Cell–biomaterial interactions: the role of ligand functionalization

Chapter 8

Cell–biomaterial interactions: the role of ligand functionalization

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8.1 Introduction

The design of new biomaterial-based devices implies the optimization of their interactions with the living cells in the host tissue. Cells are in continuous communication with adjacent cells and microenvironment in order to maintain their homeostasis and function. Cells are characterized by a plethora of cell surface receptors (e.g., integrins) that can sense biochemical and biophysical signals outside the cell membrane. When stimulated, receptors activate intracellular signaling pathways that regulate gene expression and, as a result, cell behavior [1]. Essential signals come from their environment, both from neighboring cells and from the surrounding extracellular matrix (ECM) to which cells adhere. The ECM is a highly hydrated substrate composed by different types of molecules, such as collagens, elastic fibers, glycosaminoglycans (GAGs), and adhesive glycoproteins (e.g., laminin, fibronectin) [2]. These molecules are deposed by cells themselves, and different organs and tissues are characterized by different compositional and structural organization of their ECM, giving rise to their unique ECM arrangement [3]. Nowadays, ECM is known not to function just as passive support for cells, but it influences and controls cell behavior. In more detail, it supports the diffusion of soluble cytokines, mediates cell-cell interaction, and affects cell adhesion, proliferation, differentiation and possibly apoptosis, through its physical properties and chemical composition. Variation in ECM structure can have a great impact over cell functioning [2]. The first biomaterial-based devices exploited in regenerative medicine were based on inert materials, to avoid interaction with the host tissue and to reduce the probability of rejection. Currently, the trend is to develop biomaterials able to establish a specific communication with the resident cells of the implant area [4]. Such interactions are generally mediated by cell adhesive molecules that can endorse cell attachment and activate specific cellular signaling pathways [1]. Furthermore, ligand functionalization is also exploited in nanomedicine for targeted drug release: the surface functionalization of drug-loaded nanoparticles (NPs) with selective ligands allows NP internalization by the target cells, minimizing off-target effects [5].

Considering these premises, the functionalization of biomaterials with specific ligands is of pivotal importance to impart the desired functionality to the final device. Ligands generally consist of brief peptides derived from ECM proteins. In the case of scaffolding biomaterials, the purpose is to mimic ECM stimuli for a fine control over cell adhesion, proliferation, and differentiation, enhancing tissue regeneration outcomes [6]. On the other hand, in nanomedicine, drug-loaded NPs may be decorated with surface ligands, that confer them the capability to interact with target cells, undergoing receptor mediated-endocytosis [7].

This chapter aims at presenting the most recent advances in biomaterial functionalization, with a focus on the surface functionalization of scaffolds and the bulk functionalization of hydrogels with cell adhesive specific peptides, as well as the functionalization of NPs to address cell targeting in drug release. Biomaterial development can assist the regeneration of human tissues/organs by a wide range of possible strategies; however, in this contribution our attention will be devoted to ligand functionalization of biomaterials for cardiac regeneration.

Cardiac failure is one of the primary causes of death and disability in the world [8]. A healthy heart comprises different cell types, including cardiomyocytes (CMs), cardiac fibroblasts, and endothelial cells (ECs). CMs retain the contractile activity of this organ but have an extremely low regeneration rate. Ischemic injury leads to the death of a huge number of CMs: functional tissue is replaced by cardiac scar, mainly populated by cardiac fibroblasts and composed of collagen, resulting in compromised heart function [9,10].

The currently available treatments to cardiac failure are invasive surgical interventions for implantation of ventricular assistance devices or heart transplantation, and/or pharmacological treatments unable to restore proper cardiac function.

Newly studied strategies to restore myocardial function are (1) the *in situ* grafting of CMs previously differentiated *in vitro* from patient-derived stem cells [11], (2) the stimulation of resident CMs to induce their proliferation [12,13], and (3) the generation of new CMs by the direct reprogramming of resident cardiac fibroblasts [14–16].

Tissue engineering (TE) exploits biomimetic materials to sustain cell function and cells/bioactive molecules delivery. Different supports have been developed, such as polymeric scaffolds recapitulating the mechanical, structural, and electrical properties of the native cardiac ECM [17], which can be functionalized to sustain cell growth and differentiation [18]. In addition, innovative injectable hydrogels can provide a three-dimensional (3D) environment to locally deliver cells, drugs, or biomolecules (e.g., growth factors) through a direct and non-invasive approach [17]. Finally, NPs can act as carriers to protect and deliver biomolecules to a precise site of action through specific ligand functionalization [5].

A synergic combination of biomaterials-mediated strategies with proper functionalization could improve treatments aimed at cardiac tissue regeneration.

8.2 Ligand functionalization in the design of bioactive hydrogels

In this section, general strategies for peptide functionalization of hydrogels are initially reported, followed by a description of biomimetic hydrogels for cardiac applications.

8.2.1 General functionalization strategies for hydrogels

The first application of hydrogels for biomedical purposes dates back to the 1960s, with the work published by Wichterle and Lím [19]. In its general definition, the term “hydrogel” identifies 3D polymeric networks with high swelling potential in a watery environment [20,21]. As a consequence of this high water content (hydrogels can reach swelling percentages in the order of hundreds %), these systems usually possess low stiffness and high deformability. These properties, which structurally mimic the natural ECM, make hydrogels highly promising systems for the design of scaffolds able to guide the regeneration of soft tissues. However, hydrogels often lack specific bioactive moieties and, thus, cannot exert precise control over cellular functions. For this reason, during the past few years, the design principles of hydrogels for TE have been mainly focused on making their forming materials biomimetic and bioactive. To this aim, ligand functionalization has been widely explored in the literature to graft polymers with bioactive/functional moieties, thus making them able to drive specific cell behaviors (i.e., adhesion, migration, proliferation, and differentiation).

Over the last few decades, an exhaustive investigation has been carried out on hydrogel functionalization with adhesive peptide sequences. In this regard, much research has been focused on the bulk grafting of peptides containing the adhesion motif arginine–glycine–aspartic acid (RGD) derived from fibronectin. For instance, Burdick and Anseth investigated the relationship between the RGD functionalization degree of poly(ethylene glycol) diacrylate (PEGDA) gels and the attachment of rat calvarial osteoblasts, proving that cell adhesion and

spreading were significantly driven by RGD concentration [22]. Specifically, the number of attached cells significantly increased in RGD-modified gels compared to unmodified hydrogels at each analyzed time point, confirming RGD capability to enhance cell adhesion. Furthermore, a much higher cell density was observed upon the RGD concentration increase from 0.5 to 5 mM (Fig. 8.1), while cytoskeleton organization was detected only at 5 mM peptide concentration.

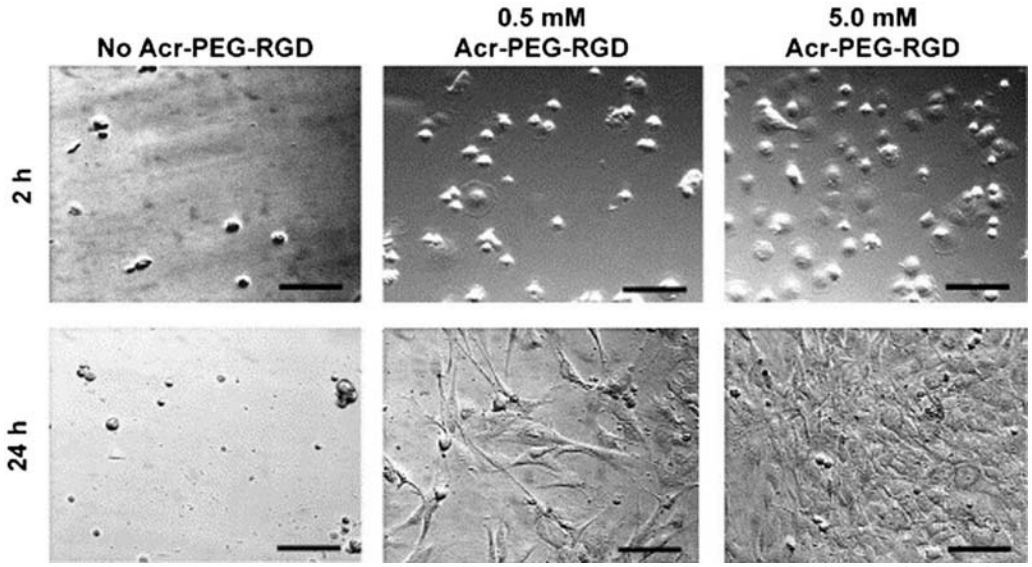

Figure 8.1 Light micrographs of attached osteoblasts after 2 and 24 h culture on PEGDA gels with no adhesive peptides (left images), with 0.5 mM RGD (central images), and with 5 mM RGD (right images). *PEGDA*, Poly(ethylene glycol)diacrylate; *RGD*, arginine–glycine–aspartic acid.
Source: Reprinted with permission from Burdick JA, Anseth KS. Photoencapsulation of osteoblasts in injectable RGD-modified PEG hydrogels for bone tissue engineering. Biomaterials 2002;23:4315–23. Available from: https://doi.org/10.1016/S0142-9612(02)00176-X. ©2002, from Elsevier.

Another important aspect is the effect of a spacer sequence to peptide exposure and capability to exert its biological function. The first outcomes on this topic date back to 1998, when Hern and Hubbel compared unspaced and PEG_{77} (3400 Da)-spaced RGD grafted to PEGDA backbone [23]. The presence of the spacer increased fibroblast spreading from 50% to 70% (only 5% spreading was observed in non-functionalized PEGDA gels), while PEGDA gels functionalized with unspaced RGD peptides did not exhibit any cell spreading when cells were cultured in serum-free conditions. Later, Wilson et al. further investigated the role of PEG spacer length on cell behavior, reporting that the concentration of RGD moieties required to support cell adhesion and spreading decreased with increasing PEG spacer length within the range PEG_5–PEG_{77}, thus suggesting that longer spacers make bioactive ligands more available for interactions with cells [24].
In addition to the spatial control on RGD sequence exposure, its temporally controlled presentation has been shown to influence cell behavior, allowing cells to exert specific functions. For instance, Lee et al. developed PEGDA hydrogels functionalized with RGD sequences modified with a light-sensitive moiety on the carboxylic terminal group of aspartic acid [25]. This caging group was successfully released upon hydrogel exposure to UV light (wavelength within 350–365 nm), thus making the RGD sequences available for interaction with the surrounding cells (Fig. 8.2).

