Peptide grafting strategies before and after electrospinning of nanofibers

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Graph Abstract

Abstract

Nanofiber films produced by electrospinning currently provide a promising platform for different applications. Although nonfunctionalized nanofiber films from natural or synthetic polymers are extensively used, electrospun materials combined with peptides are gaining more interest. In fact, the selection of specific peptides improves the performance of the material for biological applications and mainly for tissue engineering, mostly by maintaining similar mechanical properties with respect to the simple polymer. The main drawback in using peptides blended with a polymer is the quick release of the peptides. To avoid this problem, covalent linking of the peptide is more beneficial. Here, we reviewed synthetic protocols that enable covalent grafting of peptides to polymers *before* or *after* the electrospinning procedures to obtain more robust electrospun materials. Applications and the performance of the new material compared to that of the starting polymer are discussed.

Keywords

Electrospinning, Peptide-grafted electrospun fibers, Peptide-grafted polymers, Tissue engineering, Scaffolds

1. Introduction

Nanotechnology is the ability to understand, control, and manipulate the matter at atomic and molecular levels to produce systems with size and shape in the nanoscale [1]. Peptides/peptidomimetics are often used to prepare nanomaterials because amino acids (AAs) or non-coded AAs form the basis of self-organization at the molecular

level [2–8] and because they broaden the number of possible applications, ranging from biomaterials to biosensors and drug delivery systems.

Peptide nanofibers can be produced by using various techniques [9,10]. Among them, electrospinning (ESP) is a widely used technology to produce various fiber assemblies on a large scale with a tunable diameter (nano- to micrometer size) [11]. Both two-dimensional (2D) and three-dimensional (3D) structures can be produced. Studies on 3D as an alternative to conventional 2D structures have gained increasing attention due to the properties of the scaffold, which is considered superior to the 2D counterpart. Natural or synthetic polymers form the basis of material production. The first ones were extensively exploited [12–16] because natural scaffolds promise better clinical functionality; however, the use of animals as the source of origin and partial denaturation reported for some of the scaffolds have raised health concern [17]. Synthetic polymers offer several advantages such as ease of availability, processing without difficulty and with reproducible results. Usually, synthetic polymers have excellent mechanical properties but, in some cases, they show lack of cell recognition sites and lack of cell affinity due to low hydrophilicity [18].

Selected peptides represent an interesting tool to overcome some of these drawbacks, mostly in tissue engineering, where biodegradable scaffolds are required to direct tissue repair and regeneration while providing temporary structural support. In addition, peptides were shown to be very useful in the design of a well-defined milieu resembling the native environment surrounding the cells. Moreover, because of the limited length and complexity (4-15 AAs), their preparation on the large scale is easy. Advantages mostly include the possibility to tune AA sequences and produce a hydrophobic or hydrophilic surface with specificity for different biological targets. Furthermore, they do not suffer from denaturation issues and exhibit compatibility with nonaqueous solvents [19,20].

Peptides can be used in ESP according to different protocols: i) in combination with natural or synthetic polymers and ii) alone, as reported for poly- and oligopeptides [21,22]**.** Different strategies were used to integrate peptides into electrospun fibers (EFs), including electrostatic attraction and bulk or surface modification [23]. The main drawback in using peptides/polymers blend or physically adsorbed peptides on ESP is the quick release of peptides [19][24–27], which is often observed when the functionalized scaffolds are implanted *in vivo* [28]. To avoid this problem, their covalent link is more beneficial and affords more robust materials featuring mechanical properties similar to those shown by the native polymer. Considering the lack of a general overview of the exploitation of different synthetic protocols for peptide covalent grafting to polymers before or after the ESP procedures, a special focus on this issue is given (since 2010). The advantages of the methodologies and the effects and benefits of peptide conjugation and applications, mostly in tissue engineering, are reported.

Since characterization techniques for ESP products have been extensively reviewed [17], they are not examined here in detail, except in the case of an added value for the specific application.

