed by *in vitro* tissue maturation and construct implantation in the host environment. However, alternative TERM approaches exist, lacking some elements or steps of the basic

TERM paradigm. Among them, the most commonly implemented approaches in cardiac TERM are (i) cell-seeded' (*in vitro* maturation); (ii) cell injection (no scaffold and no *in vitro* maturation); and (iii) scaffolds that attract endogenous cells (no cells and *in vitro* maturation) [10]. All these approaches involve the design of a pre-formed or injectable scaffold, made using a biomaterial, able to properly interact with seeded or endogenous recruited cells. Therefore, surface functionalization can be exploited both in seeded and unseeded scaffolds.

The development of suitable biodegradable biomaterials as candidates for CTE is an active field of research [7,11]. Different fabrication methods are being continuously studied to develop three-dimensional scaffolds with a specific shape, thickness, mechanical strength and porosity to promote cell growth [7,12–14]. The specific physical properties of CTE constructs that are crucial for the success of this approach are biocompatibility, ability to foster cells, tailored degradation rate, permeability (for biomolecule diffusion), suitable mechanical properties, contractility and electrophysiological stability [15,16]. Both natural (gelatin [17], alginate [18], collagen type I [19–21] and fibrin glue [22,23]) and synthetic polymers (polyglycerol sebacate (PGS), polyethylene glycol (PEG) [24,25], polyglycolic acid (PGA), poly-L-lactide (PLA), poly(lactide-co-glycolide) (PLGA), polyvinyl alcohol (PVA), polycaprolactone, polyurethanes and poly(N-isopropylacrylamide) are being considered to develop cardiac patches. For both classes, pros and cons are summarized in table 1.

Despite various advancements made, incomplete understanding of the interactions between biomaterials and biological systems still limits the advancement of CTE in clinical settings. Indeed, specific and complex mechanisms govern the reactions that occur at the interface between the biomaterial and the cellular environment. Schematically, figure 1 describes the initial interactions between biomaterials and cells. These interactions are governed by surface energy, chemical composition, stiffness, as

**Table 2.** Techniques used to modify biomaterials (post-polymerization) to obtain better interaction with the cells.

modification methods	modified biomaterial properties
morphological modifications	coatings containing pores to enhance tissue ingrowth
	topography induced (grooves, morphology, roughness)
	fibrous materials
	porous materials
chemical modifications	glow discharge to increase surface energy and tissue adhesion
	cross-linked polymeric surfaces to decrease surface permeability and increase surface hardness
	plasma treatment with reactive gases to create new functional groups on polymer surface
	grafting macromolecules such as polyethylene glycol to reduce protein adsorption and cell adhesion
	functional groups used to produce positively or negatively charged surface
biological modifications	immobilization of biomolecules to promote cell adhesion and growth
	heparin, heparin sulfate binding peptides
	natural ECM proteins (fibronectin, laminin, collagen)
	peptide sequences (RGD)
	growth factors

well as roughness and topography of the biomaterial surface in contact with the biological environment [26].

Over the years, surface modification techniques have been adopted to enhance biocompatibility, haemocompatibility [27,28] and to promote vascularization [29] of scaffolds. The most promising synthetic materials investigated for CTE are polyurethanes [30,31] and polyesters [32,33]. However, these polymers lack cell recognition sites. Therefore, it is crucial to introduce functional groups on the surface of the scaffold that will function as cell recognition sites or may act as focal points for additional modification with bioactive molecules [34,35]. Moreover, surface modification can be useful to prevent thrombotic deposition and occlusion triggered by the activation of the coagulation cascade and platelets. Biomolecular modifications should lead to promising bioactive materials with the ability to control interactions with cell receptors (e.g. integrins) thus enhancing cell proliferation, differentiation, production and organization of the extracellular matrix (ECM).

There are basically two strategies for the biofunctionalization of polymers. The first one is pre-polymerization functionalization via polymerization of functional monomers [36] (e.g. alcohols, carboxylic acids, amines, acrylates). This procedure provides, for example, functional polyesters or polyurethanes with a defined chemical structure that allow for further modification following polymerization [37]. The second strategy is post-polymerization functionalization, which is the modification of the polymer after the polymerization process [35]. Post-polymerization techniques might be specific, targeting functional groups present in the polymer via carbodiimide or UV-initiated radical coupling, or non-specific, using azide- or glutaraldehyde-based couplings. A disadvantage of the non-specific covalent functionalization method is that it may result in the destruction of biomolecule bioactivity and/or can involve side reactions such as hydrolysis, chain-degradation or cross-linking [38].

This review focuses on the post-polymerization functionalization of scaffolds, covering different methods and techniques being investigated for application in CTE. In particular, chemical and biological modifications are reviewed in detail,

discussing relevant examples. Table 2 presents a summary of techniques for the post-polymerization functionalization proposed or being investigated to modify biomaterials to induce a desired cell response.

## 2. Bioactive molecules

Bioactive molecules can promote angiogenesis and engrafting by improving viability and survival of the grafted engineered tissue [39,40]. Scaffolds can be incorporated with bioactive molecules, such as chemicals in the form of ionic dissolution products, drugs, peptide sequences and/or growth factors (GFs). Bioactive molecules, which can be discharged from the scaffold by controlled release, diffusion or network breakdown, are capable of interacting with cells when they are released. The cells produce additional GFs that, in turn, stimulate multiple generations of growing cells to self-assemble into tissues *in situ* [41].

The bioactive molecules can be whole protein molecules such as ECM proteins or short peptide sequences (cell binding domains) isolated from ECM proteins. Short peptides are generally preferable to whole proteins, owing to the tendency of whole proteins to randomly fold, so that the receptor binding domains are not always available. Moreover, during the modification process, short peptides tend to be more stable. Short peptides can be mass-produced in laboratories. Arg-Gly-Asp (RGD), which is derived from fibronectin [42,43], is the most common short peptide sequence used.

In the next paragraphs, the attention will be focused on biomolecules already tested for CTE or that can reasonably find application in this field.

### 2.1. Extracellular matrix proteins and short peptide sequences

#### 2.1.1. Collagen

Collagen is one of the most popular bioactive coatings used [44,45]. Collagen provides a biomimetic environment for cell

growth [46–48], as collagen has a fibrous structure and appropriate mechanical properties [49]. In addition, it is highly biocompatible and biodegradable [47,49]. Collagen type I provides structural support (stiffness and resistance to deformation). Collagen type III plays an important role by linking contractile elements of adjacent myocytes together [50].

Collagen is usually incorporated onto polymer surfaces by immersion of the scaffold into a solution of the protein, to obtain a layer of collagen physically attached to the polymer surface. Park *et al.* [51] seeded neonatal rat heart cells suspended in Matrigel (a protein mixture of laminin, entactin, collagen and heparan sulfate (HS) proteoglycans) into a composite scaffold made of poly(DL-lactide-co-caprolactone), poly(DL-lactide-co-glycolide) (PLGA) and type I collagen at a density of  $1.35 \times 10^8$  cells  $\text{cm}^{-3}$  and cultivated them in cartridges perfused with culture medium for 8 days; a collagen sponge (Ultrafoam) and a PLGA sponge served as controls. Results showed improved presence of cardiac markers and contractile properties in the composite scaffolds in comparison with both controls [51]. Collagen sponge exhibited a fast degradation rate and poor mechanical properties, even over short culture periods, whereas the PLGA sponge was hydrophobic and lacked cell attachment. The composite scaffold had several advantages, including a surface that supported cell attachment, a structure that allowed seeding and nourishment of cells at higher densities, mechanical properties that supported construct contractions and tunable degradation rate.

In a related study, Bai *et al.* [52] fabricated novel alginate/collagen composite microbeads encapsulating neonatal rat cardiomyocytes cells. The composite microbeads showed the proliferation of cardiac cells, the formation of interconnected multilayer heart-like tissues, the presence of well-organized and dense cell structures, and the spontaneous synchronized contractility of the grafts after two weeks in culture (at a rate of 20–30 beats  $\text{min}^{-1}$ ).

### 2.1.2. Fibronectin

Fibronectin is a high molecular weight glycoprotein of the ECM crucial for cell adhesion, spreading and migration. Previous studies have shown that ECM proteins influence cardiac cell morphology and physiology [53]. Hidalgo-Bastida *et al.* showed that fibronectin was the optimal ECM protein to enhance cell adhesion on poly(1,8-octanediol-co-citric acid) (POC) film. This could possibly be related to the fact that a higher level of fibronectin expression is associated with cell proliferation and migration at the embryonic stage of heart development, whereas during heart growth, fibronectin levels decrease, and laminin and collagen levels increase [53,54]. In another study, fibronectin was shown to be the most appropriate component, in comparison with laminin, gelatin and collagen I, to maintain the highest cell adhesion and proliferation rate [55].