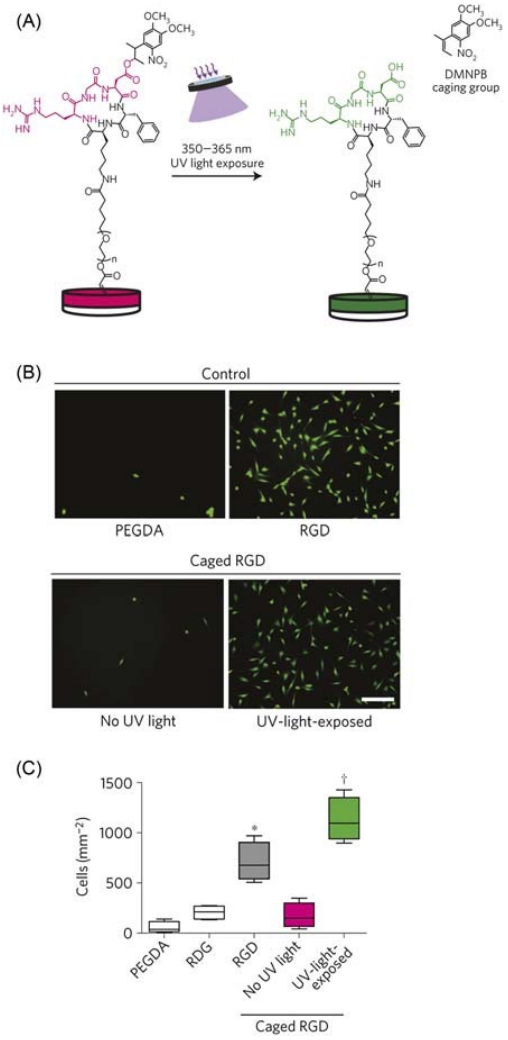

Figure 8.2 (A) Preparation of RGD-functionalized PEGDA hydrogel: removal of the photolabile protecting group 3-(4,5-dimethoxy-2-nitrophenyl)–2-butyl ester through system exposure to UV light. (B) Fluorescently labeled cells cultured on virgin PEGDA (control), free RGD-functionalized PEGDA, and caged RGD-functionalized PEGDA exposed or not-exposed to UV light. (C) Adherent cell density on virgin PEGDA, free RGD-functionalized PEGDA, free RDG-functionalized PEGDA (scrambled peptide), and caged RGD-functionalized PEGDA exposed or not-exposed to UV light. PEGDA, Poly(ethylene glycol)diacrylate; RGD, arginine–glycine–aspartic acid; RDG, arginine-aspartic acid-glycine.

Source: Reprinted with permission from Lee TT, García JR, Paez JI, Singh A, Phelps EA, Weis S, et al. Light-triggered in vivo activation of adhesive peptides regulates cell adhesion, inflammation and vascularization of biomaterials. Nat Mater 2015;14:352–60. Available from: https://doi.org/10.1038/nmat4157. ©2015, Springer Nature Publishing AG.

The potential of the approach was demonstrated by implanting the hydrogels subcutaneously in mice: successful exposure of RGD sequences was achieved through non-invasive transdermal UV-light irradiation for 10 minutes, with no skin damage. Moreover, timely triggered exposure of RGD moieties turned out to significantly impact the modulation of chronic inflammatory response and fibrosis: RGD exposure at 7 or 14 days from implantation induced the formation of a fibrotic capsule with approx. 50% lower thickness than hydrogels subjected to UV

irradiation and RGD exposure immediately after implantation. RGD exposure could also be regulated by using enzyme-cleavable peptide sequences. In this regard, Salinas and Anseth demonstrated the importance to trigger RGD sequence exposure in guiding human mesenchymal stem cell (hMSC) chondrogenesis, in a similar way as in natural stem cell niches [26,27]. hMSCs cultured in RGD-cleavable hydrogels produced 10-fold and 4-fold higher amounts of GAGs and collagen type II, respectively, compared to hydrogels with uncleavable RGD moieties [27]. To further increase hydrogel adhesiveness and to better mimic ECM composition, Gould et al. designed a new thiol-ene hydrogel exposing a combination of peptide sequences [28]. Specifically, with the final aim to assess the role exerted by biochemical cues in the formation of myofibroblasts from valvular interstitial cells (VICs), the authors developed a PEG-based hydrogel containing different amounts of P15, VGVAPG, and RGDS (glycine-threonine-proline-glycine-proline-glutamine-glycine-isoleucine-alanine-glycine-glutamine-arginine-glycine-valine-valine, valine-glycine-valine-alanine-proline-glycine and arginine-glycine-aspartic acid-serine, respectively) derived from collagen type I, elastin and fibronectin, respectively. The exposure of RGDS alone allowed a moderate α-smooth muscle expression (α-SMA), while the combination with elastin- and collagen-derived sequences significantly increased α-SMA expression, if compared to that obtained considering the whole ECM (control condition). These findings suggested that only those peptides belonging to the ECM were effectively responsible for VIC activation into myofibroblasts. Depending on the target cell behavior, other ligands have been also tested, such as the laminin-derived RKRLQVQLSIRT syndecan-1 binding ligand (arginine-lysine-arginine-leucine-glutamine-valine-glutamine-leucine-serine-isoleucine-arginine-threonine) to regulate the hemostatic functions of valve ECs [29] and the fusion proteins EphA5-Fc and EphrinA5-Fc which are involved in the regulation of both insulin secretion and β cell communication pathways [30]. With the aim of triggering cell infiltration and migration in the damaged area, matrix metalloproteinases (MMPs) activity has been usually mimicked. In detail, such enzymes are the main players in ECM degradation, allowing cells to migrate toward specific sites to organize a new tissue. A wide variety of MMP-sensitive substrates has been isolated from animal and human proteins and the possibility to finely modulate their degradation kinetics by changing their amino acid composition has been already reported by Nagase and Fields in 1996 [31]. Adhesivity and enzyme-sensitivity have thus been combined in a sole hydrogel to ensure MMP- and integrin-mediated cell homing and migration within gel network. For instance, Lutolf et al. demonstrated that human fibroblasts migrate within PEG hydrogels at a rate depending on MMP-mediated degradation, concentration of attachment ligands, and degree of crosslinking [32]. Similarly, Mann et al. designed a PEG hydrogel exposing both adhesive and proteolytically degradable peptides in order to guide hydrogel degradation by tissue formation processes [33]. Specifically, the authors selected the previously mentioned RGD sequence to improve cell adhesion and LGPA (leucine-glycine-proline-alanine) and 9-mer of alanine enzyme-sensitive peptides to achieve degradation by collagenase and elastase, respectively. Results showed that in the absence of one of these sequences, cell migration through the gel was not observed. Cell binding to adhesive peptides is necessary for cell migration and secretion of proteolytic enzymes; furthermore, the formation of pores facilitates cell migration mechanism. The same approach was exploited later by Phelps et al. that designed bioactive PEG hydrogels decorated with pendant adhesive RGD peptides and cross-linked with protease-sensitive GCRDVPMSMRGGDRCG peptide (glycine-cysteine-arginine-aspartic acid-valine-proline-methionine-serine-methionine-arginine-glycine-glycine-aspartic acid-arginine-cysteine-glycine) [34]. Kyburz and Anseth exploited a light-initiated thiol-ene reaction to design hMSC-embedded PEG-based gels of varying susceptibility to MMPs (by tuning the crosslinking degree) and adhesion properties (by modulating the amount of grafted CRGDS (cysteine-arginine-glycine-aspartic acid-serine) peptide sequences) [35]. Thiol groups of cysteine residues were exploited to react with PEG macromolecules functionalized with norbornene moieties. In order to make the gels degradable in response to biochemical

stimuli, peptide sequences susceptible to MMPs 1, 2, 3, 8, and 9 which are secreted by hMSCs were used to crosslink the systems.

Another important aspect is the design of immunomodulatory hydrogels to avoid the infiltration of proinflammatory cytokines and foreign body reaction. To this aim, antiinflammatory peptides have been used. For instance, Su et al. developed a PEG-based hydrogel for pancreatic islet encapsulation acting as a barrier against the infiltration of immunocytes and low molecular weight inflammatory factors due to the exposure of an inhibitory peptide for islet cell surface interleukin-1 (IL-1) receptor (IL-1RIP, phenylalanine-glutamic acid-tryptophan-threonine-proline-glycine-tryptophan-tyrosine-glutamine-proline-tyrosine-NH2, FEWTPGWYQPY-NH2) [36]. IL-1RIP functionalized PEG hydrogels reduced the death of loaded cells to 60% compared to not-modified gels, confirming peptide function against inflammation. Moreover, the coexposure of IL-1RIP and RGD sequence further enhanced anticytokine effects (Fig. 8.3). On the other hand, grafted peptides did not reduce cell ability to secrete insulin in response to changes in glucose concentration; the presence of IL-1RIP sequence further induced insulin secretion by encapsulated cells.

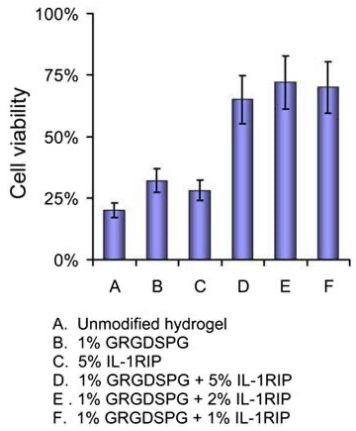

Figure 8.3 Pancreatic cells encapsulated in ligand-functionalized hydrogels to investigate anti-inflammatory peptides preservation against cell death induced by cytokines. 24 h post encapsulation, cells were treated with IL-1β, TNF-α, and INF-γ for 2 h and cell death was investigated through LIVE/DEAD assay. Cell encapsulation within RGD- and IL-1RIP-modified hydrogels significantly increased their viability compared to cells encapsulated in hydrogels exposing only one or no peptide. *RGD*, Arginine–glycine–aspartic acid; *TNF-α*, tumor necrosis factor α*; INF-γ, interferon gamma.*

Source: Reprinted with permission from Su J, Hu B-H, Lowe WL, Kaufman DB, Messersmith PB. Anti-inflammatory peptide-functionalized hydrogels for insulin-secreting cell encapsulation. Biomaterials 2010;31:308–14. Available from: https://doi.org/10.1016/j.biomaterials.2009.09.045. ©2010, Elsevier.

During the same years, Lin et al. carried out similar investigations designing a PEG-based hydrogel functionalized with the highly specific tumor necrosis factor α (TNF-α) binding sequence, WP9QY (YCWSQYLCY, tyrosine-cysteine-tryptophan-serine-glutamine-tyrosine-leucine-cysteine-tyrosine) [37]. The ability of WP9QY peptide to preserve the viability of loaded cells was demonstrated using three different cell types, that is, adrenal pheochromocytoma cells from rats (PC12s), mouse pancreatic islets, and hMSCs. Results revealed that peptide-functionalized PEG gels prolonged PC12 cell and mouse islet survival and functionality, thus demonstrating the capability of the designed systems to modulate local inflammation. For what concerns hMSC encapsulation,

WP9QY peptide grafting hindered hMSC proliferation induced by TNF-α and did not alter their potential to undergo osteogenic differentiation.

8.2.2 Peptide functionalization of hydrogels for cardiac tissue engineering

Hydrogels have been widely investigated in cardiac regeneration over the last few decades. According to the classification proposed by Reis et al. [38], injectable hydrogels are usually applied in cardiac TE to (1) induce endogenous repair through the recruitment of endogenous cells, the retention of cell survival and the stimulation of cell proliferation, differentiation and neovascularization processes; (2) promote exogenous regeneration through cell therapy approaches (the hydrogel is used as cell carrier); or (3) provide a physico-mechanical support to the injured heart region with the aim to keep and restore wall thickness and heart geometry and, as a consequence, improve heart function (acellular hydrogels). Beyond this classification, hydrogels are often designed to be multifunctional to make them able to exert more than one function.