2. Grafted peptides

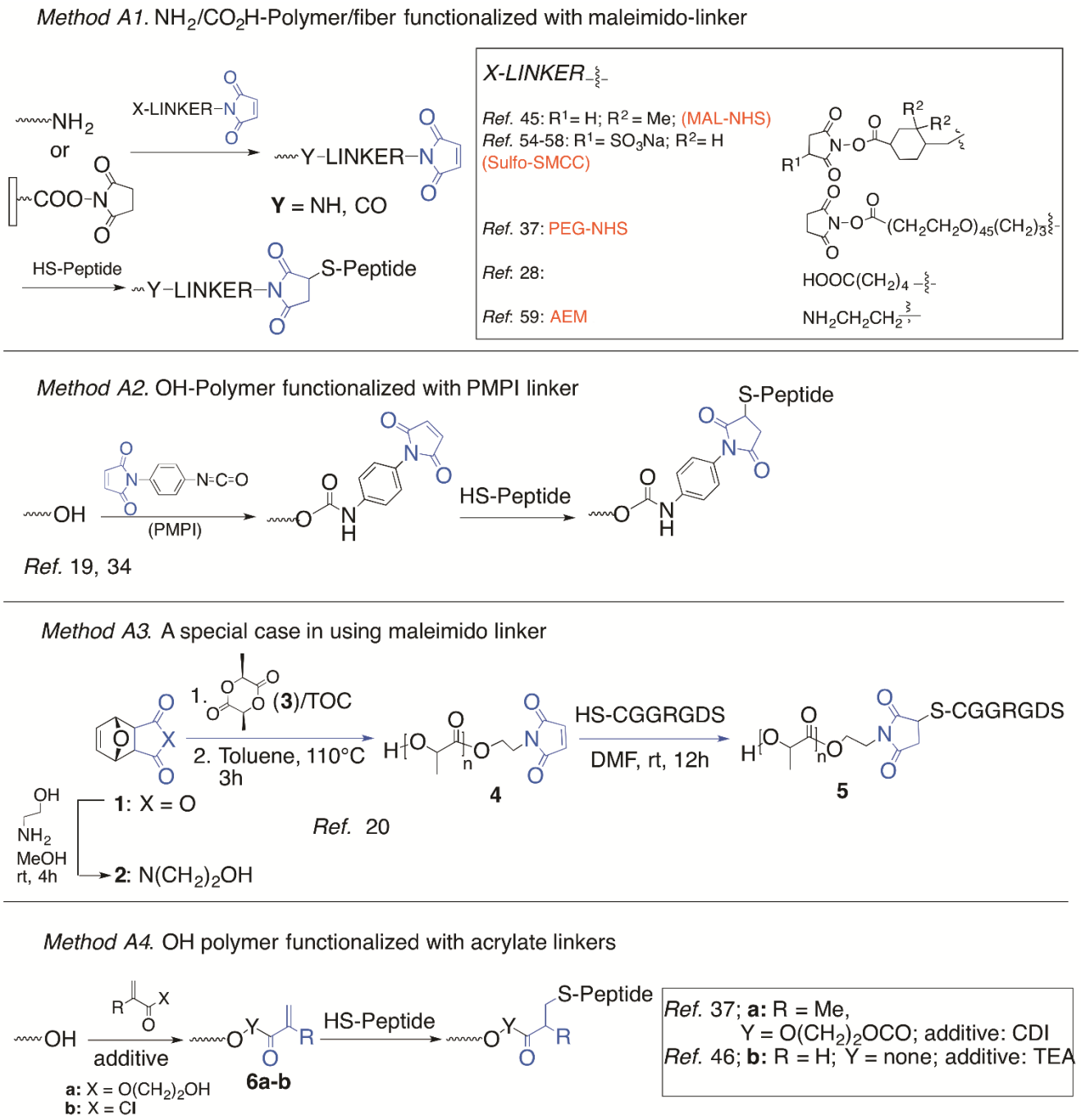
ESP of natural [16,17][29–31] and synthetic polymers [18] is extensively reviewed, and their advantages and disadvantages have been deeply discussed. Efforts were made to expand the pool of these polymers to meet requirements for more advanced applications [32]. A short overview of polymers used to link peptides is reported in Supplementary Materials. A very important pre-requisite for tissue engineering is the biocompatibility and degradability of polymers [33]. The main limitation, mainly related to their difficulty to promote the exchange of nutrients and metabolites, assuring integrity to the newly formed tissue [34,35], can be addressed by functionalization with peptides/growth factors[19][36]**.** As reported above, the covalent grafting of peptides to polymers is currently the preferred method. Accordingly, the main procedures are reported below.