### 2.1.3. Laminin

Laminin is a glycoprotein of the basal lamina and it has been demonstrated to play an important role in supporting and stimulating migration, adhesion, growth and differentiation of various cell types [56–60]. Because cardiac muscle is made of highly aligned fibres, lanes of laminin have been microcontact-printed onto non-adhesive surfaces to direct the organization of cultured cardiomyocytes to more closely

resemble that found *in vivo* [61]. Adherent cardiomyocytes responded to the spatial constraints by forming elongated, rod-shaped cells whose myofibrils aligned parallel to the laminin lanes [61]. With the final aim of better matching the mechanical properties of the native tissue, a similar study but using a different substrate was carried out. Similar cardiomyocyte patterns were achieved on biodegradable polyurethane (PU). PU scaffolds are known for their elastomeric behaviour, which is a desired property for the regeneration of myocardial tissue [62]. Thin films based on PU synthesized from poly( $\epsilon$ -caprolactone) diol, 1,4-butandisocyanate and L-lysine ethyl ester dihydrochloride chain extender were patterned by microcontact printing of laminin moieties and seeded with neonatal rat cardiomyocytes. Seeded cells produced a multi-layered, dense and highly aligned tissue, with the ability to contract within the thin films [63]. Alperin *et al.* [64] seeded embryonic stem cell derived cardiomyocytes on PU films coated with laminin or collagen IV and the coated surfaces showed higher numbers of contracting cells compared with unfunctionalized PU controls, thus showing the potential of PU elastomers for the repair of damaged heart. POC scaffolds coated with fibronectin, collagen and laminin showed better proliferation of the mouse cardiac muscle cell line HL-1 than untreated scaffolds. In particular, fibronectin-coated films showed the highest proliferation rate, compared with collagen and laminin coating [53]. In a related study, LaNasa *et al.* [65] seeded neonatal rat cardiomyocytes on flexible two-dimensional hydrogels consisting of covalently bounded collagen I, laminin and cell adhesive oligopeptides (RGD). Cells were sensitive to the different modified substrates: whole proteins, collagen and laminin, were effective in promoting cardiomyocyte interaction with hydrogels and cardiomyocyte maturation, whereas RGD did not provide adequate ECM cues for cardiomyocytes.

### 2.1.4. Nephronectin

Nephronectin (Npnt) is an ECM protein investigated for use as a natural adhesive material for CTE. It contains an N-terminal signal peptide followed by EGF-like repeats, an RGD sequence and a C-terminal MAM domain [66]. Nephronectin is expressed in cardiomyocytes throughout the heart and it is secreted into the cardiac jelly, which is in direct contact with cardiomyocytes and endocardial cells [67]. Neonatal rat cardiomyocytes were seeded on nephronectin-coated glass coverslips, demonstrating the excellent properties of nephronectin for cardiomyocyte adhesion and function [68]. Nephronectin enhanced cell-to-cell communication, sarcomere maturation and alignment and synchronized contractions.

Other studies have shown that all major cellular components of the heart, such as cardiomyocytes, cardiac fibroblasts, vascular smooth muscle cells and endothelial cells, can attach and spread on Npnt. Cardiomyocytes have been shown to attach and spread markedly faster on Npnt than on gelatin [67]. This was in part due to the presence of the integrin-binding RGD sequence in Npnt [69]. In addition, cardiomyocytes grown on Npnt exhibited a matured contractile apparatus with well-aligned sarcomeres. Connexin 43 expression data suggest, moreover, that the cells were electrically coupled. Consequently, cardiomyocytes on Npnt contracted synchronously and exhibited a higher beating frequency than cardiomyocytes on gelatin or fibronectin. Npnt was shown to maintain differentiation, promote sarcomere maturation as well as electrical signal propagation and consequently cardiomyocyte contractility [70–73].

### 2.1.5. Gelatin

Gelatin is derived from collagen by denaturation of the triple-helix structure. Gelatin is highly biodegradable and biocompatible, in addition it exhibits excellent non-antigenicity, antithrombogenicity and it is cost effective [74–76].

Miskon *et al.* [77] reported that a gelatin-coated surface has a high ability not only to maintain the cardiac phenotype, but also to enhance cardiac differentiation. In another study, gelatin was demonstrated to maintain most of the cellular characteristics of cardiomyocytes in comparison with collagen I, laminin and fibronectin [55].

### 2.1.6. Short peptide sequences

RGD peptide is one of the most physiologically ubiquitous binding motifs commonly used, which is found in many natural adhesive proteins such as fibronectin, vitronectin, laminin and collagen type I [78]. The intracellular cytoskeleton is linked by cellular integrins to the ECM via RGD. Many studies with different cell types cultured on synthetic scaffolds immobilized with RGD demonstrated good adhesion and often comparable results to those obtained using entire proteins [79].

Silicone surfaces modified with both RGD and YIGSR have shown the same degree of adhesion obtained with native proteins (fibronectin and laminin, respectively) [80]. Furthermore, RGD-modified chitosan hydrogels were developed and C2C12 myoblasts proliferated and differentiated on these hydrogels and not on the unmodified ones [81]. In another study, three-dimensional alginate scaffolds modified with RGD peptide promoted neonatal rat cardiac fibroblasts viability *in vitro* [82]. Moreover, it was observed that tissue culture surfaces coated with RGD enhanced cardiomyocyte contractility and doubled cell viability compared with fibronectin-coated wells [83]. In a comparative study, collagen I, laminin and RGD were covalently bound to two-dimensional flexible hydrogels: RGD-modified hydrogels demonstrated good bioactivity when tested with skeletal myoblasts; however, when neonatal rat myocytes were cultured on RGD-modified hydrogels, the myocytes were self-associated and formed aggregates that exhibited a disorganized cytoarchitecture, suggesting that RGD does not provide adequate ECM cues for cardiomyocytes [65]. In another study, alginate was immobilized with cell adhesion peptides, containing the sequences RGD and YIGSR, or with a non-specific peptide (RGE) and implanted 7 days after infarction [84]. After 60 days, peptide-modified alginate reduced the therapeutic effects when compared with unmodified alginate, as revealed by the extent of scar thickness and by LV dilatation and function [84]. These findings were explained by structural changes occurring in alginate following covalent attachment of the peptides. The apparent viscosity of the cross-linked alginate, when modified with RGD/YIGSR or RGE peptides, increased by four- to sevenfold. The distribution of the biomaterial in the infarct region depends on solution viscosity, thus the peptide-modified biomaterial covered a smaller scar area in comparison with unmodified alginate. The influence of surface modification on myocardial microenvironment and the effect on myocardial function was also investigated *in vitro* and *in vivo* (in a rat model of ischaemic cardiomyopathy) [85]. *In vitro*, RGD-modified alginate improved the proliferation and adhesion of human umbilical vein endothelial cells (HUVECs) when compared with non-modified alginate [85]. Results also demonstrated that both modified and unmodified

alginate improved heart function when injected into the infarct area of rats five weeks post-MI. RGD-modified alginate and unmodified alginate increased the arteriole density; however, the greatest angiogenic response was shown by the RGD-modified alginate [85].

The RGD motif has been also shown to enhance cell adhesion and contractility. For example, RGD immobilized on collagen scaffolds has been shown to improve cardiomyocyte viability, differentiation and contractile performance [86]. *In vitro* studies of encapsulated human mesenchymal stem cells (hMSCs) in RGD-modified alginate microspheres demonstrated that RGD-modified alginate improved cell attachment, growth and increased angiogenic growth factor expression [87]. Surface modification combined with microencapsulation technique was also able to maintain the LV geometry, preserve LV function, increase angiogenesis and improve cell survival. Moreover, immobilization of macroporous alginate scaffolds with RGD peptide has been proven to be a key parameter in promoting the formation of functional cardiac muscle tissue and in enhancing the preservation of the regenerated tissue in culture [88]. Neonatal rat cardiac cells were cultured onto unmodified, RGD-modified and heparin-binding peptide-RGD-modified (HBP/RGD) alginate porous scaffolds. The HBP/RGD-modified scaffolds revealed the best features of a functional cardiac muscle tissue, demonstrating isotropic myofibre arrangement [89]. Cells spread extensively on such RGD-modified scaffolds, with the F-actin fibres well stretched and showing many focal adhesion points. The strong adhesion and spreading inhibited the cell organization process into the myofibril structure. Thus, the introduction of HBP was shown to alter the effect of RGD, reducing cell spreading and promoting proper cell organization [89]. In addition to RGD tripeptide, a great number of longer peptides containing the RGD sequence have been investigated. In particular, GRGDS and GRGDSP peptides were shown to promote the adhesion of several cell types, including myoblasts, and they were found to stimulate integrins that are relevant in early cardiac development [90,91].

In related studies, *in vitro* cell culture tests on three-dimensional matrix metalloproteinase (MMP)-sensitive PEG-based hydrogels, modified with different quantities of GRGDSP peptide have been performed using P19 embryonal carcinoma cells, as a model of pluripotent cardioprogenitors [92]. It was demonstrated that indicators of cardiac maturation were promoted by GRGDSP-mediated stimulation of integrins that are relevant in early cardiac development, with a sixfold increased amount of myosin heavy chain-positive cells when compared with cells in suspension [92].

## 2.2. Growth factors

GFs are small, soluble, natural cell-signalling polypeptides, which are secreted by cells and can stimulate specific activities in a biological environment such as cell growth, proliferation and differentiation. Usually, GFs have slow diffusion and short half-life and as a result they act locally. Typical GFs are listed in table 3. GFs promote myocardial healing and repair at the infarct site [103]. Examples of GFs are granulocyte colony-stimulating factor (G-CSF) [104,105], stromal-derived growth factor (SDF-1) [106], leukaemia inhibitory factor [107], insulin-like growth factor (IGF-1) [108], vascular endothelial growth factor (VEGF) and erythropoietin (EPO) [109]. GFs-based regenerative therapy is a promising therapeutic strategy for MI.

**Table 3.** Different GFs and their functions in cardiac tissue engineering.

growth factor	function	critical issues	reference
VEGF	migration, proliferation and survival of endothelial cells; endothelial capillary formation	rapid degradation due to short half-life; excessive amounts cause vascular leakage	[93–96]
bFGF	heparin-binding protein; proliferation of endothelial cells, smooth muscle cells; endothelial capillary formation	rapid diffusion, requiring controlled release; mitogen for a wide variety of cell types	[95,97,98]
HGF	mitogen for hepatocytes and other cell types; growth of endothelial cells	short half-life, rapid diffusion; large amounts of protein required for response	[99]
PDGF	mitogen for connective tissue cells, released from platelets; recruitment of smooth muscle cells to endothelial linings; vessel maturation	vessel destabilization for high levels; increased activity linked with several diseases	[100–102]

### 2.2.1. Vascular endothelial growth factor

VEGF is a dimeric glycoprotein, secreted and synthesized by many cell types. VEGF binds to the ECM through indirect interaction via linker molecules (heparin sulfate proteoglycans), so that ECM regulates the bioavailability of VEGF. As a result of hypoxic or inflammatory stimuli, VEGF is secreted by cardiomyocytes [110–112], in order to regulate the formation and maturation of blood vessels, acting mainly on vascular endothelial cells [113]. VEGF promotes angiogenesis and coronary collateral formation in infarcted myocardium, inducing the growth of new blood vessels, by promoting the dissolution of existing blood vessels, the migration and proliferation of endothelial cells and the formation of tubes from endothelial cells [114].