Hydrogels aiming at inducing and guiding endogenous or exogenous cardiac regeneration are usually based on bioactive biomaterials provided with specific moieties along their backbone or able to release biomolecules (e.g., angiogenic, antiapoptotic, immunomodulatory molecules [39]) to the injured area. Bioactive biomaterials are usually obtained by grafting properly selected peptide sequences to native polymer backbone through functionalization procedures. The variety of peptides investigated to functionalize hydrogels for cardiac application can be categorized into three main classes: (1) antiapoptotic and cardioprotective sequences (e.g., QHREDGS (glutamine-histidine-arginine-glutamic acid-aspartic acid-glycine-serine), glutathione); (2) adhesive and proangiogenic peptides (e.g., RGD, GFOGER (glycine-phenylalanine-hydroxyproline-glutamic acid-arginine), YPHIDSLGHWRR (tyrosine-proline-histidine-isoleucine-aspartic acid-serine-leucine-glycine-histidine-tryptophan-arginine-arginine, -RoY) peptide); and (3) cardiac phenotype inducers (e.g., Notch1 ligand Jagged1 mimicking peptide).

In cardiac TE, cellular therapies exploiting cardiac progenitor cells (CPCs) represent a promising strategy to induce infarcted cardiac tissue restoration due to their ability to differentiate toward the cardiac, endothelial, and vascular smooth muscular phenotypes as well as their paracrine effects [40]. However, poor retention of injected cells and the low survival in the hostile infarcted environment have limited the use of CPCs in the clinics. Bioactive hydrogels could overcome these drawbacks, providing the cells with a friendly biomimetic environment. In this context, self-assembling peptide hydrogels were functionalized with a peptide mimicking the Notch1 ligand Jagged1 (RJ, H2N-CDDYYYGFGCNKFCRPR-OH, H2N-cysteine-aspartic acid-aspartic acid-tyrosine-tyrosine-tirosine-glycine-phenylalanine-glycine-cysteine-asparagine-lysine-phenylalanine-cysteine-arginine-proline-arginine-OH) to trigger the Notch signaling pathway, which is activated in the early stages of cardiac development as well as in CPC survival and differentiation pathways [41,42]. Injection of CPC-loaded or -free RJ-functionalized hydrogels in murine model of myocardial infarction improved heart functionality recovery (improvements in cardiac output, ejection fraction, stroke volume, stroke work), contractility, and neovascularization of the infarcted area, accompanied with decreased fibrosis and increased CM cell cycle activity (increased expression of Ki67) compared to untreated animals or rats subjected to injection of virgin hydrogels or hydrogels grafted with a~~n~~ nonfunctional peptide sequence (RS, H2N-RCGPDCFDNYGRYKYCF-OH, H2N-arginine-cysteine-glycine-proline-aspartic acid-cysteine-phenylalanine-aspartic acid-asparagine-tyrosine-glycine-arginine-tyrosine-lysine-tyrosine-cysteine-phenylalanine-OH) (Fig. 8.4).

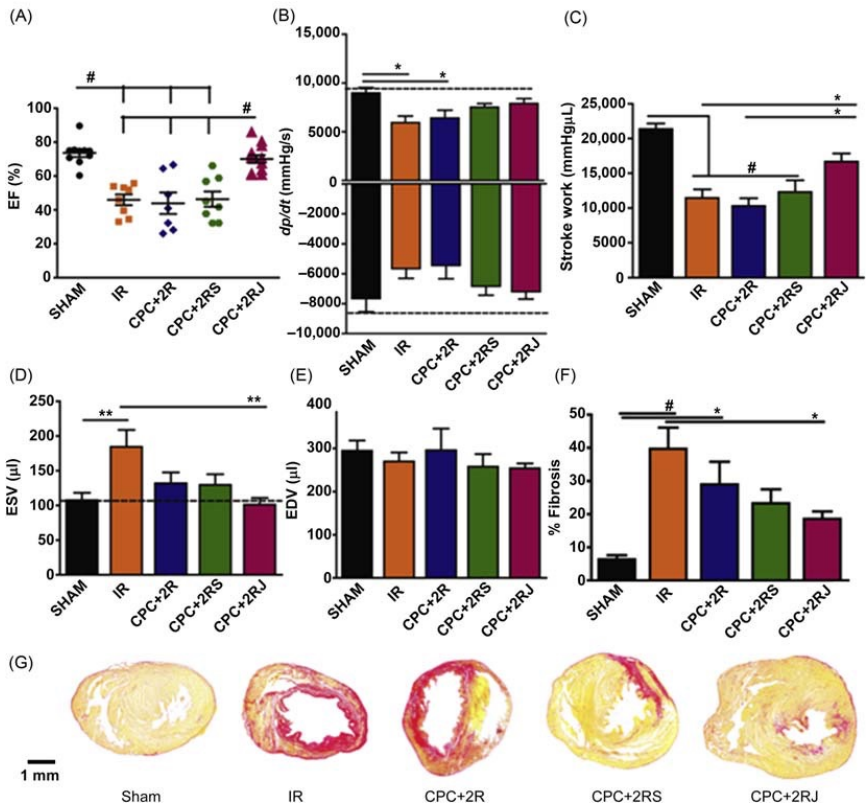

Figure 8.4 Injection of CPC-loaded SAP hydrogel (2% w/v) functionalized or not-functionalized with peptides (2-R: not-functionalized, 2RJ: functionalized with RJ, 2RS: functionalized with RS). Comparison with sham-operated animals (SHAM, i.e., subjected to placebo surgery) or animals subjected to ischemia-reperfusion procedure (IR). (A) EF%, (B) rate of left ventricular pressure increase (*dP*/*dt*), (C) stroke work, (D) ESV, (E) EDV, (F) percentage of fibrosis (% Fibrosis), (G) Pico-Sirius red-stained heart sections. *CPC*, Cardiac progenitor cell; *EDV*, end diastolic volume; *EF*, ejection fraction; *ESV*, end systolic volume; *SAP*, self-assembling peptide. *Source: Reprinted with permission from Boopathy AV, Che PL, Somasuntharam I, Fiore VF, Cabigas EB, Ban K, et al. The modulation of cardiac progenitor cell function by hydrogel-dependent Notch1 activation. Biomaterials 2014;35:8103–12. Available from: https://doi.org/10.1016/j.biomaterials.2014.05.082. ©2014, Elsevier.*

With the aim to enhance CPC retention within the hydrogels, thus protecting them from the hostile environment of the infarcted area, Bhutani et al. [43] encapsulated CPCs into PEG-based hydrogels grafted with the RGD and the GFOGER (collagen-mimetic peptide) sequences. Both RGD- and GFOGER-grafted gels exhibited higher cell adhesion compared to hydrogels grafted with the nonadhesive RDG sequence used as control (approx. five-fold increase in cell adhesion). Surprisingly, *in vitro* CPCs underwent cardiac differentiation accompanied by a reduction in the secretion of reparative paracrine factors when cultured in GFOGER-grafted hydrogels, probably as a consequence of their biomimetic mechanical properties and the biological signaling pathways initiated by GFOGER sequence. However, unexpectedly, *in vivo* the best recovery in cardiac function was observed upon injection of RDG-exposing hydrogels loaded with CPCs, which also showed the best retention of transplanted cells. These findings have thus opened a new chapter in the field, suggesting that (1) cellularized hydrogels exposing adhesive peptides along their backbone could elicit a higher immune response upon injection *in vivo* leading to hydrogel degradation and possible cell death, and (2) grafted adhesive peptides could block CPC integrins thus limiting their engagement with the surrounding tissue and engraftment. Although the role of

adhesion ligands on hydrogels for cell delivery to the infarcted area has been criticized, their use in acellular hydrogels has been reported to favor ventricular function recovery and angiogenesis (significantly higher arteriole density compared to unmodified gel) as a consequence of integrin-ligand interactions that stimulate tissue regeneration [44].

The capability of RGD-functionalized hydrogels to interact with cell receptors and enhance cell adhesion has been also exploited by Plouffe et al. to design a new RGD-functionalized alginic acid coating able to control the capture and release of cardiac fibroblasts within a microfluidic system [45]. RGD exposure improved the capture of cardiac fibroblasts flowing within the system (two-fold higher compared to unmodified alginic acid hydrogel), meanwhile hydrogel dissolution under mild conditions made cell release easy, thus providing viable cells for further applications.

The goal of improving angiogenesis and cardiac repair upon myocardial infarction was also achieved through functionalization of hydrogel forming material with the so-called RoY peptide [46]. RoY peptide has been reported to interact with GRP78 receptor which is overexpressed by vascular ECs in hypoxia conditions (a typical condition of the infarcted heart), thus activating cell survival and proliferation pathways. As a matter of fact, RoY-functionalized chitosan (CH) chloride-based gels promoted the survival and proliferation of human umbilical vein ECs as well as their organization into tubular constructs. Results obtained *in vitro* were further confirmed *in vivo* upon gel injection in a myocardial infarction rat model: animals treated with RoY-grafted hydrogels showed increased angiogenesis and, as a consequence, improved recovery of cardiac function compared to rats subjected to injection of saline solution or virgin gel (Fig. 8.5).

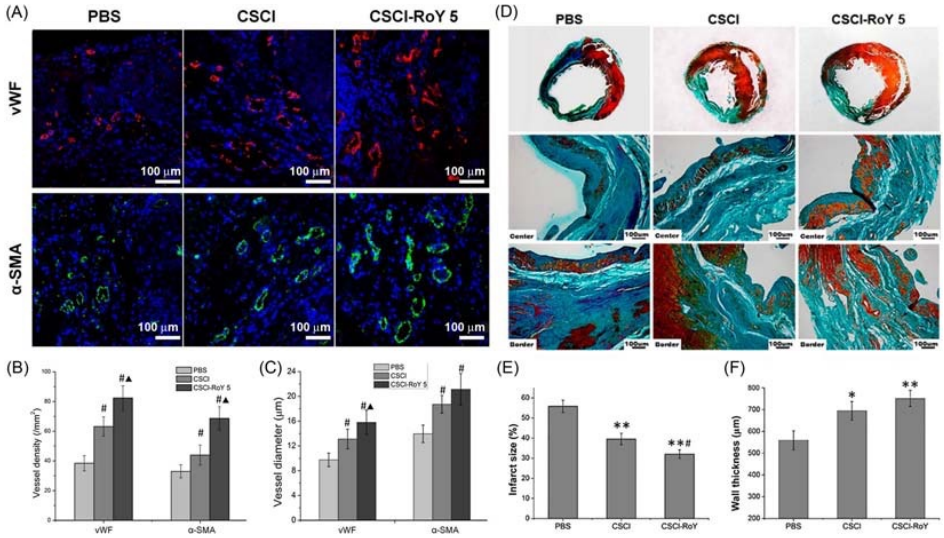

Figure 8.5 Angiogenesis in the infarcted area of rats 28 days postsurgery: (A) myocardial sections from hearts of animals treated with saline (PBS, phosphate buffered saline, pH 7.4), virgin CSCl, and CSCl-RoY 5 stained with vascular specific antibodies [vWF (red) and α-SMA (green)]; (B and C) vessel density and diameter. Myocardial structures of the infarcted region 28 days postsurgery: (D) cardiac tissue stained with Masson's trichrome staining; (E and F) quantitative evaluation of infarct size and infarct wall thickness. *CSCl*, Chitosan chloride gel; *CSCl-RoY 5*, RoY-functionalized chitosan chloride gel; *vWF*, Von Willebrand factor; *α-SMA*, α-smooth muscle expression.