2.1 General methods for grafting peptides

Two main strategies are currently used to covalently bind peptides, the first one consisting of the modification of the polymer prior to ESP. During ESP, the functional segment of the modified polymer is driven to the fiber surface to functionalize and fabricate the scaffold in a single step [37]. Advanced ESP techniques are often combined with this strategy to control both the amount and spatial presentation of functional groups within the scaffold. A possibile problem could be the buring of the bioactive species in the nanofiber, thus making them biologically unavailable to the target cell population [38]. In the second approach, the electrospun polymer surface is modified with the desired biomolecule. A blank canvas of fibers is produced to which tailored surface properties can then be given to suit a range of specific applications [39]. This approach adds many processing steps and may affect bulk scaffold properties [32]. Moreover, it is difficult to control the concentration and orientation of the biomolecule on the surface.

Different methodologies were used to link peptides, many of them common to both strategies. A short description of the main methods used in both approaches is given below.

Both electron-poor (*Method A*; Scheme 1) and electron-rich (*Method B*; Scheme 2) double bonds are the key elements to link the peptide *via* a SH-group.



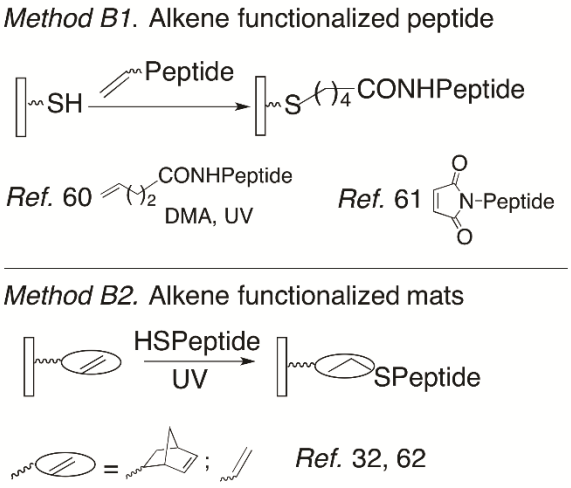
Scheme 1. *Method A*. Electron-poor alkene tools to graft thiol-containing peptides.

Covalent bond is formed through a nucleophilic (Michael reaction) or radical mediated addition. The Michael reaction is fast and applicable at low concentration of reagents. Furthermore, it is highly selective, which ensures oriented and homogeneous peptide immobilization without compromising materials properties. The second one is efficient, high yielding, and flexible. The cytocompatibility of the used conditions makes this process highly interesting [40]. The Michael addition is applied before or after peptide grafting, and it requires a cysteine-functionalized peptide. The second one, used to graft

peptide on EFs, takes advantage of a thiol-functionalized fiber or a cysteine-functionalized peptide as SH-donors.

According to *Methods A1-2*, maleimido (MAL) scaffold is the most popular and generally linked to the polymer/EFs *via* different linkers. In the third approach (*Method A3*), the functionalized polymer was obtained starting from the furan-protected MAL **1** that is reacted with 2-aminoethanol affording **2**. After reaction of **2** with L-lactide **3,** polymerization is performed [$Sn(Oct)_2$ catalyst (TOC)]. Removal of the furan protective group in a retro-Diels-Alder reaction (toluene, reflux) affords poly(L-lactic acid) (PLLA)-functionalized MAL conjugate **4**. Acrylates are also used to link peptides (*Method A4)*. In a general approach, the hydroxyl groups of a polymer are reacted with an acrylate derivative affording the functionalized polymer **6**. The electron-poor double bonds of MAL or acrylate scaffolds are key elements to link cysteine-containing peptides *via* a Michael addition.