Ozawa *et al.* studied the effect of VEGF dosage on the morphology and function of newly formed blood vessels in adult mice muscles implanted with retro virally transduced myoblasts that constitutively express VEGF [115]. The study showed that high levels of VEGF may lead to the growth of abnormal blood vessels and haemangiomas, whereas when VEGF concentration is maintained between low-to-medium levels, it leads to normal angiogenesis. Thus, this issue should be taken into consideration while designing a cardiac tissue construct incorporating VEGF.

### 2.2.2. Granulocyte colony-stimulating factor

G-CSF is a hormone crucial in regulating the proliferation, differentiation and survival of myeloid progenitor cells. Moreover, G-CSF showed a significant increase in the release of haematopoietic stem cells into the peripheral blood circulation. It has been recently demonstrated that G-CSF stimulates healing and repairing, improving cardiac function [104], and reduces mortality after acute MI [116,117]. The mechanism by which G-CSF alters cardiac dysfunction, however, is not fully understood. One possible rationale is that G-CSF regenerates cardiac myocytes and blood vessels through mobilization of bone marrow stem cells [117].

### 2.2.3. Erythropoietin

EPO is a hormone of importance for erythrocyte differentiation and survival. EPO has the ability to maintain vascular autoregulation and reduces primary (apoptosis)

and secondary (inflammation) causes of cell death. Calvillo *et al.* [109] showed that EPO decreased cardiac myocyte loss by 50% in a rat model of infarction, which is sufficient to normalize haemodynamic function.

### 2.2.4. Insulin-like growth factor

IGF-1 is a GF that can delay cardiomyocyte ageing and death [118]. IGF-1 in an injured muscle can enhance cell homing, healing and regeneration [119].

GFs have been supplied to cells via different methods, including addition to the culture medium, loading in controlled release systems and covalent immobilization onto the biomaterial. Immobilization of GFs onto biomaterials protects them against cellular inactivation and digestion, sustaining their activity [120]. Moreover, GFs immobilization overcomes diffusional limitation of soluble GFs [121].

One of the classical means to functionalize biomaterials is to incorporate GFs, such as VEGF, basic fibroblast growth factor (bFGF) and many others, as summarized in table 4 [93,94,101,139,140]. Some GFs act as angiogenic growth factors (AGFs) which provide an efficient means for stimulating localized vessel recruitment to the scaffold. By improving the vascularization of transplanted engineered tissues, AGFs improve cell survival and function. The local delivery of GFs prevents serious unfavourable effects such as hyper permeability, oedema, hypotension and accelerated atherosclerosis [141]. It has been demonstrated that the incorporation of VEGF in CTE scaffolds facilitates blood vessel growth within the scaffold [142].

An overview of the different methods used for GFs inclusion in scaffolds will be provided in §3.2.

## 2.3. Synthetic chemicals

In addition to protein-based cytokines and GFs, synthetic chemical compounds have also demonstrated ability to promote cardiomyogenic differentiation *in vitro*. Synthetic chemicals present some advantages with respect to protein-based cytokines and GFs; they have a longer half-life in solution, permitting *in vitro* cell culture to be prolonged over several days or even weeks, and, being manufactured by chemical reactions, are more structurally and chemically defined. However, it is known that they may negatively affect cell genomic stability, limiting their potential application in clinical practice.

**Table 4.** Summary of cardiac scaffolds with immobilization of GFs developed in the last years.

incorporation method	polymer	angiogenic factor	method of preparation	model	result	reference
porous composite scaffold loaded with microspheres	PLGA microspheres alginate	bFGF	spray phase inversion method	myocardial implantation in rat	controlled release of bFGF promoted vascularization	[102]
composite scaffold	poly(ether)urethane—poly-(di-methylsiloxane) fibrin	VEGF bFGF	double injection extrusion	subcutaneous implant and unilateral hindlimb ischaemia model in rat	enhanced angiogenesis	[122]
hollow fibre	cellulose acetate	VEGF	precipitation method	subcutaneous implant model in mice		[123]
covalently immobilized GF	collagen	SIP VEGF Ang-1	commercial scaffold (Ultrafoam collagen sponge)	chicken chorioallantoin membrane angiogenesis assay	enhanced angiogenesis compared with soluble VEGF and Ang-1	[124]
polymeric injectable carrier	poly(trimethylene carbonate)	VEGF		subcutaneous injection in rat	local GF delivery	[125]
dual-layered scaffold	poly(trimethylene carbonate)	VEGF HGF	cross-linking	<i>in vitro</i> HAECS	release of dual GFs at similar and controlled rates	[126]
fibrous membranes	dextran PLGA	VEGF	coaxial electrospinning	<i>in vitro</i>	promoted cell proliferation	[127]
alginate hydrogel	alginate	VEGF	cross-linking	Hindlimb ischaemia in mice		[128]
hydrogel with surface cross-linked heparin	star-PEG	VEGF FGF-2	cross-linking	HUVECs <i>in vitro</i> and chicken chorioallantoin membrane angiogenesis assay	enhanced angiogenic activity compared with signal factor immobilization	[129,130]
covalently immobilized VEGF	collagen	VEGF	commercial scaffold (Ultrafoam collagen sponge)	replacement after resection of the right ventricle	promoted tissue formation	[131]
biometric hydrogel with adhesive peptide sequence RGD	PEG diacrylate	VEGF	photopolymerization	HUVECs and hMECs <i>in vitro</i>	promoted EC proliferation, migration and viability	[132]
surface cross-linked heparin	polycaprolactone	VEGF	solvent casting particulate leaching	subcutaneous implant model in mice	improved VEGF efficacy	[133]
temperature-sensitive injectable hydrogels	PVL-b-PEG-b-PVL	VEGF	metal free cationic method	myocardial implantation in rat	stabilization of the infarct	[134]

(Continued.)

**Table 4.** (Continued.)

incorporation method	polymer	angiogenic factor	method of preparation	model	result	reference
collagen patch	collagen	VEGF		myocardial implantation in rat	improved LV function enhanced vascularization	[135]
	PEG	EPO		rat model	improvement in fractional shortening LV end diastolic diameter LV end systolic diameter	[136]
	poly( <i>N</i> -isopropylacrylamide-co-propylacrylic acid-co-butyl acrylate) (p(NIPAAm-co-PAA-co-BA))	bFGF		rat model	increase in vessel density and fractional shortening	[137]
fibrous membranes	PLLA	G-CSF	electrospinning	C2C12 <i>in vitro</i>	cell elongation and appearance of cellular junctions	[138]

Among the synthetic chemicals that are known to promote differentiation towards the cardiac phenotype there are dimethyl sulfoxide (DMSO), all-trans retinoic acid (RA), dynorphin B, ascorbic acid and 5-aza-2'-deoxycytidine (5-aza-dC). 5-aza-dC is a synthetic nucleoside that is commonly used as an inhibitor of DNA methylation. It has been demonstrated that it is a potent inducer of cardiomyogenic differentiation in both embryonic [143] and adult stem cells, in particular bone marrow-derived mesenchymal stem cells [144–148].

Both ascorbic acid (vitamin C) and retinoic acid (derivative of vitamin A) have been shown to promote cardiomyogenic differentiation of embryonic stem cells [149,150]. DMSO is a commonly used cryoprotectant but it has also been shown to induce cardiomyogenic differentiation in both embryonic stem cells [151] and embryonal carcinoma cells [152,153]. Additionally, the cardiomyogenic differentiation of embryonal carcinoma cells has been shown to be stimulated by dynorphin B, which is a naturally occurring  $\kappa$ -opioid [151].

### 3. Functionalization strategies

#### 3.1. Surface chemical modification

Cell adhesion is affected by surface hydrophilicity, surface charge density, surface micro-morphology, free energy and specific chemical groups present on the surface of the scaffold. Influencing cell spreading and signalling, material surface properties regulate cell growth, migration, differentiation, synthesis of ECM components and tissue morphogenesis. Given that surface chemistry is crucial for the biocompatibility of the scaffold, specific surface modifications are requested to inhibit unfavourable effects and to enhance specific biological responses. Different surface modification methods can be exploited to change surface property or to add functional groups to be used for biomolecule grafting (figure 2).