Source: Reprinted with permission from Shu Y, Hao T, Yao F, Qian Y, Wang Y, Yang B, et al. RoY peptide-modified chitosan-based hydrogel to improve angiogenesis and cardiac repair under hypoxia. ACS Appl Mater

Interfaces 2015;7:6505–17. Available from: https://doi.org/10.1021/acsami.5b01234. ©2015, American Chemical Society.

The same bulk material was also used to design antioxidant hydrogels with scavenging activity toward the reactive oxygen species overproduced after a myocardial infarction, which represent one of the main obstacles for a successful cardiac regeneration [47]. In detail, scavenging potential was provided to CH chloride by grafting glutathione (glutamic acid-cysteine-glycine tripeptide), which has been reported to favor cell adhesion and protect cells from oxidative stress [48,49].

Cardioprotective and prosurvival features were also obtained by grafting CH with the integrin-binding motif of angiopoietin-1 growth factor (QHREDGS) that was found to promote CM adhesion and survival in a similar way as the full-length molecule. QHREDGS-grafted CH hydrogels promoted CM survival and adhesion at similar levels as RGDS-grafted hydrogels (used as positive control). However, these newly designed hydrogels showed a superior ability of preserving cells from apoptosis, promoting both elongation and contractile apparatus assembly [50,51]. *In vivo*, a peptide concentration-dependent response was observed: a higher number of recruited myofibroblasts and viable CMs were detected after subcutaneous injections of CM-loaded hydrogels prepared from CH grafted with a higher amount of QHREDGS (approx. 650 nmol of peptide per gel mL) [52]. In addition, a higher number of beating CMs were obtained with increasing peptide grafting to CH chains. Hydrogels injected in a myocardial infarction rat model remained *in situ* for approximately 3 weeks and induced significant improvements in cardiac morphological and functional features compared to control conditions (i.e., animal injected with PBS) and not-functionalized hydrogels). In more detail, scar thickness, fractional shortening, and ejection fraction improved by 53%, 35%, and 62%, respectively, meanwhile a 34% decrease in fractional scar area was observed [53].

All the above studies demonstrated the key role of ECM-like hydrogels in properly guiding the fate of encapsulated and host cells. To this purpose, one widely adopted strategy is to graft bioactive ligands to natural and synthetic hydrogels, exploiting their exposed functional groups, as summarized in Table 8.1.

Table 8.1 Widely adopted peptide ligands to mimic the natural extracellular matrix in the design of functional hydrogels.

Peptide sequence	Biological function	Hydrogel material	application
General applications			
RGD	Adhesiveness	PEG	Hydrogel loaded with osteoblasts [22]
RGD	Adhesiveness	PEGDA	Evaluation of spacer length on peptide efficacy [23]
RGD	Adhesiveness	PEGDA	Hydrogels with biological function controlled through UV irradiation [25]
RGD+MMP-13 linker	Adhesiveness and enzymatic sensitivity	PEGDA	Hydrogel sensitive to enzymatic degradation for hMSC differentiation in chondrocytes [27]
GRGDSPC+GCRDVPMSMRGGDRCG	Adhesiveness and enzymatic sensitivity	PEG	Hydrogel sensitive to protease enzymatic activity [34]

RGDS+VGVAPG+P15	Adhesiveness	PEG	Hydrogels exposing multiple peptide sequences for VIC activation into myofibroblasts [28]
RKR	Hemostatic control	PEGDA	Controlled hemostatic function of valve endothelial cells [29]
EphA5-Fc EphrinA5-Fc	Control over insulin secretion	PEG	Controlled insulin secretion from encapsulated β cells [30]
RGD+LGPA	Adhesiveness and enzymatic sensitivity	PEG	Hydrogels sensitive to collagenase [33]
RGD+9AK	Adhesiveness and enzymatic sensitivity	PEG	Hydrogels sensitive to elastase [33]
RGD+KCGPQG↓IWGQCK	Adhesiveness and enzymatic sensitivity	PEG-norbornene	hMSC-loaded hydrogels with increased adhesiveness and controlled degradation [35]
RGD+IL-1RIP	Adhesiveness and antiinflammatory properties	PEG	Antiinflammatory hydrogels for pancreatic islet transplantation [36]
WP9QY	Antiinflammatory properties	PEG	Cell-loaded hydrogels with enhanced protection against TNF-α-induced damage [37]
Cardiac applications			
RGD	Adhesiveness	Alginic acid	Adhesive coating for microfluidic systems [45]
Jagged 1-mimetic peptide	Trigger for Notch	Self-assembling peptides	Self-assembling peptide hydrogels to activate Notch signaling pathway [42]
RoY peptide	Angiogenesis	Chitosan chloride	Injectable bioactive hydrogels to stimulate angiogenesis and improve cardiac function after myocardial infarction [46]
Angiopoietin-1 peptide QHREDGS	Cardioprotection and prosurvival	Photocrosslinkable azidobenzoic acid modified chitosan	Hydrogels for heart cells attachment and survival [51]
		Chitosan/collagen blend	Injectable hydrogels for cardiac cell culture and delivery [52]
			Acellular hydrogel for cardiac functional and morphological recovery upon myocardial infarction [53]
RGD	Angiogenesis	Alginate	Cell-free injectable gels with proangiogenic properties [44]

GFOGER	Adhesiveness	PEG	CPC-loaded hydrogels to be locally injected in the infarcted region [43]
RGD	Adhesiveness	PEG	CPC-loaded hydrogels to be locally injected in the infarcted region [43]
Glutathione	Reactive oxygen species scavenging	Chitosan	Injectable hydrogel to suppress oxidative stress damages [47]
GRGDSPC+GCRDVPMSMRGGDRCG	Adhesiveness, enzymatic sensitivity, triggered growth factor release	PEG	Hydrogels sensitive to protease cleavage for localized delivery of growth factors in infarcted area [34]

CPC, Cardiac progenitor cell; *hMSC*, human mesenchymal stem cell; *MMP*, matrix metalloproteinase; *PEG*, poly(ethylene glycol); *PEGDA*, poly(ethylene glycol)diacrylate; *RGD*, arginine–glycine–aspartic acid; *TNF-α*, tumor necrosis factor α; *VIC*, valvular interstitial cell; *GRGDSPC,* glycine-arginine-glycine-aspartic acid-serine-proline-cysteine; *GCRDVPMSMRGGDRCG*, glycine-cysteine-arginine-aspartic acid-valine-proline-methionine-serine-methionine-arginine-glycine-glycine-aspartic acid-arginine-cysteine-glycine; *RKR*, arginine-lysine-arginine; *KCGPQG↓IWGQCK*, lysine-cysteine-glycine-proline-glutamine-glycine↓isoleucine-tryptophan-glycine-glutamine-cysteine-lysine; *P15*, glycine-threonine-proline-glycine-proline-glutamine-glycine-isoleucine-alanine-glycine-glutamine-arginine-glycine-valine-valine; *VGVAPG*, valine-glycine-valine-alanine-proline-glycine; *RGDS*, arginine-glycine-aspartic acid-serine; *RKRLQVQLSIRT*, arginine-lysine-arginine-leucine-glutamine-valine-glutamine-leucine-serine-isoleucine-arginine-threonine; *LGPA*, leucine-glycine-proline-alanine; *WP9QY*, tyrosine-cysteine-tryptophan-serine-glutamine-tyrosine-leucine-cysteine-tyrosine); IL-1RIP, phenylalanine-glutamic acid-tryptophan-threonine-proline-glycine-tryptophan-tyrosine-glutamine-proline-tyrosine-NH2; *Jagget 1-mimetic peptide*, H2N-cysteine-aspartic acid-aspartic acid-tyrosine-tyrosine-tirosine-glycine-phenylalanine-glycine-cysteine-asparagine-lysine-phenylalanine-cysteine-arginine-proline-arginine-OH; *Roy*, tyrosine-proline-histidine-isoleucine-aspartic acid-serine-leucine-glycine-histidine-tryptophan-arginine-arginine; *QHREDGS*, glutamine-histidine-arginine-glutamic acid-aspartic acid-glycine-serine; *GFOGER*, glycine-phenylalanine-hydroxyproline-glutamic acid-arginine; glutathione, glutamic acid-cysteine-glycine; *9AK*, alanine-alanine-alanine-alanine-alanine-alanine-alanine-alanine-alanine-lysine.

Finally, in a different approach, ligand-receptor interactions have been also exploited to drive the sol-to-gel transition [54] and peptide sequences have been used as crosslinking moieties among polymer chains [34,35,55]. This latter approach has been exploited to design hydrogels susceptible to a triggered degradation by specific enzymes with the final aim of locally releasing cells or biomolecules. For instance, Salimath et al. loaded hepatocyte and vascular endothelial growth factors (HGF and VEGF, respectively) into PEG hydrogels grafted with RGD moieties and crosslinked with a protease-cleavable peptide sequence (GCRDVPMSMRGGDRCG, glycine-cysteine-arginine-aspartic acid-valine-proline-methionine-serine-methionine-arginine-glycine-glycine-aspartic acid-arginine-cysteine-glycine) and delivered them to the border zone of the infarcted region in rat myocardium [55]. HGF and VEGF release from the hydrogel increased angiogenesis and stem cell recruitment from the surrounding tissue, and significantly decreased fibrosis compared to the injection of a single growth factor (either encapsulated in the hydrogel or delivered as free growth factor solution), virgin PEG hydrogel, or HGF/VEGF mixture (Fig. 8.6).

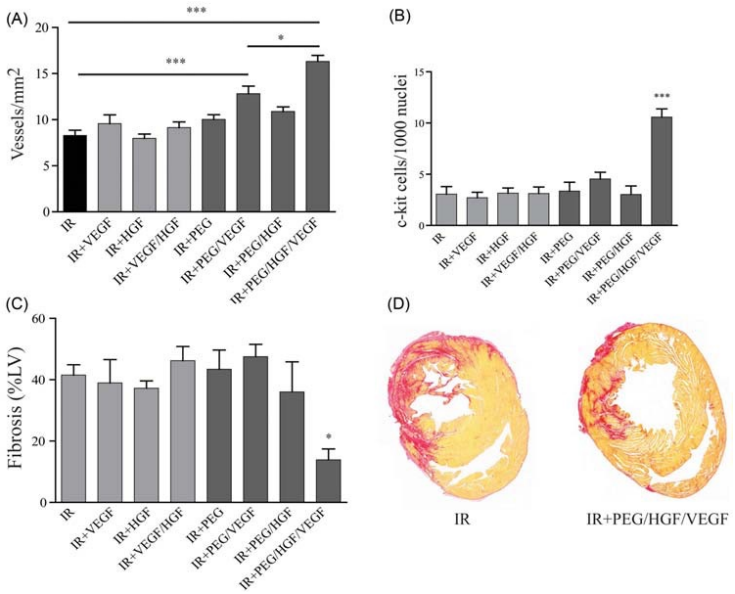

Figure 8.6 Evaluation of angiogenesis (expressed vessels/mm^2) (A), c-kit positive cells (i.e., progenitor cells) (B), and fibrosis (C) expressed as percentage of the total LV area in the infarcted region of rats, assessed 21 days post myocardial infarction. (D) Representative heart sections stained for collagen (index of fibrosis) with picrosirius red (*red stain*, 206×). Group nomenclature: IR: rats subjected to IR surgery (control); IR+VEGF: IR-rats treated with VEGF aqueous solution; IR+HGF: IR-rats treated with HGF aqueous solution; IR+VEGF/HGF: IR-rats treated with VEGF/HGF aqueous solution; IR+PEG: IR-rats treated with PEG gel; IR+PEG/VEGF: IR-rats treated with VEGF-loaded PEG gel; IR+PEG/HGF: IR-rats treated HGF-loaded PEG gel; IR+PEG/VEGF/HGF: IR-rats treated with VEGF/HGF-loaded PEG gel. *HGF*, Hepatocyte growth factor; *IR*, ischemia-reperfusion; *LV*, left ventricular; *PEG*, poly(ethylene glycol); *VEGF*, vascular endothelial growth factor.