Reactions of simple alkenes are applied only when starting from EFs, adopting different strategies (Scheme 2): *i)* the peptide functionalized at *N*-terminus with an alkenyl group is conjugated to the HS-functionalized fibers by a radical thiol-ene reaction [*Method B1*; radical photoinitiator 2,2-dimethoxy-2-phenyl acetophenone (DMPA)/UV irradiation]; *ii)* cysteine-containing peptides are "clicked" (UV) on the fibers (*Method B2*).

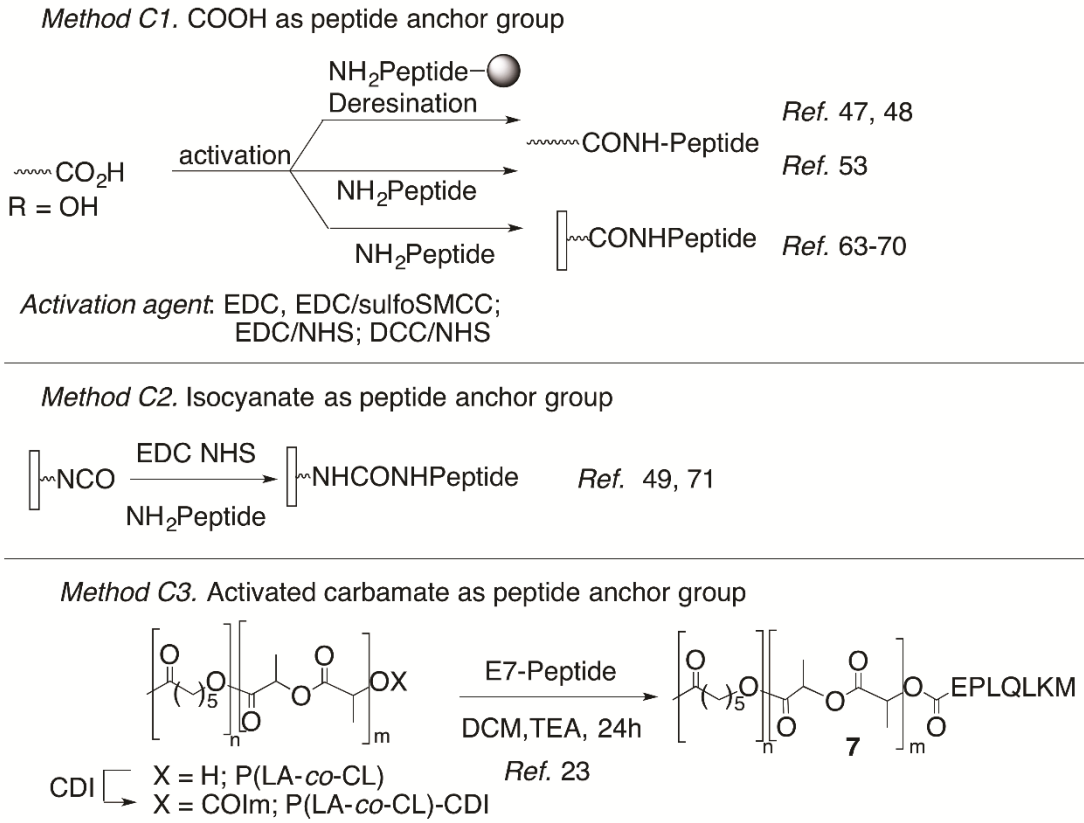


Scheme 2. *Method B.* Electron-rich alkene as linker.

Several examples are reported taking advantage of peptide conjugation *via* its *N*- or *C*-terminus.