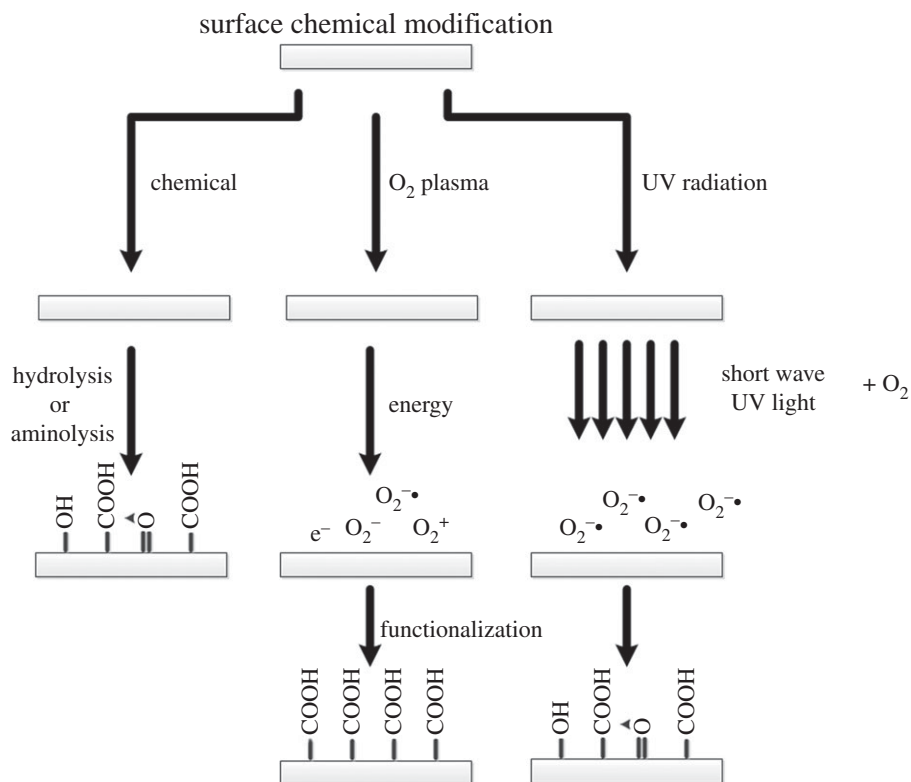
##### 3.1.1. Chemical methods

*Chemical modification* plays a key role in the design of functional biosurfaces by chemical grafting of functional groups (acetylation, fluorination, silanization, incorporation of sulfonate groups) or by modifications of existing functional groups (oxidation, reduction) [12–14].

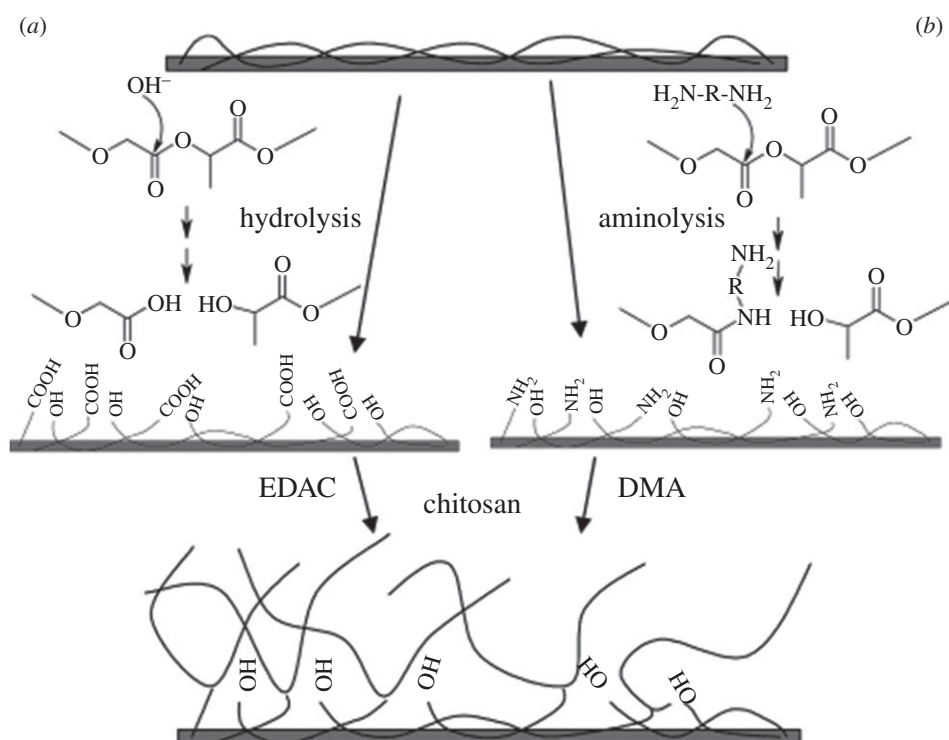
Numerous studies have demonstrated that chemical modifications enhance the cytocompatibility of polymers as presented in this section. In one study, the influence of NaOH treatment on fibroblast compatibility was investigated. Cells cultured on untreated films for 6 and 24 h showed more rounded morphology compared with that observed on treated films, on which cells assumed a more stretched morphology. It has been demonstrated that biodegradable polymer surfaces can be modified for better cell attachment in an alkaline solution to provide hydrophilic, rough surfaces [154,155]. Biodegradable aliphatic polyesters degrade in water, owing to scission of the main ester bonds. Degradation results in the generation of carboxylic acid and hydroxyl groups at the incised chain ends.

In addition, surface chemical treatments are performed to introduce functional groups on the polymer surface for a subsequent modification of biomaterial surfaces with biomolecules. In one study, two wet techniques were used to introduce carboxylic acid by hydrolysis and primary and





**Figure 2.** Schematic diagram showing three different surface chemical modification methods that can be exploited to modify the surface properties to add functional groups to the surface.



**Figure 3.** Schematic diagram showing two methods for the functionalization of PLGA surface, first activated (a) by hydrolysis (b) aminolysis and then further functionalized by covalently binding chitosan using EDAC or DMA, respectively. Reproduced with permission from ref. [156].

secondary amine groups using aminolysis, on PLGA surfaces [156] (figure 3). For the hydrolysis process, films were immersed in a suitable concentration of aqueous sodium hydroxide (NaOH) for a desired period of time [157–159]. For aminolysis, films were immersed in various concentrations of ethylenediamine (ED) or *N*-aminoethyl-1,3-propanediamine (AEPDA) in either water or isopropyl alcohol for a desired period of time [156,160,161].

Hydrolysis is a simple and frequently used method; however, it is pH dependent and might lead to unwanted degradation of the polymer surface. Moreover, carboxylic acid groups first need to be ‘activated’ by turning the acid (anhydride, acid halide) into a more reactive derivative or by using a coupling agent. If a wrong agent is used, it can lead to racemization. Aminolysis is a very simple reaction, which however could produce a salt of the organic acid

and base. High temperatures (more than 200°C) could be used to overcome salt formation, but the polymer could decompose at these elevated temperatures.

### 3.1.2. Photo-induced grafting methods

Photo-induced grafting and photo-induced polymerization are well-established low-cost methods for surface modification [162]. These are modification methods with mild reaction conditions and local surface chemistry modification, without affecting the bulk polymer properties. UV-induced photo polymerization has been used to modify PLLA increasing the hydrophilicity of its surface, by grafting a single monomer or a combination of two hydrophilic monomers (vinyl acetate, acrylic acid and acrylamide) [163]. When copolymerized with vinyl acetate or acrylic acid, acrylamide contributes to the hydrophilicity of the polymer surface. The contact angle of the modified surface varied with the feed composition [163]. Other functional groups such as hydroxyl, carboxyl and amide groups, have also been introduced onto PLLA surfaces, using the photo-induced grafting method, by grafting hydroxyethyl methacrylate, methacrylic acid (MAA) or acrylamide, respectively [164].

### 3.1.3. Plasma grafting and plasma treatment

Plasma grafting and plasma treatment are effective methods for surface modification.

Plasma strongly interacts with polymer surfaces leading to chemical and physical modifications of the biomaterial surface via electrons, ions, radicals and neutral molecules. Cell culture devices such as Petri dishes, microcarriers and membranes are modified to enhance cell adhesion and growth using plasma treatment. It has been demonstrated that plasma treatment generally acts locally on the surface and it does not alter bulk polymer properties [165].

Moreover, if plasma treatment is applied using non-polymerizing gases, functional groups on the biomaterial surface are generated such as amine or carboxyl groups. For example, a hydrophobic surface was made hydrophilic by oxygen plasma treatment, whereas a hydrophilic surface was modified into a hydrophobic one by using tetrafluoromethane (CF<sub>4</sub>) plasma [166]. In another study, a PLGA surface was modified using oxygen treatment [167]. Analysis showed that contact angles of plasma-treated samples decreased from 78° to 45° after 2 min treatment, meaning that the hydrophilicity of PLGA increased after oxygen plasma modification. The treatment also resulted in increased surface roughness, owing to the formation of peaks and valleys. In addition, cell culture tests performed on the treated PLGA surface showed enhanced attachment of mouse NIH 3T3 fibroblasts [167].

Plasma grafting is a surface graft polymerization, covalently binding functional groups to the polymer surface. It allows the modification of the polymer surface, through a choice of different monomers, to obtain desired properties. Bulk properties of the polymer are not altered through the grafting of high-density chains, in a precise location on the polymer surface. In addition, the chemical bond formed between the polymer surface and the grafted functional group results in a more durable functionalization in comparison with the physical coating [168,169]. Plasma grafting is restricted to localized surface areas and to a depth from several hundred angstroms to 10 nm [154]. In one study, PLLA and PLGA nanofibres were chemically modified using oxygen

plasma treatment and by *in situ* grafting of hydrophilic acrylic acid to add carboxyl groups on the surface. The modified scaffolds showed improved fibroblast attachment and proliferation *in vitro* [170]. Brown *et al.* investigated plasma grafting of acrylic acid on PLGA followed by activation of carboxylic groups by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) for the coupling of fibronectin. They demonstrated better spreading of neonatal rat cardiomyocytes and myofibril development on modified PLGA with respect to unmodified PLGA [171]. In another study, derived hMSCs, cultured on PLGA supports, plasma grafted with fibronectin strips, showed highly elongated morphology and markers associated with myogenesis. It was also observed that protein surface micropatterns can highly influence the differentiation process [172].

## 3.2. Surface biological modification with bioactive molecules

Surface biological modification is achieved by adsorption or chemical bonding of biomolecules to the polymer surface in order to stimulate a specific cell response. Different modifications are discussed in this section.

### 3.2.1. Chemical incorporation

There are two main groups of methods to chemically incorporate GFs into polymeric scaffolds: (i) non-covalent incorporation by physical adsorption, owing to protein–protein hydrogen bonding or protein–protein hydrophobic interaction with an intermediate molecule and (ii) covalent incorporation of GF.