Source: Adapted from Salimath AS, Phelps EA, Boopathy AV, Che P, Brown M, García AJ, et al. Dual delivery of hepatocyte and vascular endothelial growth factors via a protease-degradable hydrogel improves cardiac function in rats. PLoS One 2012;7:e50980. Available from: https://doi.org/10.1371/journal.pone.0050980.

8.3 Ligand surface functionalization in the design of scaffolds and implants

Scaffolds play a crucial role in TE and act as a support for the growth of new tissues, promoting cell adhesion and proliferation. For this reason, TE is aimed at designing and fabricating scaffolds mimicking the ECM of the tissue to be engineered. Biomimetic mechanical properties, chemical composition, and architecture are the target requirements for TE scaffolds [56]. Considering the ECM composition, different natural polymers, such as proteins and polysaccharides, have been used as scaffold materials; however, they suffer from poor mechanical properties and stability in physiological environment. On the other hand, the main advantages of synthetic polymers are their superior mechanical properties, tailored degradation rate, and processability; however, they do not possess any functionality recognized by the cells for integrin activation [57]. "Bioartificial materials" are materials based on synthetic and natural polymers or bioactive peptides combining their properties. Surface modification approaches are among the possible methods to introduce bioactive molecules on the surface of synthetic polymer substrates, without affecting the material bulk properties [58].

As discussed in the previous sections, RGD sequence has been widely investigated with the aim to enhance cell adhesion in many biomedical fields, such as bone and cardiovascular applications [59]. Titanium (Ti) and its alloys are important materials in orthopedic implant surgery, due to their biocompatibility with tissues and excellent

mechanical properties; however, in clinical practices the osteointegration of orthopedic implants is often incomplete, resulting in a high risk of implant loosening over time. RGD peptide may improve the implant osteointegration [60]. Chua et al. [61] designed a multilayered coating able to combine antibacterial properties and cell adhesion. They performed a layer-by-layer coating on Ti substrate surface using hyaluronic acid (HA) and CH as polyelectrolytes. After the deposition of five HA/CH bilayers, with CH as the outermost layer, they covalently grafted RGD peptide via carbodiimide chemistry. Results demonstrated that the HA/CH coating showed antibacterial efficacy and, only in the case of RGD covalent grafting, the surface modification showed a positive influence on osteoblast adhesion and proliferation.

This section will be focused on the surface functionalization of cardiovascular implants, including coronary stents and scaffolds for myocardial regenerations.

Surface-induced thrombosis and in-stent restenosis cause the major clinical failures of cardiovascular stents. The formation of a functionally intact endothelium on the implant could inhibit growth of neointimal tissue after percutaneous coronary intervention and prevent thrombosis [62]. Many efforts were addressed to enhance and accelerate stent re-endothelialization, by surface functionalization with specific peptides. Li et al. [63] synthesized a Gly–Arg–Gly–Asp–Ser–Pro (GRGDSP) peptide coupled with photoactive 4-benzoylbenzoic acid, that was grafted on the surfaces of poly(carbonate urethane)s (PCUs) by UV irradiation, with the purpose to improve re-endothelialization of small-diameter vascular grafts. The proliferation and spreading of adherent ECs on modified PCU surfaces increased with increasing the concentration of the peptide. Moreover, the retention of ECs on the functionalized PCU was higher compared to the uncoated PCU under flow shear stress conditions. In conclusion, GRGDSP grafted on the surface of small-diameter vascular grafts and functional tissue engineered small-diameter blood vessels was demonstrated to be effective in enhancing re-endothelialization. However, RGD-peptide is recognized by approximately half of the integrin cell receptors and it has been found to promote platelets, ECs~~s~~, and smooth muscle cells (SMCs~~s~~) adhesion. Therefore RGD-based peptides are not able to support *in vivo* selective adhesion and proliferation of ECs [64]. The REDV (arginine–glutamic acid–aspartic acid–valine) fibronectin-derived peptide is recognized by α4β1 integrins and has been reported to selectively promote EC adhesion and spreading over SMCs and platelets [65]. Ceylan et al. [66] developed a peptide-based self-assembled nanofibrous coating functionalized with REDV. Their results showed that REDV functionalization provided selective growth of ECs on the stainless steel surface, as shown in Fig. 8.7. Plouffe et al. [67] exploited the ability of REDV peptide toward selective EC attachment in polydimethylsiloxane microfluidic devices. Microfluidic devices coated with REDV were used for the adhesion-based separation of ECs from heterogeneous suspensions containing ECs, SMCs, and fibroblasts. The adhesion of ECs on REDV-coated devices was significantly higher ~~with respect to~~ than the other cell types. Therefore REDV was confirmed to be a selective peptide favoring EC adhesion respect to SMCs. Other studies demonstrated that REDV may hinder the adhesion of platelets [68].

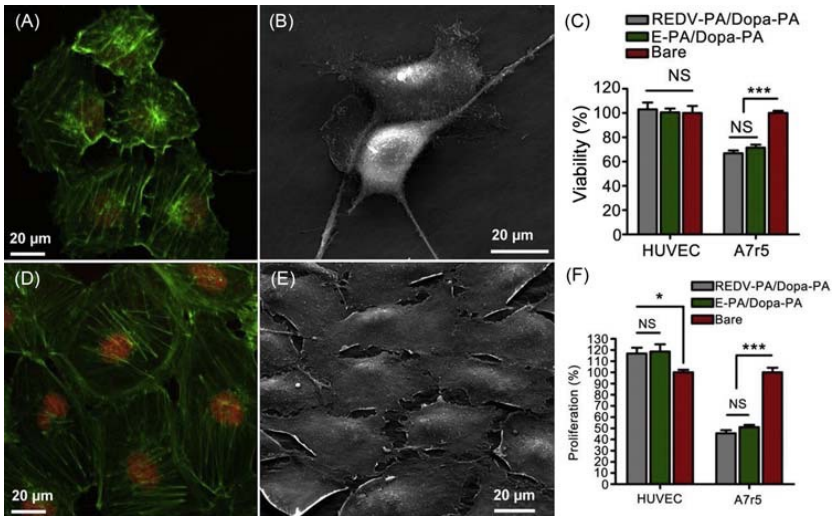

Figure 8.7 (A,B,D,E) HUVECs preserved their morphology and formed filamentous actin-based stress fibers after 24 and 72 h on REDV-PA/Dopa-PA network. (C) HUVECs were completely viable on both PA surfaces compared to the bare steel surface. On the contrary, A7r5 rat aortic smooth muscle cells showed decreased viability on coated steel surfaces compared to the bare steel surface. (F) HUVECs proliferation was higher on both PA-coated surfaces while the proliferation of A7r5 cells decreased significantly on the PA networks. ***$P<.0001$, *$P<.05$. *Dopa*, 3,4-Dihydroxyphenylalanine; *HUVECs*, human umbilical vein endothelial cells; *PA*, peptide-based self-assembled.

Source: Reprinted from Ceylan H, Tekinay AB, Guler MO. Selective adhesion and growth of vascular endothelial cells on bioactive peptide nanofiber functionalized stainless steel surface. Biomaterials 2011;32:8797–805. Available from: https://doi.org/10.1016/j.biomaterials.2011.08.018, ©2011, with permission from Elsevier.

Unlike the RGD peptide, which is present in many ECM proteins and binds a large number of cellular integrins, including αIIbβ3 on platelets as well as αvβ3 and α5β1 on ECs, CRRETAWAC is a non-~~un~~natural peptide, identified from a phage display library for its interaction with human α5β1 integrin [69,70]. Larsen et al. [71] functionalized polytetrafluoroethylene (PTFE) vascular grafts with CRRETAWAC. In addition, Meyers et al. [72] used CRRETAWAC as a bioactive stent coating. Dudash et al. [73] reported the cross-reactivity of the cell adhesive peptide CRRETAWAC between human and porcine ECs (hECs and pECs). In this study, they demonstrated that CRRETAWAC peptide is capable of binding pECs specifically, with pECs growing then similarly compared to hECs. *In vitro* validation of the porcine model is critical for ensuring effective validation for *in vivo* testing of CRRETAWAC-coated PTFE vascular grafts.

Concerning scaffolds for cardiac regeneration, functionalization with RGD which is present in several adhesion proteins, such as fibronectin, vitronectin, laminin, and collagen type I, is of crucial relevance. Schussler et al. [74] investigated an *in vitro* method to improve the contractile properties of CMs seeded on a collagen scaffold. In particular, they modified commercially available collagen scaffolds (Avitene Ultrafoam hemostat sheets) with GRGDS by covalent coupling. As shown in Fig. 8.8, results indicated that contractility in cell-seeded collagen scaffolds was significantly improved by the covalent grafting of GRGDS to collagen scaffolds, probable due to the increased availability of ligands for the αvβ5, αvβ3, and α5β1 integrins. Moreover, the improvement of cell adhesion, survival, growth, and differentiation of CMs in GRGDS scaffolds enhanced mechanical performance of the constructs. Such scaffolds appeared promising for future clinical applications.

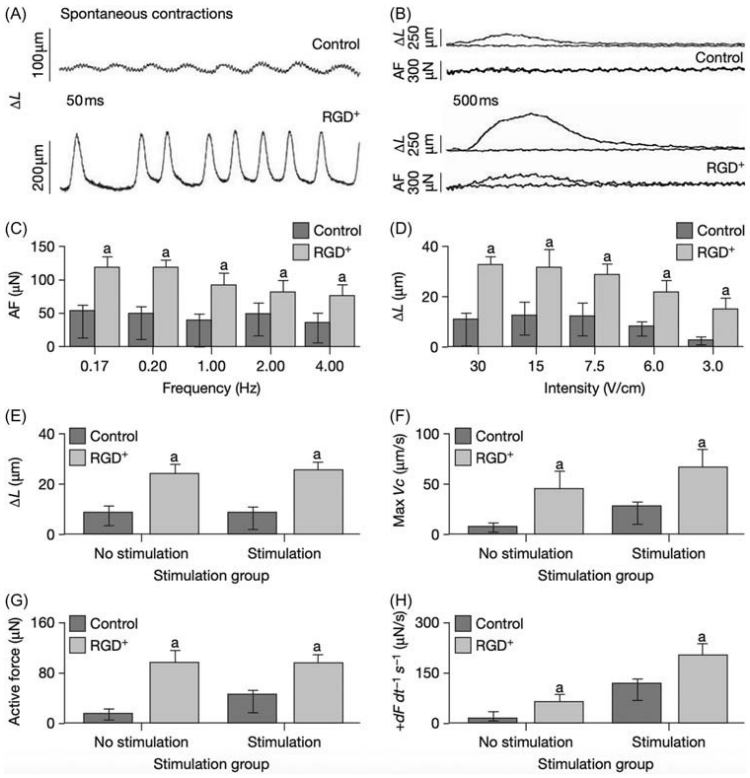

Figure 8.8 Contractile performance in GRGDS (RGD+) functionalized and control scaffolds seeded with cardiomyocytes. (A) Muscle shortening length as a function of time. (B) Muscle shortening length and AF of contractions at 0.17 Hz electrical stimulation frequency. (C) Effects of electrical stimulation frequency on AF. (D) Effects of electrical stimulation intensity on maximum extent of muscle shortening (Δ*L*). (E) Contractile performance expressed as maximum extent of muscle shortening. (F) Contractile performance expressed as maximum shortening velocity (V_c) at preload. (G) Contractile performance expressed as AF. (H) Contractile performance expressed as positive peak of the force derivative ($+dF\ dt^{-1}\ s^{-1}$). Values are means±std. dev., (A) $P<.05$. *AF*, Active force; *RGD*, arginine–glycine–aspartic acid *GRGDS*, glycine–arginine–glycine–aspartic acid–serine–proline.