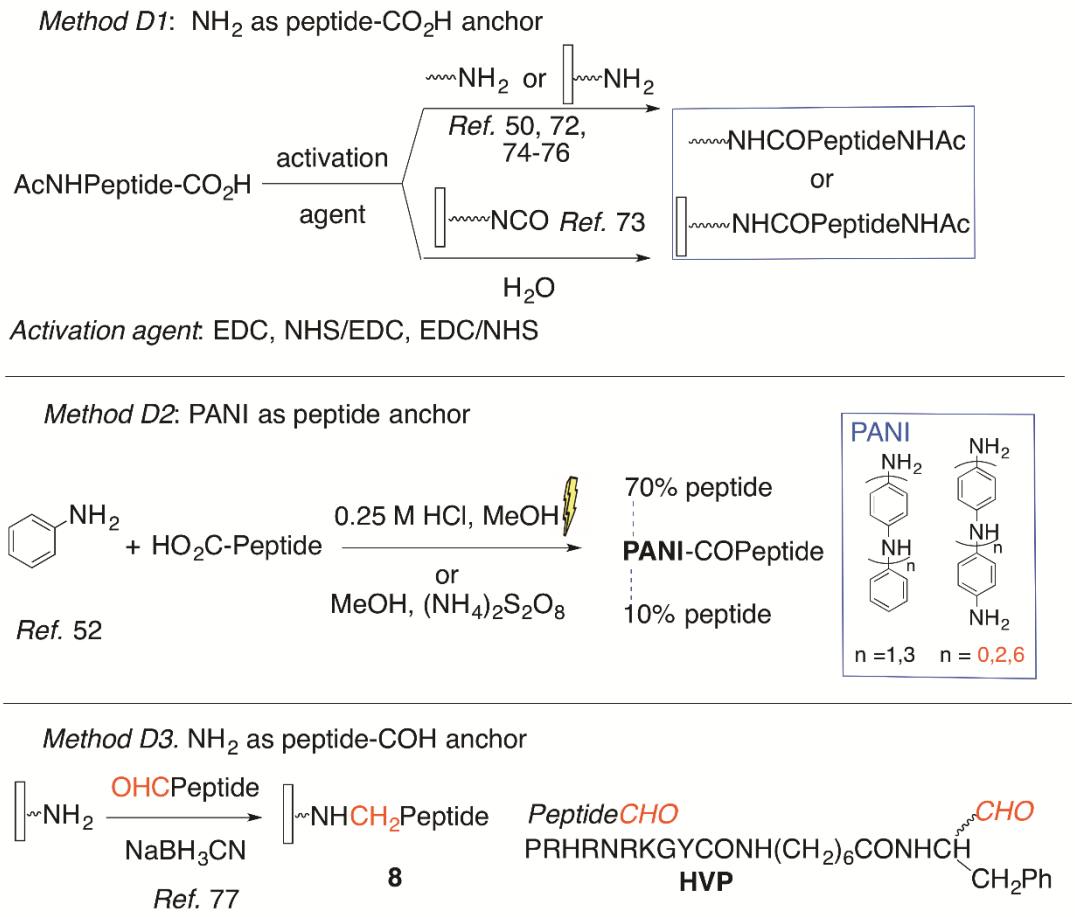
According to *Method C* (Scheme 3), amide, urea, or carbamate bonds can be obtained starting from selected CO_2H, NCO-functionalized polymers or EFs. *Method C1* is the mostly used method to link peptides to CO_2H-functionalized materials. Solid phase peptide synthesis (SPPS with Fmoc-chemistry) is useful for polymer-peptide conjugate preparation. The peptide sequence is built, the polymer is linked, and after the cleavage, the final construct is generated. As an alternative, the peptide can be directly linked to the CO_2H-activated polymer (condensing agent). The same protocol can be applied to functionalize EFs. In a different approach, isocyanate-functionalized fibers are linked to the *N*-terminus

of peptides to obtain a urea function (*Method C2)*. Carbamate **7** was obtained by direct grafting of a lactic acid (LA)-containing co-polymer, activated with carbonyldiimidazole (CDI) to the *N*-terminus of the peptide *(Method C3).*



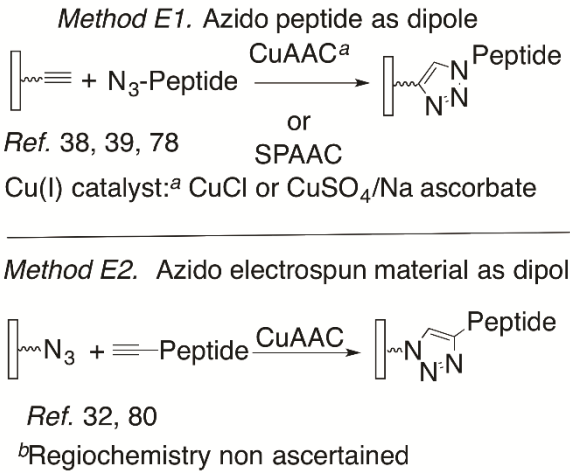
Scheme 3. *Method C. N*-terminus-grafted peptides.