#### 3.2.1.1. Covalent surface bonding

In covalent surface bonding, biomolecules are chemically bonded to the scaffold surface exposing relevant functional groups. Covalent surface bonding results in a more efficient coating, with the biomolecules being retained over a longer period of time when compared with physical adsorption [173]. To accomplish covalent coating two steps are required: the first is the exposure of functional groups and the second is the covalent binding of the biomolecules to the exposed functional groups.

Carboxyl and amine groups are the most common functional groups exploited in surface functionalization. Carboxyl groups in polymers such as PLLA and PCL can be exposed on the surface by hydrolysis [158,159], amine groups by aminolysis [160,161]. In addition, plasma treatment can be applied to graft functional groups on the surface for further covalent grafting [174], as previously mentioned.

Attachment of biomolecules requires the activation of functional groups on both polymer surface and on biomolecules. Hydrolysed films have been activated by treatment in MES buffer solution containing EDC for an adequate period [174–176]. By adding NHS, EDC efficiency is improved [174,175]. On the other hand, aminolysed films were activated by treatment in triethanolamine buffer containing glutaraldehyde and 1-hydroxybenzotriazole (HOBt), followed by EDC exposure for an adequate period of time [160,176]. After a preliminary chemical treatment, the surface is modified by immobilization of the biomolecules via an amide bond between the amine group/carboxylic group of the biomolecule and the exposed carboxylic group/amine group on the biomaterial surface, respectively [177].

Linker molecules are used when functional molecules cannot be directly grafted onto the polymer surface. A linker

molecule is a chemical substance capable of reacting with both the polymer and the functional molecules. In one study, di-amino-poly(ethylene glycol) (di-NH<sub>2</sub>-PEG) was used as a linker molecule to add functional amine groups to previously exposed carboxyl groups, by soaking PLLA nanofibres in NaOH solution [178]. Using a similar strategy, carboxyl functional groups were grafted on polyethersulfone by using a linker molecule (polyacrylic acid) introduced on the polymer surface by photo polymerization [179]. Moreover, polyacrylic acid was used as linker molecule to covalently bond collagen in the form of lamellae or sheets on PMMA and other polymers [180]. It is very important to consider the properties of the linker molecules before using them, as several studies have demonstrated that linker molecule properties influence the interaction between cells and the immobilized biomolecules, such as cell adhesion ligands [179,181,182].

Covalent bonding is more complex and may limit the type of biomolecule that can be attached owing to the harsh conditions sometimes required to gain a satisfactory functionalization. For instance, biomolecules such as GFs can be deactivated by the organic solvents used during covalent bonding procedures. Alternatively, combined techniques employing both covalent bonding and physical adsorption can be exploited to biofunctionalize polymer surfaces. These techniques will be discussed in detail later.

Covalent immobilization is a promising approach to bind GFs to biomaterials [124,183]. The covalent attachment is achieved by a reaction between functional groups of the polymer and GFs amino acids such as lysine and cysteine [184]. Enhanced cell survival and spreading of MSC have been demonstrated on a PEG scaffold covalently modified with VEGF [185]. GFs can be also covalently immobilized on scaffolds by homo- and heterobifunctional cross-linking agents. A homo-bifunctional cross-linker (disuccinimidyl-disuccinate-polyethyleneglycol (SS-PEG-SS)) was used to immobilize VEGF on collagen matrices. It was demonstrated by *in vitro* endothelial cell growth and *in vivo* vessel growth in chorioallantois membrane that VEGF covalently immobilized on collagen matrix produced increased angiogenic effects compared with free VEGF matrices [186].

In one study, VEGF was first modified by introducing a cysteine tag in its structure and then immobilized onto a fibronectin-coated surface using free sulfhydryl group [187]. In a different approach, EDC chemistry was applied to immobilize GFs onto scaffold surfaces, for instance, VEGF and fibronectin were immobilized on PLLA surfaces, demonstrating improved growth of HUVECs. [188]. Collagen scaffolds have also been incorporated with VEGF using EDC chemistry [121]. As a result of VEGF immobilization, survival and proliferation of D4T endothelial cells were enhanced [121]. In a related study, Miyagi *et al.* demonstrated increased angiogenesis and patch stability in defective rat hearts upon transplantation of scaffolds uniformly immobilized with VEGF, compared with VEGF-free controls [131]. In another study, VEGF and angiopoietin-1 (Ang-1) were co-immobilized onto collagen scaffolds [124]. The co-immobilized scaffolds showed increased cell growth and proliferation *in vitro* compared with unfunctionalized controls without GF. Furthermore, increased vessel density was found when scaffolds were implanted in chicken chorioallantoic membrane [124]. VEGF and Ang-1 were also covalently immobilized onto porous collagen scaffolds using EDC chemistry. The effect of different reaction buffers (PBS, distilled water and MES) on

covalent immobilization was investigated. PBS showed the highest VEGF and Ang-1 immobilization, leading to the highest proliferation rate and lactate metabolism [189].

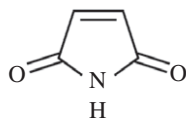
### 3.2.2. Physical adsorption

Physical adsorption is one of the simplest methods to biofunctionalize biomaterials, by incubating the scaffold in solutions containing biomolecules. The biomolecules attach to the material surface owing to surface interactions, such as Van der Waal forces, electrostatic forces, hydrophobic interactions and hydrogen bonds. The physical adsorption efficiency can be enhanced by treating the material with air plasma to increase its hydrophilicity. Generally, hydrophilic surfaces tend to improve adhesion strength, biocompatibility and other pertinent properties [190,191]. Surface functionalization via physical adsorption has the advantage of being a simple and gentle procedure accompanied by limited damage to fragile structures and biomolecules; on the contrary, biomolecule binding to scaffold surfaces is relatively weak.

Non-covalent immobilization is based on electrostatic interactions. For example, ionic complex of gelatin and transforming growth factor-1 (TGF-1) can be obtained when gelatin microparticles loaded with TGF-1 are encapsulated in oligo[poly(ethylene glycol) fumarate] hydrogels at pH 7.4 [192]. The interactions between gelatin and TGF-1 occur because of negatively charged chemical groups on the gelatin surface and TGF-1 positive charge [192]. Typical adsorption of a GF onto a polymer is either a charge to charge or a secondary interaction between the polymer surface and the GF. This result can be also achieved through an indirect interaction using an intermediate biomolecule [193–195].

### 3.2.3. Combined techniques

Chemical reagents and solvents used for chemical conjugation can damage biomolecules (proteins and peptides) such as GFs that are susceptible to inactivation or denaturation. An alternative approach for biomolecule binding is the use of molecules that bind to both the biomolecule and the biomaterial surface. Therefore, intermediate molecules such as glycosaminoglycans, ECM proteins, small oligopeptides mimicking ECM proteins and avidin–biotin molecules can be chemically or physically deposited onto the scaffolds to offer biological sites for GF incorporation. It has been proven that GF binding via ECM linkers regulates GF activity [196]. For example, several groups of GFs interact with ECM proteins, heparin or HS, including IGF, fibroblast growth factor (FGF), TGF-1,2,3, endothelial growth factor (EGF), VEGF, PDGF and HGF [95,196]. As a result, stable and strong covalent bonds are formed between the biomaterial surface and the linker molecules, avoiding the exposure of biomolecules to harsh solvents that could damage their bioactivity. In addition, thanks to the presence of the linker molecules the surface of the material exhibits a strong charge or hydrophobicity favouring the attraction of biomolecules [197]. Combinational techniques are also fast, and do not require substantial incubation time for the biomolecule to attach to the functionalized surface [198]. Moreover, they can be applied using a wider choice of buffer systems in comparison with chemical bonding, where some amine-based buffers (Tris, glycine) cannot be used [198]. The only drawback is that combinational techniques involve many steps.



**Figure 4.** Chemical formula of maleimide investigated to attach heparin to PEG hydrogels [203].

### 3.2.3.1. Interaction with glycosaminoglycans

Heparin and HS are linear sulfated polysaccharides with high negative charge, which bind to different proteins, such as FGF and their tyrosine kinases receptor, TGF, bone morphogenetic proteins (BMPs), chemokines, interleukins, enzymes, enzyme inhibitors, lipases, apolipoproteins, ECM and plasma proteins [199]. The specific sulfation motifs function as molecular recognition elements for GFs. The existence of a ‘sulfation code’ has been proposed, whereby glycosaminoglycans encode functional information in a sequence-specific manner analogous to that of DNA, RNA and proteins [200]. GFs become more resistant to proteolysis and thermal denaturation when bound to HS or heparin [201]. GAGs can be therefore used in binding, modulation and sustained release of GFs. GAGs have been incorporated in different polymers. For example, heparin-conjugated PLGA nanospheres (HCPNs), trapped in cross-linked fibrin gel, were developed for long-term and zero-order delivery of bFGF. Heparin was immobilized on PLGA nanospheres via a coupling reaction in the presence of EDC and the bFGF was physically bonded to heparin [202]. The release kinetics of bFGF from HCPNs suspended in fibrin gel was of zero order, sustained for three weeks with no initial burst effect. The release rate was inversely proportional to the fibrinogen concentration in the fibrin gel. *In vitro* cell culture demonstrated that bFGF released from HCPNs supported HUVEC growth for 15 days. *In vivo* tests (implantation in a mouse limb ischaemia model) also offered successful results [202]. The use of maleimide reactive groups (figure 4) was demonstrated to be a possible strategy to attach heparin to PEG hydrogels [203]. PEG was immobilized with heparin by the reaction between PEG terminal thiol and maleimide-functionalized low molecular weight heparin. bFGF incorporated in such system exhibited controlled release. The release kinetics of bFGF were the same as the matrix erosion kinetics and the delivery lasted several days [203].