Source: Reprinted from Schussler O, Coirault C, Louis-Tisserand M, Al-Chare W, Oliviero P, Menard C, et al. Use of arginine-glycine-aspartic acid adhesion peptides coupled with a new collagen scaffold to engineer a myocardium-like tissue graft. Nat Clin Pract Cardiovasc Med. 2009;6:240–9. Available from: https://doi.org/10.1038/ncpcardio1451, ©2009, with permission from Springer Nature.

Rosellini et al. [75] covalently grafted on poly(ε-caprolactone) (PCL) substrates two penta-peptides: GRGDS from fibronectin, and YIGSR (Tyrosine-Isoleucine-Glycine-Serine-Arginine) from laminin. GRGDS peptide was shown to promote the adhesion of C2C12 myoblasts, to stimulate integrin receptors relevant in early cardiac development (α5β1, αvβ3) and to promote cell proliferation. On the other hand, YIGSR mainly promoted C2C12 myoblast differentiation, as shown by the appearance of multinucleated myotubes even in the absence of a differentiation medium.

Hayoun-Neeman et al. [76] developed functionalized alginate scaffolds able to induce the differentiation of human embryonic stem-derived CMs in order to obtain functional cardiac tissues. The macroporous alginate scaffolds were modified with two different peptides: RGD and heparin-binding peptide, to mediate cell–matrix

interaction by both an integrin-dependent and independent mechanism, respectively. The authors demonstrated that the presence of both peptide types was needed for functional tissue development.
The same approach based on alginate functionalization with RGD peptide was successfully investigated by Shachar et al. [77]. They obtained 3D porous scaffolds through freeze–drying technique using both neat alginate and alginate grafted with RGD via carbodiimide chemistry. They demonstrated that the immobilization of RGD peptide into 3D porous alginate scaffolds enhanced the formation of functional cardiac tissue.
Table 8.2 summarizes relevant examples of scaffolds and implants surface-functionalised with peptide-ligands for different applications, including the cardiovascular field.

Table 8.2 Widely adopted peptide ligands in the surface functionalization of scaffolds and implants to guide cell behavior.

Peptide sequence	Biological function	Material	Application
General applications			
RGD	Adhesiveness	Ti substrate	Multilayered HA/CH coating with antibacterial and cell adhesive properties [61]
Vascular applications			
GRGDSP	Proliferation and spreading of ECs	Poly(carbonate urethane)s	Reendothelialization of small-diameter vascular grafts [63]
REDV	Selectivity for ECs attachment	Self-assembled functional peptides used as coatings for stainless steel	Reendothelialization of metal vascular implants [66]
REDV	Selectivity for ECs attachment	PDMS microfluidic devices	Adhesion-based separation of ECs from heterogeneous cell suspensions [67]
CRRETAWAC	Selectivity for ECs attachment	PTFE vascular grafts	Reendothelialization of vascular prostheses [71]
CRRETAWAC	Selectivity for ECs attachment	Preliminary coating of culture plates	Reendothelialization of stents [72]
CRRETAWAC (incorporated a FSP)	Selectivity for ECs attachment	Self-assembled monolayers of perfluorosilanes on glass slides	Validation of the ability of CRRETAWAC to bind both porcine and human ECs [73]
Cardiac regeneration			
GRGDS	To increase availability of ligands for the αvβ5, αvβ3 and α5β1 integrin receptors in cardiomyocytes	Collagen scaffold (Avitene Ultrafoam hemostat sheets)	To improve the contractile properties of cardiomyocytes [74]
GRGDS, YIGSR	To improve cell adhesion, proliferation, and differentiation	PCL-based substrates	C2C12 myoblast attachment, proliferation and differentiation [75]

RGD and HBP	To induce the differentiation of human embryonic stem-derived cardiomyocytes	Alginate scaffolds	To achieve cell–matrix interaction mediated by an integrin-dependent and independent mechanism [76]
RGD	To increase the formation of functional cardiac tissue	Freeze–dried alginate scaffolds	Cardiac regeneration tested using neonatal rat cardiac cells [77]

CH, Chitosan; *ECs*, endothelial cell; *FSP*, fluorosurfactant polymer; *HA*, hyaluronic acid; *HBP*, heparin-binding peptide; *PCL*, polycaprolactone; *PDMS*, polydimethylsiloxane; *PTFE*, polytetrafluoroethylene; *RGD*, arginine–glycine–aspartic acid.

8.4 Ligand functionalization of nanoparticles for cell targeting

In the last few decades, nanomedicine has been widely exploited for different applications, including the diagnosis, prevention, and treatment of diseases. NPs can encapsulate different types of molecules (even combined), protect them from degradation, transport them at the target site, and even deliver them to specific cell types. For these reasons, nanocarriers have been extensively employed in cancer diagnosis and treatment [78–80]. However, nanomedicine also covers different therapeutic fields, including the treatment of cardiovascular diseases [10,81].

NP size is in the order of few hundred nanometers or less, allowing them to elude renal clearance [82]. They can be prepared from diverse materials, both organic and inorganic, or even by organic–inorganic combinations [83,84]. Examples of carrier materials for NPs applied in cardiac regenerative medicine include lipids (liposomes) [85,86], polymers such as PEG–PLA (PEG-b-poly(d, l-lactide)) [87] or dextran [88], gold [89,90], and iron oxide [91].

Besides the small size and the versatile composition, the feature that mostly prompted their application in biomedicine is their high surface-to-volume ratio. Their large available surface can be modified and functionalized with molecules able to direct NPs to the target tissue, where they finally deliver their cargo (drugs or other bioactive molecules) exerting the desired medical function. Based on that, NPs can establish new pharmacodynamics and pharmacokinetics of the therapeutic molecules, carrying them to a precise site of action [92].

Particularly, functionalization of NPs is essential to achieve an efficient cell delivery and even an active targeting. By attaching specific ligands, it is possible to direct NPs toward cell receptors (glycoproteins or GAGs–based): as an example, cells in diseased tissues may overexpress surface receptors (disease markers) which may be targeted by NPs for a specific drug delivery. By this approach, NPs may enter the cells via receptor-mediated endocytosis [93].

In the treatment of cardiac diseases, scientific literature offers different examples and strategies for NP functionalization.

Proper surface tailoring of NPs may enhance their biocompatibility properties and ability for cell uptake. For example, Ornelas-Soto et al. [94] covalently modified the surface of mesoporous silica NPs (MSN) with oleic acid (OA) and evaluated NPs internalization by myocardial cells *in vitro*. NPs were first chemically functionalized with (3-aminopropyl)triethoxysilane to expose primary amines which can react with organic acids. In this case, cis-9-octadecenoic acid was added to obtain aminopropyl/OA-modified MSN particles (MSN-OA). These NPs were tested *in vitro* on rat myocardial cell line H9c2, and showed an increased and dose-dependent uptake compared with nonfunctionalized silica particles, with low levels of cytotoxicity when compared with the nonfunctionalized NPs [94].

Another approach to ameliorate NP uptake consists of ~~is through~~ the tuning of their external electrical charge. Di Mauro et al. [95] prepared calcium phosphate (CaP) NPs for drug delivery to CMs. NPs were produced through biomineralization-inspired one-pot synthesis using citrate as stabilizer and were characterized by a negatively charged surface that can help the CM membrane crossing. Polarized excitable cells (like CMs) were found to have a selective affinity for negatively charged NPs, which can also facilitate the formation of not-harmful nanopores for NP internalization [96]. In this work, CaP NPs did not show *in vitro* toxicity on HL-1 cell line and primary mouse CMs and did not affect cell functionality, such as Na^+, Ca^{2+},and K^+ ionic currents. Moreover, NPs encapsulating synthetic microRNAs (in this case not-mammalian cel-miR-39–3p from *Caenorhabditis elegans*) were systemically injected *in vivo* in mice showing a significant accumulation in the left ventricle [95].

The same CaP NPs loaded with therapeutic peptides were also proposed for heart targeting through inhalation and tested *in vivo* initially in a rodent model of diabetic cardiomyopathy, followed by a porcine large animal model [97].

As an additional strategy, peptide surface functionalization was exploited to promote active targeting, and one of the first works applying this concept on cardiac cells was published by Dvir et al. [98]. The authors exploited the overexpression of angiotensin II type-1 (AT1) receptor by the infarcted heart [99]. PEGylated liposomes with 142 nm average size were prepared and surface functionalized with a short peptide composed by 4 glycine residues (serving as spacer) followed by 8 residues of angiotensin II, the specific ligand for the overexpressed receptor. AT1 binding NPs, labeled with a fluorescent dye, demonstrated to recognize cardiac cells both *in vitro* and *in vivo*. *In vitro*, primary cardiac cells isolated from neonatal rats showed higher uptake of AT1 binding NPs compared to nonspecific NPs bearing a peptide with scrambled aminoacids (52% vs 27%, respectively). NP specificity was confirmed by exposing NPs to the same cells after 48 h in hypoxia conditions (5% O_2): targeted cells percentage increased from 52% to 83%. *In vitro* results are shown in Fig. 8.9. *In vivo*, AT1 binding NPs were systemically injected into mice after induction of myocardial infarction and demonstrated to accumulate mainly in the left myocardium and less in the other organs; they were also administered to healthy mice but they did not accumulate in the heart [98].

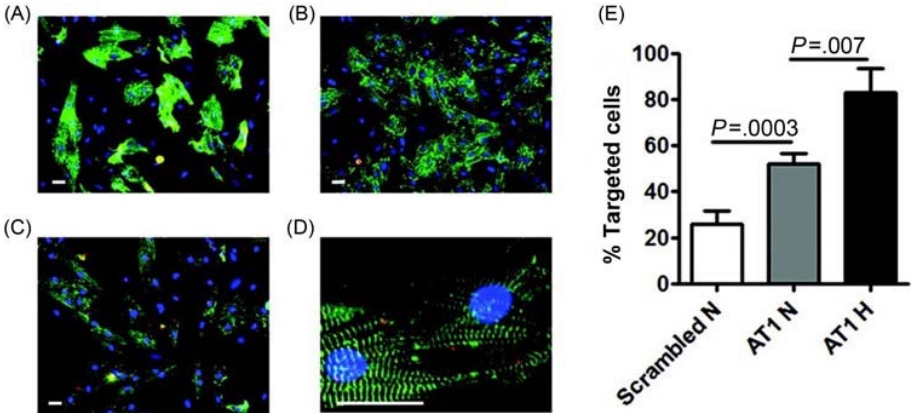

Figure 8.9 (A–D) Fluorescent images of *in vitro* cardiac cell-targeting, comparing: (A) targeting with NPs conjugated with nonspecific scrambled peptide; (B) targeting with AT1 binding NPs; (C–D) targeting cardiac cells under hypoxic conditions with AT1-binding NPs. In all panels ~~are shown~~ sarcomeric actinin (green), NPs (red) and nuclei (blue) are shown; Scale bar=20 μm. (E) Percentage of targeted cells. *NP*, Nanoparticle.