C-terminus-grafted peptides were prepared according to *Method D* (Scheme 4). Starting from the NH_2-containing polymers/EFs or isocyanate-functionalized fibers, the *C*-terminal end of the peptide, after activation of the peptide carboxylic function, is grafted to form an amide bond (*Method D1*). A special approach uses polyanilines (PANI), giving covalently linked PANI-peptides (*Method D2*; Section 2.2 for details). Peptides with an aldehyde function at the *C*-terminus can be used to link the amino-containing EFs *via* reductive amination, as shown in *Method D3, to* produce **8**.



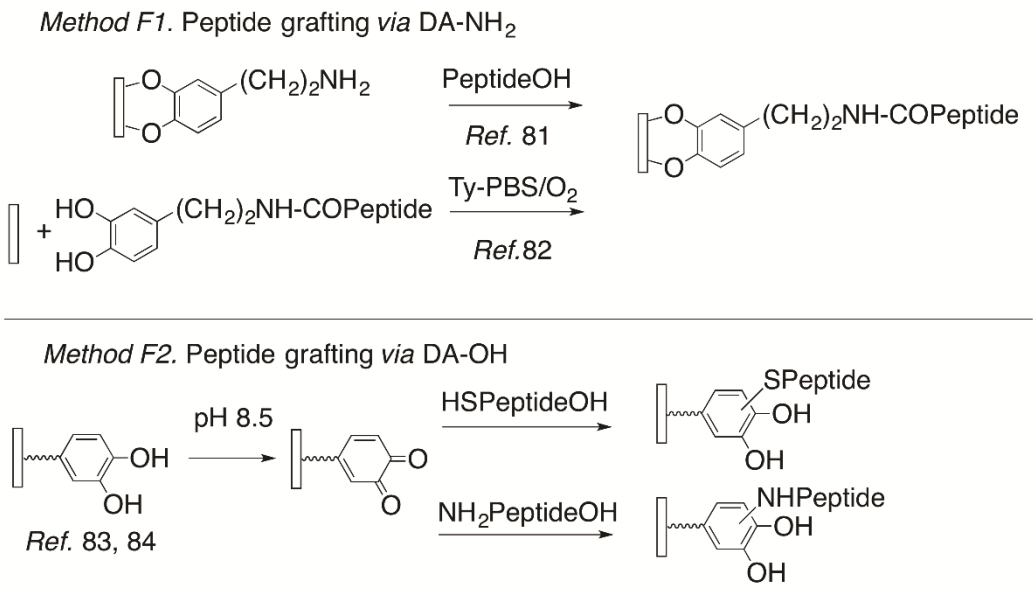
Scheme 4. *Method D*: *C*-terminus-grafted peptides.

Click chemistry is an interesting tool to covalently graft peptides to spun fibers (*Method E*; Scheme 5*)* for its high specificity; each peptide is linked to a defined site that allows a rational orientation and conformation of the attached ligand [39]. Two different protocols can be used: copper-catalyzed (CuAAC) or strain-promoted azide/alkyne cycloaddition reactions (SPAAC) [41–43]; the latter is selective for cyclic alkynes and does not require a catalyst. Selective linkage of different azides to the same scaffold presenting both cyclic and acyclic alkynes with different reactivities can be achieved [38]. Both reactions proceed in high yields in aqueous conditions, producing a linkage insensitive to hydrolysis or other degradation mechanisms. Click reactions can occur starting from *i)* an alkyne-functionalized EF with the azido peptide (*Method E1*) and/or *ii)* an azido-functionalized EF with an alkyne-substituted peptide (*Method E2*). In general, the regiochemistry is not defined, but usually, the 4-substituted triazole derivative is obtained [32].



Scheme 5. *Method E.* Peptide grafting *via* click chemistry[a].