In order to prolong and slow down the release of VEGF, which stimulates both angiogenesis and vasculogenesis, a novel self-assembling system was developed, where the heparin-binding domain sequence was attached to a self-assembling peptide [204]. Results of *in vivo* tests in infarcted rat model demonstrated myocardial protection, sufficient angiogenesis and improvement in cardiac function [204]. In one study, heparin was covalently bonded to the biomaterial, and then the biomolecule was attached to heparin by physical adsorption, as shown in figure 5 [178]. A different approach for covalent attachment of heparin was also investigated, where heparin was attached by modifying its carboxyl groups to obtain thiol groups [205]. Thiol groups reacted with PEG diacrylate, undergoing Michael addition to form thioether linkages. *In vivo* studies demonstrated that fibroblast cells encapsulated in the bFGF–heparin-modified PEG remained viable after gelation, showing enhanced proliferation [205].

### 3.2.3.2. Interaction with extracellular matrix components

Gelatin is a charged biopolymer which can interact with GFs. Gelatin can be negatively charged (acidic gelatin) or positively

charged (basic gelatin), depending on the extraction process. Acidic gelatin can be used to incorporate basic proteins, whereas basic gelatin can be used to incorporate acidic proteins under physiological conditions [206]. A study carried out by Layman *et al.* [207] has shown the inclusion of bFGF into acidic gelatin hydrogels, comparing the release profile with those from basic gelatin and PAA anionic hydrogels. In this study, the effect of solution temperature and ionic strength on the absorption and desorption was investigated. It was demonstrated that the bFGF absorption increased with increasing the number of carboxylic groups in acidic gelatin hydrogel. This study also demonstrated also the possibility of delivering multiple GFs from gelatin hydrogels. bFGF and G-CSF were immobilized in these hydrogels and a controlled release for several days for both GFs was reported [207].

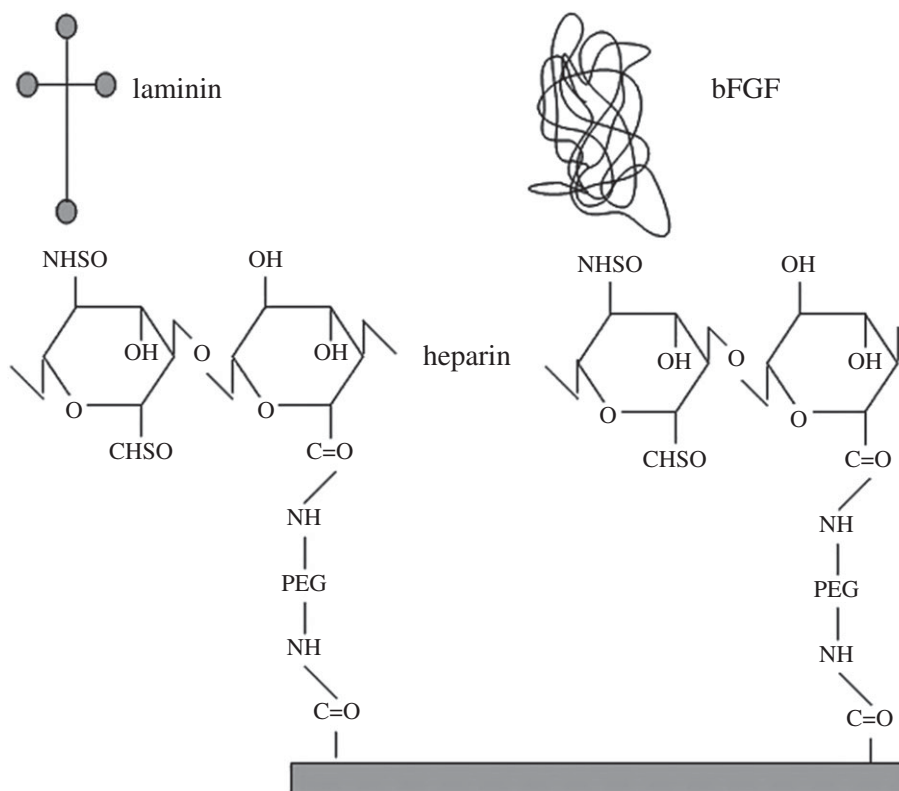
Collagen is a ubiquitous protein in the human body able to interact with GFs. For example, bFGF binds to collagen type I both *in vitro* and *in vivo*, obtaining a protection effect. Incorporation of bFGF in collagen matrices showed a prolonged release of the GF, depending on the degradation rate of the matrix, leading to controlled local delivery with angiogenic activity. Therefore, collagen type I is a suitable candidate material to be used as a reservoir for bFGF delivery [208].

### 3.2.3.3. Functionalization by the avidin–biotin binding system

Avidin is a tetrameric glycoprotein found in egg white (MW 68 kDa) with a size of 5 nm, which exhibits a specific and extraordinarily high interaction with biotin (also known as vitamin H; MW 244.3 kDa). Avidin contains four biotin binding sites and therefore, the two molecules make a highly specific and stable complex, with an affinity constant of  $10^{15} \text{ M}^{-1}$ . Avidin–biotin binding approaches have been extensively applied to biotechnology, such as affinity chromatography, histochemistry, diagnostic, immunoassay and drug delivery [209].

The feasibility of introducing an avidin–biotin binding system in biomedical relevant approaches for increasing cell adhesion has been also demonstrated. The conjugation of biotin on the cell membranes of non-adhesive cells of the Ehrlich ascites carcinoma cell line produced their adhesion to avidin-coated substrata [210]. Similarly, biotinylation of endothelial cells was used to enhance the initial cell attachment to avidin-treated surfaces, in order to improve *in vivo* patency of vascular grafts [211–215]. The application of this strategy in cell culture and tissue engineering was also explored. It was demonstrated that biotinylated chondrocytes adhered to avidin-coated substrates (tissue culture polystyrene or flat films based on biodegradable polymers), more quickly than untreated chondrocytes to bare substrates [216,217]. The efficiency of the avidin–biotin binding system was also shown for the initial attachment of a biotinylated human hepatoma cell line to avidin adsorbed on the surface of two-dimensional and three-dimensional PLLA scaffolds [218]. Using this approach, avidin-, streptavidin- and neutravidin-modified molecules could be introduced onto scaffold surfaces.

Davis *et al.* [219] conjugated biotinylated IGF-1 with streptavidin and then bound it to biotinylated self-assembling peptides. The biotin–streptavidin–biotin system enabled IGF-1 attachment to the peptides, without effecting their self-assembly behaviour. After injection into rat myocardium, biotinylated IGF-1 nanofibres provided sustained IGF-1 delivery for 28 days, and delivery of IGF-1 *in vivo* increased activation of protein kinase B in the myocardium. Moreover,



**Figure 5.** Immobilization of bFGF and laminin on PLLA using di-NH<sub>2</sub>-PEG and heparin as linkers. Reproduced with permission from [178].

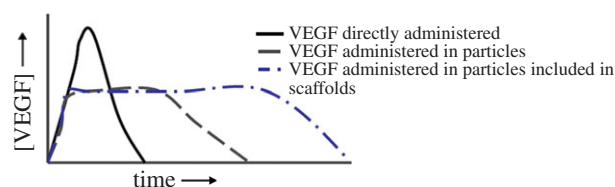
cardiomyocytes added to biotinylated IGF-1 nanofibres showed improved systolic function, a decrease in caspase-3 cleavage by 28% and an increase in the myocyte cross-sectional area by 25%, compared with cells embedded within nanofibres alone or with untethered IGF-1.

### 3.3. Bulk biological modification with biomolecules

Bulk biomolecule incorporation is another method to introduce biomolecules into polymers during the fabrication process. There are three common techniques for bulk biomolecule incorporation: direct mixing, coaxial electrospinning and self-assembling (amphiphile) peptide nanofibres. When compared with surface functionalization, bulk biomolecule incorporation enables more biomolecules to couple onto the scaffolds. Blending is considered a rapid and simple modification technique in comparison with covalent immobilization and physical adsorption that involve several steps to achieve modification of the scaffold. In addition, the presence of biomolecules both on the surface and inside the material can provide the necessary signals for cell interaction as the synthetic polymer degrades [173]. The biomolecules are embedded in the bulk material and therefore they can be continuously released by diffusion through the polymer or, in the case of biodegradable polymers, by degradation of the matrix.

#### 3.3.1. Direct mixing of biomolecules

Direct mixing or incorporation of biomolecules into the polymer bulk during fabrication is possible if both are soluble in the same solvent. Biomolecules can be dissolved directly into the polymer solution [220], incorporated as a suspension by dissolution in a miscible solvent [221,222], and they can be incorporated as an emulsion by dissolution in an immiscible solvent [223–225]. Alternatively, a multicomponent system can be used when two miscible solvents are not available



**Figure 6.** Simplified diagram showing the modulation of VEGF release profile acting on the carrier used to administer it (direct administration, encapsulation in nanoparticles, encapsulation in nanoparticles embedded in a scaffold). Reproduced with permission from [230]. (Online version in colour.)

for a particular polymer–biomolecule system. For example, a three-component system was used for the incorporation of water-soluble heparin into dichloromethane (DCM)-soluble PCL. Heparin was dissolved in water, which is miscible with methanol, which is miscible with DCM [226]. Direct loading is one of the simplest techniques used to incorporate proteins into polymeric matrices. As a drawback, incorporation of proteins using direct loading without further modification usually leads to a fast burst release during the initial swelling phase, as a significant amount of protein originally trapped in the gel network is released [184]. Therefore, controlled release cannot generally be achieved using the direct loading technique, and the release rate is diffusion-controlled [227].