Source: Reprinted with permission from Dvir T, Bauer M, Schroeder A, Tsui JH, Anderson DG, Langer R, et al. Nanoparticles targeting the infarcted heart. Nano Lett 2011;11:4411–4. Available from: https://doi.org/10.1021/nl2025882. ©2011, American Chemical Society.

In another work, porous silicon (PSi) NPs were functionalized with three different peptides to specifically target cardiac cells. The first peptide was a circulating cardiac hormone (atrial natriuretic peptide, ANP), known for its cardioprotective properties, antiapoptotic action, and its capacity to inhibit hypertrophy; it is also able to bind a receptor expressed in both CMs and cardiac fibroblasts. The other two peptides used (named P2 and P3) were selected with a phage display approach, focused on identifying sequences able to target ischemic myocardium. The three peptides were covalently bonded to the free carboxyl groups by EDC/NHS (1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide hydrochloride/*N*-hydroxysuccinimide) chemistry. The three different pools of functionalized NPs were tested for biocompatibility and cellular uptake *in vitro* in H9c2 cell line (cardiomyoblast cells), primary CMs, and non-muscle cells to cover all heart cell types. Compared to non-modified NPs, functionalized NPs showed cytocompatibility at concentrations up to 50 μg/mL for all cell types, as shown in Fig. 8.10. On the other hand, the uptake of all the peptide-modified NPs significantly increased in H9c2 cell line, while primary CMs showed high uptake also of negatively charged non-functionalized NPs, probably due to a nonspecific binding. Subsequently, specificity of NPs for cardiac cells was demonstrated *in vivo*: in rat models of isoprenaline-induced infarct, radiolabeled particles were inoculated via tail vein and proved to accumulated in the heart 10 minutes after administration [100].

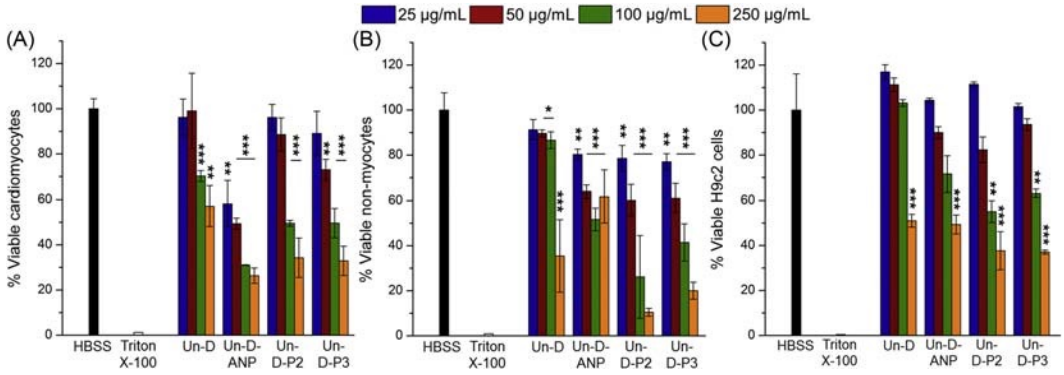

Figure 8.10 Cytotoxicity profiles of (A) primary cardiomyocytes, (B) primary non-myocytes, and (C) H9c2 cells after exposure to different concentrations of non-functionalized (Un-D) and peptide-functionalized nanoparticles (Un-D-ANP/P2/P3). All conditions are normalized to the negative control (cell treated with HBSS, pH 7.4). **P*<.05, ***P*<.01, and ****P*<.001. *HBSS*, Hank's balanced salt solution.
Source: Reprinted from Ferreira MPA, Ranjan S, Correia AMR, Mäkilä EM, Kinnunen SM, Zhang H, et al. In vitro and in vivo assessment of heart-homing porous silicon nanoparticles. Biomaterials 2016;94:93–104. Available from: https://doi.org/10.1016/j.biomaterials.2016.03.046, ©2016, with permission from Elsevier.

The same authors exploited ANP hormone in acetylated dextran (AcDX) NPs carrying two model drugs, CHIR99021 and SB431542, useful in increasing the efficiency of direct reprogramming of fibroblasts into CMs [101,102]. In this work, spermine-modified AcDX was loaded with the hydrophobic drugs using an oil-in-water emulsion procedure to produce NPs. Then, their surface was functionalized with PEG and ANP hormone using carbodiimide chemistry.

Functionalized NPs demonstrated *in vitro* cytocompatibility with cardiac fibroblasts even at high concentrations, (up to 250 µg/mL), while toward CMs they were non-toxic only at low concentrations (up to 25 µg/mL). Remarkably, in this work, NPs were allowed to carry two water insoluble drugs; moreover the pH responsiveness of AcDX allowed the triggering of drug release from the endosomes, after NP uptake by the cells [88].

Other peptides selected through phage display technique were applied for targeted heart treatment. One significant example is represented by the short linear peptide CRPPR (cysteine-arginine-proline-proline-arginine), which specifically binds to the heart endothelium [103]: CRPPR peptide was conjugated to phospholipid-based liposomes using a PEG molecule (3600 Da) as spacer to expose the peptide [104]. Liposomes were loaded with a fluorescent dye as model drug and systemically administered *in vivo* in a murine model of myocardial disease (male C57BL/6 mice, 2-month old). Such particles demonstrated to accumulate more in the cardiac ECs compared to the surrounding tissues, and accumulation increased with time after the MI event [105].

Another targeting strategy involves NP functionalization with peptides binding heparan sulfates (HS) in the GAG molecules on the cell surface. Generally, NPs are coupled with cell-penetrating peptides (CPP), known for their capacity of facilitating cell endocytosis of extracellular cargoes without affecting cell viability and proliferation [106]. As an example, in the work published by Osman et al. [107], they utilized this approach to develop a new tool for gene delivery with the prospective to treat genetic disease, such as cystic fibrosis, where a mutation of the *CFTR* gene causes lung failure in the long-term. In this work, DNA NPs were functionalized with a HS-binding sequence, precisely a 16-aminoacid sequence derived from fibroblast growth factor 2, coupled with an amphiphilic sequence (identified as LK15), to help DNA condensation ability and intracellular trafficking, and an octaarginine (8 R) as CPP. The resulting peptide (FLR) was covalently coupled with PEG maleimide chains (5 kDa) via a thioether linkage after addition of a N-terminal cysteine. The resulting cationic peptide facilitated encapsulation of DNA molecules in the NP core, while PEG chains formed a layer on NP surface that helped to inhibit particle aggregation. Fig. 8.11 shows DNA-loaded NP composition and structure.

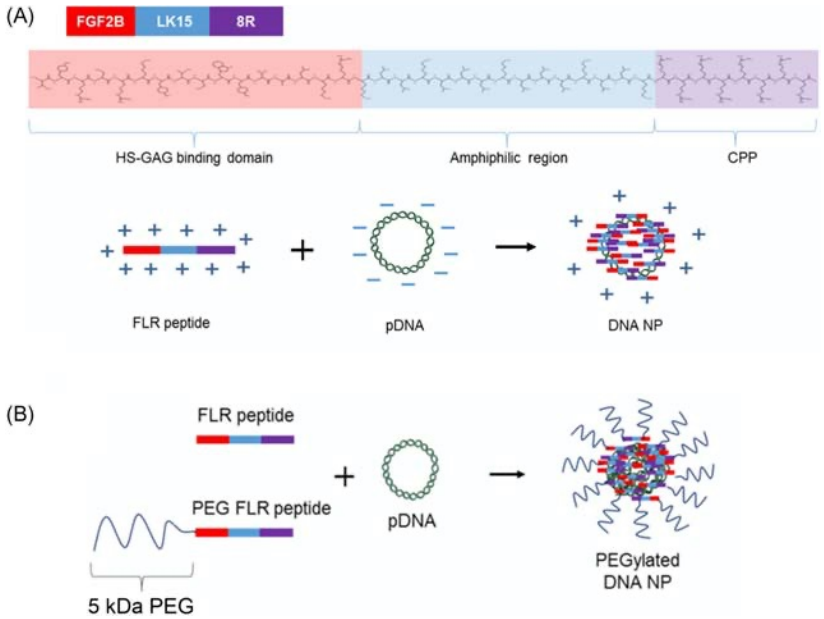

Figure 8.11 Schematic representation of DNA-loaded NP structure. (A) FLR sequence, a multidomain peptide composed of a HS GAG-binding domain (red), an amphiphilic region (blue), and CPP (purple). When FLR peptides are mixed with DNA, they establish electrostatic interactions with the phosphate groups (negatively charged) of the plasmid leading to NPs formation through self-assembly. (B) Final NP structure, after

PEGylation of FLR sequence. *CPP*, Cell-penetrating peptide; *GAG*, glycosaminoglycan; *HS*, heparan sulfates; *NP*, nanoparticle.

Source: Adapted from Osman G, Rodriguez J, Chan SY, Chisholm J, Duncan G, Kim N, et al. PEGylated enhanced cell penetrating peptide nanoparticles for lung gene therapy. J Control Release 2018;285:35–45. Available from: https://doi.org/10.1016/j.jconrel.2018.07.001, ©2018, with permission from Elsevier.

The PEGylated NPs were tested for their delivery *in vivo* in mice lungs by local administration with an intratracheal microspray apparatus. The DNA used consisted of a plasmid encoding for a luciferase reporter that enables noninvasive gene expression quantification, through bioluminescence measurement. Mice treated with PEG–DNA complexes showed higher transgene expression compared to treatment with DNA alone or combined with a polymer-based vector [107].

GAG-binding peptides can also be combined with magnetic NPs (Nanomag-D dextran shell/iron oxide core), by covalently coupling the peptide through -COOH functional groups with EDAC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide)/NHS chemistry. In this case, the GAG-binding peptide was composed of 21 residues coming from heparin-binding epidermal growth factor (HB-EGF) and again by 8-arginine residues. NP uptake was demonstrated *in vitro* in NIH3T3 fibroblast cell line, through light microscopy Prussian blue iron-staining [106].

Table 8.3 summarizes relevant examples of NP functionalization in cardiac regenerative medicine, classified by targeting implementation.

Table 8.3 Examples of nanoparticle surface functionalization classified by target type.