This observation is exemplified in a study reported by Kanematsu *et al.* [228], in which GFs were added to hydrogels based on collagen type 1. The hydrogels were prepared in aqueous solutions containing bFGF, hepatocyte growth factor (HGF), platelet-derived growth factor-BB (PDGF-BB), VEGF, IGF-1, or heparin-binding epidermal growth factor-like growth factor (HB-EGF). Each GF showed a distinct release profile, as a consequence of the fact that the interaction mechanism between collagen and the different GFs

varies. However, in general, the release lasted for some days, and a burst effect was observed [228,229]. Figure 6 schematically shows that VEGF release profile can be modified according to the incorporation method [230].

Structural and surface modifications, such as variations of cross-linking density of the polymer network, change the release profiles [184,230]. For example, degradation time and storage modulus could be varied and controlled for dextran hydrogels (which were formed by mixing aqueous solutions of dextran vinyl sulfone conjugates and tetrafunctional mercapto-poly(ethylene glycol)), varying the composition of vinyl sulfone and dextran molecular weight [231]. bFGF was loaded into dextran hydrogels by mixing the protein with polymer in aqueous solution and it was released with first-order kinetics in 28 days, without showing a burst effect [231].

In recent years, there has been an increasing number of experimental approaches to regenerate the infarcted myocardium by using injectable scaffolds loaded with GFs. Iwakura *et al.* [232] delivered bFGF via injectable gelatin microspheres, with an average diameter of 10  $\mu\text{m}$ , and reported increased angiogenesis as well as improved cardiac function. An alternative concept was reported by Hsieh *et al.* [233], who employed self-assembling peptides as a delivery vehicle for PDGF. They reported sustained delivery for 14 days, which decreased cardiomyocytes death and preserved cardiac function, compared with either peptides or GFs alone. Moreover, they demonstrated reduction in infarct size.

In another relevant study, chitosan-gel-containing bFGF was injected into the ischaemic myocardium of rabbits with chronic MI [234], which was found to induce angiogenesis and improve the collateral circulation in the infarcted area. Synthetic hydrogels mimicking the ECM have been also created by cross-linking a thiol-modified analogue of heparin with thiol-modified hyaluronan or chondroitin sulfate with poly(ethylene glycol) diacrylate [235]. The covalently bound heparin provided a cross-linkable analogue of an HS proteoglycan, thus leading to a multivalent biomaterial capable of controlled release of bFGF. This system was found to increase the neovascularization in mice models. The sequential release of VEGF and PDGF from alginate gels was investigated in Fischer rats [236]. The results obtained demonstrated an increased capillary density and blood vessel formation with improved cardiac function in the case of dual delivery compared with the individual delivery of VEGF or PDGF [236]. More recently, the potential application of an affinity binding alginate gel for controlled delivery of HGF was studied in a hindlimb ischaemia model. The HGF-containing alginate gel was demonstrated to induce matured blood vessel network formation [237]. A thermoreversible scaffold, forming a gel at body temperature, has been also developed for VEGF delivery [238]. Such gel exhibited temperature-dependent reversible sol–gel transitions near body temperature and it was found useful for cell/protein delivery in a minimally invasive manner. Not only growth and angiogenic factors, but also other bioactive agents have been loaded into polymeric supports for CTE application. For example, increased neovascularization was reported by delivering a plasmid encoding the angiogenic growth factor pleiotrophin in fibrin glue [239]. Increased neovasculature formation, reduced infarct size and improved cardiac function have been reported when EPO was delivered intramyocardially through  $\alpha$ -cyclodextrin/MPEG–PCL–MPEG hydrogel [137]. In another study, an MMP responsive hydrogel delivering thymosin, a pro-angiogenic and pro-survival factor, was

investigated [240]. The gel was able to substitute the degrading ECM in the infarcted myocardium of rats and to promote structural organization of native endothelial cells, whereas some of the delivered hESC-derived vascular endothelial cells formed de novo capillaries in the infarct zone. Moreover, the bioactive hydrogel preserved contractile performance, attenuated LV dilation and decreased infarct size, when compared with infarcted rats treated with PBS injection as a control. In a study published by Ito *et al.* [241], VEGF was immobilized in photoreactive gelatin. Gelatin and VEGF were mixed in water, cast and photoirradiated. Samples with immobilized VEGF showed growth of HUVECs in comparison with the non-treated surface. Growth of HUVEC increased significantly with an increase in the amount of immobilized VEGF. Micropatterning of HUVEC cultures was also achieved using micropattern-immobilized VEGF using a photomask. In direct incorporation of proteins into hydrogels, the difficult and challenging step of loading the protein has to be considered. If, for example, chemical cross-linking is involved in hydrogel preparation, it is important to evaluate the chemical integrity of the added proteins. Additionally, if loading is performed after cross-linking, the even distribution of the biomolecule within the gel must be verified [184].

Another approach for the direct incorporation of GFs is by electrospinning for the fabrication of fibrous cardiac scaffolds. For example, PLLA electrospun cardiac scaffolds functionalized with G-CSF have been produced. G-CSF was added to a PLLA polymer solution (13 w/w % in DCM) at a concentration of 250 UI  $\text{g}^{-1}$ . C2C12 murine skeletal myoblasts were seeded on G-CSF modified PLLA electrospun scaffolds. Results demonstrated morphological alteration and induction of Cx43 expression, resembling the usual cardiomyocyte arrangement, characterized by cell elongation and appearance of cellular junctions [138].

In another study, Gao *et al.* [135] investigated collagen membranes loaded with a fusion protein (consisting of VEGF and a collagen binding domain, CBD-VEGF), natural VEGF and phosphate-buffered saline (PBS), in a rabbit model. The CBD-VEGF/collagen group could effectively induce more cells (endothelial cells and myocardial cells) to penetrate into the collagen membrane after four weeks and promoted vascularization in infarcted myocardium after 12 weeks, compared with control groups. Echocardiography and haemodynamic studies both showed cardiac function improvement in the CBD-VEGF/collagen group. The study showed that CBD-VEGF could be retained longer on the collagen membrane when compared with VEGF, which was rapidly released and diffused by blood flow and body fluid *in vivo*. It was reported that VEGF concentration plays a crucial role during vascularization [242]. It was shown that the CBD-VEGF/collagen group leads to better results, thanks to a higher content of VEGF than controls.

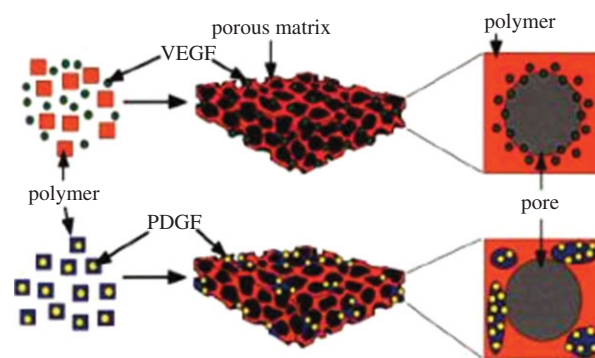
### 3.3.2. Carrier systems

When a long-term immobilization of proteins is required, carriers can be added to the scaffold to retain the bioactive molecules for long-term application [243]. This can be achieved by the immobilization of microscopic carriers or drug-releasing implants into the scaffolds [184]. Such delivery systems provide local delivery of biomolecules (local concentration  $C$ ) in the form of temporal gradients ( $d[C]/dt$ ) and spatial gradients ( $d[C]/dx$ ). Moreover, by using carrier systems, proteins can be combined with other biomolecules [244]. Carriers can also

provide protection to proteins against inactivation occurring in the biological environment contributing to preserving their bioactivity. Such carriers can be designed in shapes such as micro- or nanoparticles and matrices, to respectively achieve zero- or first-order release kinetics for different biological applications [133]. There are different methods and techniques to prepare biopolymer microparticles, for example the water in oil in water (W/O/W) emulsion. In this method, an aqueous solution of GF is prepared that is emulsified in an organic solution containing the polymer. This primary emulsion is mixed with an external aqueous solution to obtain a double emulsion. Organic solvents are then extracted from the resulting double emulsion to obtain the particles [245]. Both degradable and non-degradable materials can be used in the fabrication of carriers; however, only degradable polymers are being used in CTE.

Biodegradable polymer systems have attracted wide interest as matrices for GFs delivery. In particular, thermoplastic aliphatic polyesters, such as PLA and PLGA, have been investigated largely owing to their demonstrated biocompatibility [34,246]. The degradation of the polymeric matrix determines the release of the bioactive molecule. Indeed, the incorporation of degradable microparticles embedded with GF inside a matrix has been shown to lead to controlled protein release upon microparticle degradation [184]. An example of such a system is a novel scaffold based on degradable PLGA microspheres loaded with bFGF, which were incorporated in a porous alginate matrix [102]. The bFGF led to enhanced proliferation of cardiac fibroblast. The microsphere system was capable of controlling the release of bFGF, thereby promoting vascularization by boosting the formation of large and mature vessels [102]. In another study, heat shock protein 27 (HSP27), fused with transcriptional activator (TAT) derived from human immunodeficiency virus (TAT-HSP27), was loaded in a combination system made of PLGA microspheres and alginate hydrogel. Alginate was cross-linked by calcium ions after the incorporation of TAT-HSP27-loaded PLGA microspheres. It was possible to control the delivery behaviour for a long period of time by changing the microsphere/hydrogel ratio [247]. Apoptotic pathways of cardiomyoblasts cultured under hypoxic conditions were effectively inhibited by the release of TAT-HSP27.

In another relevant development, human embryonic stem cells were encapsulated in dextran-based hydrogel enriched with VEGF-loaded PLGA microparticles [248]. A double emulsion solvent evaporation technique was used to produce PLGA microparticles, and the effect of GF release on vascular differentiation was studied. VEGF-loaded microparticles release kinetics showed a burst effect in the initial phase followed by a lower steady-state release. The release of VEGF from the loaded microparticles was assessed by evaluating the VEGF effect on the survival of an endothelial cell line *in vitro* [248]. Indeed, biodegradable polymers provide controlled delivery of bioactive molecules enabling specific release concentrations over relatively long periods of time. Furthermore, this strategy gives the opportunity to deliver more than one bioactive agent at different pre-programmed rates, according to the needs of a specific application [246]. Following this approach, a system has been proposed, in which two or more GFs could be delivered with a controlled dose and rate of delivery [249]. VEGF was incorporated by mixing with polymer particles before processing them into a scaffold structure, whereas PDGF was pre-encapsulated



**Figure 7.** Schematic representation of the scaffold fabrication process with dual GF delivery. Growth factors were incorporated into polymer scaffolds by either mixing with polymer particles before processing into scaffolds (VEGF), or pre-encapsulating the factor (PDGF) into polymer microspheres used to form scaffolds according to Richardson *et al.* Reproduced with permission from [249]. (Online version in colour.)

into polymeric microspheres. To incorporate the two GFs into the same scaffold, particles encapsulating PDGF and lyophilized VEGF-containing particles were mixed together before scaffold fabrication (figure 7). When implanted in the subcutaneous pockets of the dorsal side of rats, the scaffolds showed enhanced formation of a mature vascular network owing to the dual delivery of VEGF and PDGF [249]. This dual GF system permitted rapid release of VEGF, promoting blood vessel formation and a slower release of PDGF, for blood vessel maturation.

Another example of a dual-release system was a scaffold with TGF-1 and IGF-1 immobilized in oligo [poly (ethylene glycol) fumarate] (OPF) hydrogel-containing gelatin microspheres. The GFs were loaded either in the OPF gel or in the gelatin microspheres phase [250,251]. Release profiles were successfully controlled by altering the phase of GF loading and the degree of microparticle cross-linking.

### 3.3.3. Coaxial incorporation (electrospinning)

In coaxial electrospinning, core shell nanofibres are formed using a special coaxial nozzle, in which one material makes up the core and the other forms the shell. This represents an alternative method when compared with direct mixing of biomolecules in the electrospinning solution [252]. Through a special nozzle, multiple solutions are fed in a concentric extruder, forming a two-phase liquid electrospun jet. As the solvent evaporates, solid core shell nanofibres are formed. Biomolecules can be incorporated inside the nanofibre core, avoiding exposure to the toxic harsh solvents used in the production of the shell [253].

### 3.3.4. Incorporation of bioactive molecules into self-assembling (amphiphile) peptide nanofibres

Another strategy for bulk incorporation of biomolecules is the incorporation of biofunctional peptide sequences into amphiphile peptides (RAD16-I and RAD16-II), which self-assemble into nanofibres. Several studies have demonstrated that direct injection of biomaterials into the infarcted myocardium prevents deleterious remodelling and reduces cardiac dysfunction [21,254,255]. Using intramyocardial injection of self-assembling peptide nanofibres, a highly biocompatible and biodegradable material was obtained [233,256]. In both small [219,257] and large [258] animals, self-assembling peptide

nanofibres have been used as a platform for controlled local delivery of GFs. If GFs or cells are directly injected, the efficacy is reduced by the rapid diffusion from the injection site.

Lin *et al.* [258] injected mini pigs at the site of infarction with autologous bone marrow mononuclear cells in saline solution or RAD16-II peptide nanofibres or a combination of both. Injection of cells with the nanofibres improved both diastolic and systolic function. More recently, self-assembling RAD16-I peptides were used for dual delivery of PDGF and FGF-2 into a rat infarct, showing a reduction in infarct size, and an increase in capillary and arteriole density, at four and eight weeks post-MI [259]. In another study, PDGF-BB was delivered into rat-infarcted myocardium using injectable self-assembly peptides [233]. PDGF-BB is an endothelium-secreted paracrine factor known to have direct anti-apoptotic effects on cardiomyocytes. The study showed a decrease in cardiomyocyte death and better preservation of systolic function after MI, in comparison with the injection of PDGF-BB or peptide nanofibres by themselves [233].

#### 4. Molecular imprinting technology: an innovative functionalization strategy

Innovative functionalization strategies are gaining increasing interest in the literature. An interesting approach for the creation of advanced synthetic support structures for cell adhesion and proliferation is based on molecular imprinting technology [260–262]. This technology permits the introduction, into a polymeric material, of recognition sites for specific molecular species (templates) through the polymerization of a monomer in the presence of a template [263,264], or through the dissolution of a pre-formed polymer in a solution containing the molecule to be recognized [265]. In both cases, the spatial arrangement is maintained by the polymer, after template extraction, and confers a selective ‘memory’ towards this molecule.

Molecular imprinting technology is a valid alternative to molecular recognition features present in biological systems, such as those activated by antibodies [266]. Macromolecular matrices prepared with this procedure, in fact, can be stable even in critical chemical and physical conditions [267], they can have a life of several years without any apparent reduction in performance and can be used repeatedly without any alteration to the ‘memory’. The most extensively studied applications of molecularly imprinted polymers are mainly in the field of affinity chromatography [268–270], and also in determining the level of drugs in human serum in substitution of natural antibodies. Other applications are in the field of biomimetic sensors [260,261] and intelligent polymers [271]. Interesting developments in the biomaterial field in general [272,273] and in tissue engineering in particular [274,275] have been proposed. Intelligent matrices usable in the field of tissue engineering should be characterized by their particular capacity for molecular recognition of extracellular proteins (collagen, fibronectin, laminin, vitronectin, etc.) that favour cellular processes. These polymeric matrices will be able to improve the adhesion and growth characteristics of cells onto the scaffolds, thanks to the presence of sites that are complementary and selective towards extracellular proteins, recognized by integrins. Molecular imprinting is preferentially performed on biostable polymers in order to guarantee the stability of the recognition sites over

time. Considering that biodegradable polymers are a fundamental requisite for tissue-engineered scaffolds, imprinted nanoparticles based on biostable polymers can be used to modify degradable scaffolds. The fate of these particles, upon scaffold degradation, has not been completely ascertained. However, considering the very small amount of particles used, simple elimination by the normal metabolic pathways is likely to occur.

The use of entire proteins as template molecules has often been often unsuccessful because of large molecular dimensions and flexibility of the chains, which limit molecular recognition capacity and selectivity. To overcome these limitations, stable short peptide sequences, representative of accessible fragments of larger proteins of interest are used. These fragments (epitopes) are located in the receptor domains or in other parts directly involved in the molecular recognition process. Therefore, if the material can recognize a peptide that represents the exposed part of a protein structure, it will also be able to bind the entire protein [276].

Two different molecular imprinted polymer systems (MIPs), capable of recognizing, respectively, a pentapeptide segment of an exposed part of a fibronectin functional domain and a pentapeptide segment of an exposed part of a laminin functional domain, were synthesized and characterized by some of the authors of this review [274,275]. The pentapeptides H–Gly–Arg–Gly–Asp–Ser–OH (GRGDS) and H–Tyr–Ile–Gly–Ser–Arg–OH (YIGSR) were used as template molecules. GRGDS is a short peptide sequence from fibronectin, the prototype of cell adhesive and bioactive matrix proteins. It has been shown to regulate cell growth, cell shape, cytoskeletal organization, differentiation, migration and apoptosis of almost all cells, including cardiac ones [277–279]. Moreover, GRGDS is able to stimulate integrins ( $\alpha 5\beta 1$ ,  $\alpha v\beta 3$ ) that are relevant in early cardiac development [90,91]. YIGSR derives from laminin, the most bioactive component in the basement membrane [280,281]. It is able to increase the ability of stem cells to differentiate into beating cardiomyocytes. The polymers were prepared by polymerization of MAA (in the presence of the template molecule). An appropriate cross-linking agent, trimethylolpropane trimethacrylate or pentaerythritol triacrylate, was added during polymerization to create stable recognition sites in the polymer structure. The obtained materials were characterized for morphological and physico-chemical properties. For both formulations, polymers in the form of densely fused microgels were obtained. The template molecules were removed from the polymers by solvent extraction, and the rebinding ability was verified. An epitope effect was observed, as the extracted polymer was able to recognize both the pentapeptide used as template and larger protein fragments containing the template sequence. Both MIPs showed good performance in terms of recognition capacity and selectivity. Cytotoxicity tests showed viable C2C12 myoblasts cultured in MIPs eluates. MIPs were used for surface modification of tissue engineering supports. The deposition of nanoparticles did not alter their specific recognition and binding behaviour. Functionalized films showed higher quantitative binding than free nanoparticles, suggesting the creation of a preferred microenvironment for the rebinding process. The most remarkable result was obtained in the biological characterization: bioactive scaffolds were able to promote C2C12 myoblast proliferation. These results can be considered very promising and suggested that MIP can be used as an innovative functionalization technique to prepare bioactive scaffolds with an effective capacity of improving tissue regeneration.



## 5. Conclusions

Infarcted myocardial tissue cannot be treated by conventional methods, thus tissue engineering represents a promising approach. In a common tissue engineering strategy, for the regeneration of cardiac tissue, a scaffold (patch) is required to host the cells and mechanically support the infarcted heart. An ideal CTE scaffold should promote cell attachment and mimic the native tissue, both biologically and mechanically. Many synthetic polymers fit the required mechanical aspect but they lack effective cell–material interaction ability. Therefore, surface and bulk functionalization strategies have been extensively investigated. This review summarizes different functionalization techniques for cardiac tissue scaffolds,

based on different physical, chemical and biological modification strategies. Biological modification is essential to enhance cell attachment by incorporating cell recognition sites in or on the material. Analysis of the literature has also revealed that often a combination of different techniques is required to achieve optimal results. The significant progresses and the promising results achieved as documented in the analysed literature indicate that the area of CTE will continue to attract major research efforts in future.

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