Surface molecule	Application	NP material	Type of functionalization	Molecules loaded/labeling	Reference
NP tailoring for increased biocompatibility and cell uptake					
cis-9-octadecenoic acid	Biocompatible silica-based particles for delivery to myocardial cells (H9c2 cell line)	Mesoporous silica	Functionalization with APTES to obtain amino groups for oleic acid grafting	FITC (staining)	Ornelas-Soto et al. [94]
Calcium phosphate	To release therapeutics to cardiomyocytes	Calcium phosphate	No functionalization was used: the intrinsic negative charge of NPs was exploited to preferentially target polarized cardiomyocytes	MicroRNAs	Di Mauro et al. [95]
				Short therapeutic peptides	Miragoli et al. [97]
Cell targeting by peptide functionalization					
Peptide with 4 glycine residues (spacer) and 8 angiotensin II residues (DRVYIHPF)	To target infarcted heart tissue by binding the AT1 receptor, overexpressed after hypoxia	PEGylated liposomes	Grafting via carbodiimide chemistry	DyLight649 probe (staining)	Dvir et al. [98]

Peptide sequences selected by phage-display approach	To target selectively ischemic heart	Porous silicon	Grafting via carbodiimide chemistry	Alexa488 (staining) ^{111}InCl 3 (labeling)	Ferreira et al. [100]
ANP	To target infarcted heart tissue by binding to a tissue-specific cardiac receptor	Porous silicon	Grafting via carbodiimide chemistry	Alexa488 (staining) ^{111}InCl 3 (labeling)	Ferreira et al. [100]
ANP	Direct reprogramming of cardiac fibroblasts	Acetylated dextran	PEG and ANP grafting on NP surface and crosslinking chemistry	SB431542 CHIR99021	Ferreira et al. [88]
Short peptide (CRPPR) identified by phage-display approach	To target heart with efficient trans-endothelial transport	PEGylated liposomes	Liposome preparation using Lipo-~~Peg~~PEG-Peptides	Alexa555 (staining) Radioactively labeled lipid, 18F-FDP (labeling)	Zhang et al. [105]
Cell targeting by polysaccharide functionalization					
Multifunctional FLR peptide (Fig. 8.11)	Target lung cells through HS for gene delivery	Plasmidic DNA	NPs are prepared by complexation through FLR self-assembly (Fig. 8.11)	DNA labeled with Cy3 or Cy5, encoding for firefly luciferase	Osman et al. [107]
Peptide with 21 residues of EGF and 8 Arginine residues	Delivery of molecules for cell labeling or cell targeting	Nanomag-D (Dextran shell/iron oxide core)	Grafting via carbodiimide chemistry	None	Dixon et al. [106]

18F-FDP, 18F-fluorodipalmitin; *ANP*, atrial natriuretic peptide; *APTES*, (3-aminopropyl)triethoxysilane; AT1, angiotensin II type-1; *Cy3/5*, cyanine dye 3/5; *EGF*, epidermal growth factor; *FITC*, fluorescein isothiocyanate; *HS*, heparan sulfates; *NP*, nanoparticles; *PEG*, poly(ethylene glycol).

To the best of our knowledge, NP decoration with peptides binding HS on the cell surface has not been exploited for cardiac delivery and deserves investigation.

The functionalization methods reported in this section are only a few examples of how nanomaterial design can be tuned to the final application. As already shown in the last examples, many works in the literature report applications of functionalized nanocarriers to treat different types of disorders, from neurodegenerative to chronic infectious diseases, such as Alzheimer's disease [108], hepatic cirrhosis [109], or HIV [110,111], among the others.

The multitude of applications is possible since NPs' interaction with proteins and cells can be controlled by tuning their size, shape, composition, external functionalization, and electrical properties to achieve different aims [112].

8.5 General discussion and conclusion

Bioactive biomaterials can be obtained by biomaterial functionalization with peptides able to specifically modulate their interaction with cells and the host environment (Fig. 8.1). Depending on the therapeutic purpose, hydrogels, scaffolds, or NPs are used in regenerative medicine, and all of them can be functionalized with peptide ligands to achieve specific biological functions.

The functionalization of biomaterials with bioactive peptides (Tables 8.1–8.3) should follow specific general rules to achieve the desired biological functionality. First of all, the bioactivity of the peptides can be enhanced by specific flanking amino acids, which help the peptide to assume a more biomimetic conformation for improved ligand-receptor interaction [69]. Although RGD triplet is the minimal peptide sequence allowing integrin binding, it is generally used in combination with flaking amino acids to improve its effectiveness: as an example, RGDS [28] and CRGDS [35] have been frequently employed, instead of RGD.

Furthermore, spacer units have been widely used to expose bioactive peptides and to increase their conformational degrees of freedom with the final aim to enhance receptor-ligand binding [23,24]. A spacer unit based on ethylene glycol oligomers has been frequently employed to exert an additional antifouling function, avoiding nonspecific protein adsorption, as it could hinder cell interaction with the bioactive peptide [23,24].

Cell adhesion, spreading, and cytoskeletal organization increase as a function of bioactive peptide concentration with a sigmoidal trend whereas, at fixed peptide density, a clustered distribution of the peptide further enhances cell attachment compared to random peptide distribution [69]. On the other hand, cell migration rate has shown a bell-shaped trend as a function of the bioactive peptide concentration [69]. In general, cell binding to adhesive peptides is necessary to generate the forces required for cell migration and to secrete proteolytic enzymes, then leading to progressive degradation into an increasingly porous structure [32].

In conclusion, as a general consideration, peptide chemistry (including flanking amino acids and spacer chains), as well as spatial distribution and density should be regulated to efficiently achieve the target biological functionality of the substrate (hydrogel or scaffold/implant). However, some specific considerations depend on the substrate used for the functionalization.

Biomimetic hydrogels should mimic natural ECM behavior, including a control in the temporal presentation of the bioactive peptides as to regulate ECM deposition by the cells. For instance, this can be obtained by photo-driven removal of a caged group to activate receptor-ligand interactions ([25]; Fig. 8.2) or by enzymatic cleavage of bioactive peptides to decrease cell attachment [27]. MMP-sensitive moieties are also fundamental to achieve cell migration and infiltration within the hydrogels [31]. For this reason, hydrogels have been frequently provided with both adhesive and enzymatically cleavable peptide sequences [33,34]. Furthermore, hydrogel degradation and/or cleavage of adhesive peptides can stimulate cells to produce their own ECM, remodeling the hydrogel [27].

In some cases, hydrogels have been functionalized with combinations of peptides to optimize their biological properties. As an example, PEG-based hydrogels have been grafted with RGDS, VGVAPG and P15, derived from fibronectin, elastin, and collagen-1 respectively, to mimic fibrotic microenvironment, stimulating VIC activation into myofibroblasts [28].

Currently, there is no agreement on the use of adhesive peptides in hydrogels for cell release: a few authors showed they may stimulate immune response, decreasing cell viability [43], while others have demonstrated their positive effect on cell delivery [52].

In addition, antiinflammatory peptides, such as the IL-1RIP peptide sequence ([36]; Fig. 8.3) or the highly specific TNF-α binding sequence~~,~~ WP9QY [37]~~,~~ may preserve cell viability within cellularized hydrogels.

Literature agrees on the use of adhesive peptides as a tool to stimulate cell recruitment in endogenous regenerative strategies. Finally, hydrogel progressive degradation through functionalization with cleavable peptides is fundamental for their functionality: it causes progressive cell release from hydrogels designed for cell therapy, while it allows hydrogel remodeling by recruited cells in the case of endogenous regeneration strategies. Specifically, for cardiac regenerative medicine, therapeutic hydrogels may be functionalized with different bioactive peptides (Table 8.1) depending on the final application. In detail, acellular hydrogels for endogenous cardiac regeneration should be functionalized with peptides able to stimulate angiogenesis, cell recruitment, and ventricular function recovery, while cellularized hydrogels should have cardioprotective, pro-survival, and antioxidant properties and additionally be able to promote cardiac phenotype development and maturation of delivered stem cells. A few examples of relevant peptide sequences for therapeutic cardiac hydrogels include: RGD, able to favor cardiac fibroblast recruitment [45] and cell adhesion (Fig. 8.6 [55]; RoY peptide (YPHIDSLGHWRR), stimulating angiogenesis (Fig. 8.5) [46]; glutathione, supporting cell adhesion and protecting cells from oxidative stress [47–49]; QHREDGS, conferring cardioprotective and pro-survival properties, promoting CM attachment and contraction [50–53], as well as myofibroblast recruitment [52]; Jagged1-mimicking peptide, activating early cardiac development, as well as survival and differentiation of CPCs ([41,42]; Fig. 8.4).

Synthetic polymer scaffolds and implants have been widely used for cardiovascular applications due to their advantageous mechanical properties. However, as they lack ligands for interaction with integrin receptors, they generally need surface functionalization to meet specific biological requirements (Fig. 8.7). Cardiovascular stents or vascular prostheses should stimulate a rapid endothelialization process to avoid thrombosis. For this reason, several authors have proposed surface functionalization with adhesive peptides [63,66,71,72]. However, RGD is not an optimal choice for this application as it also stimulates platelet adhesion and activation. For this reason, the natural REDV and unnatural CRRETAWAC peptides have been proposed to stimulate selective EC attachment versus smooth muscle cells and platelets adhesion [67,73]. Surface functionalization of cardiovascular implants makes use of covalent strategies and benefits from the co-functionalization with antifouling molecules, avoiding unspecific protein absorption, which may lead to thrombus formation.

On the other hand, several types of scaffolds for cardiac regeneration have been prepared from synthetic and natural polymers. In both cases, peptide functionalization has been frequently performed to improve cell adhesion, proliferation, and differentiation (Fig. 8.8). RGD has been one of the most used ligands in the field [76,77]. In addition, more specific peptides have also been proposed depending on the target biological function: particularly, laminin-derived peptides have been demonstrated to stimulate stem cell differentiation into cardiac phenotype [75].

Finally, nanomedicine tools represent a promising approach for the management of cardiac diseases, by directly supplying drugs, hormones, and oligonucleotide-based molecules to targeted cells, with the aim to implement successful new advanced therapeutic strategies, such as gene therapy and cell reprogramming (Figs. 8.9–8.11). NPs have the advantage of allowing minimally invasive intravenous administration through the systemic circulation, or they can either be delivered locally using injectable gel carriers, avoiding the need for invasive surgical procedures. Another option is the administration through inhalation which allows a rapid translocation of NPs from the pulmonary tree to the bloodstream and to the myocardium, where their cargo can be quickly released [97]. Functionalization with ligands specifically targeting cardiac tissue may reduce systemic toxicity and increase therapeutic outcomes [98].

Table 8.3~~2~~ collects relevant examples of peptide ligands for ~~a specific~~ targeting of cardiac cells: specific protein receptors of cardiac cells can be selected as the targets for ligand molecules, while heart-specific heparan sulfate functionalities in the proteoglycan receptors could be a new target to investigate. Generally, surface

functionalization of NPs with ligands also involves the use of antifouling surface molecules avoiding unspecific protein adsorption.

The continuous progress in nanomedicine through the discovery of new ligands for targeting specific cell populations may reduce the time to the clinical translation of several investigated approaches. Interestingly, precision nanomedicine could potentially allow clinical translation of *in vivo* gene therapies through safer alternatives to viral vectors, which ~~their~~ use has been associated with different drawbacks, including immune response, safety issues, and nontargeting properties [113]. In this context, new emerging strategies such as the direct reprogramming of cardiac fibroblasts into CMs [14,15,114] or the stimulation of cell-cycle reentry by CMs [13] could benefit from precision nanomedicine tools.

As a conclusion, peptide ligand type, temporal~~,~~ and spatial distribution, as well as combination with other peptides and/or molecules (e.g., antifouling molecules) strongly affect the biological behavior of medical devices, including nanosized particles, scaffolds, implants, and hydrogels. Proper functionalization may finely tune the substrate biological properties, paving the way to the clinical translation of new emerging approaches, such as gene therapies for cardiac regeneration, and to the design of in vitro models of human cardiac tissue for the effective preclinical testing of innovative approaches.

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