Dopamine (DA) was extensively used as a coating of spun fibers to enable covalent reactions with peptides. Polydopamine (pDA) [44] containing catechol and amino groups is useful for peptide linking (Scheme 6, *Method F*).

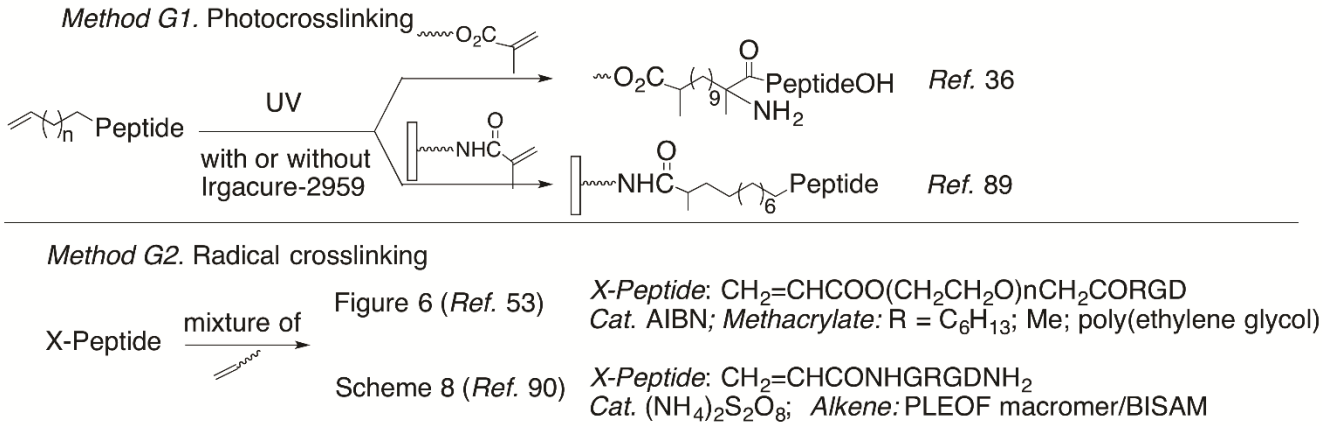


Scheme 6. *Method F.* Dopamine-functionalized EFs for peptide linking.

The reaction mechanism between peptides and pDA is not fully elucidated, and the exact bonding of the peptide to the material has not been specified in several cases. In *Method F1*, the free NH_2 links the peptide *C*-terminus *via* an amide bond *i)* on pDA already deposited on the EFs or *ii)* directly on DA followed by catechol oxidation to quinone by tyrosinase (Ty) and deposition onto the fiber. In *Method F2*, spontaneous conversion from catechol functionalities of pDA-coated material to *o*-quinones (pH 8.5) allows peptide immobilization *via* Michael (NH_2- or HS-peptide) addition.

Finally, photo-crosslink or radical-catalyzed reactions are used to prepare complex matrices starting from different substrates, all functionalized with a double bond, with one of them containing a peptide (*Method G*;

Scheme 7). According to *Method G1*, an alkenyl chain-functionalized peptide reacts with a polymer or a spun fiber bearing a methacrylate moiety. The mixture of both materials was irradiated (UV), with or without a catalyst. A radical crosslinking procedure, performed in the presence of electron-poor alkenes, is reported (*Method G2*) from a peptide-polymer construct or a peptide functionalized with an acrylate.



Scheme 7. *Method G.* Double bonds in photo- or radical-crosslink reactions.

Other specific methods are also reported and discussed in Section 2.3.

An overview on the advantages of the methodologies and effects/benefits of the peptide conjugation before or after electrospinning process and applications are summarized in Tables 1 and 2.

The literature overview given below is divided according to the adopted methodologies.

2.2 Peptides grafted before electrospinning

As mentioned earlier, peptide-polymer conjugates offer a unique solution to produce, in a single step, functionalized scaffolds, thus avoiding additional stages of polymer modification and preventing, in most cases, changes to the mechanical properties of the matrices.

In several cases, a polymer is functionalized with an electron-poor double bond useful to link the peptide via SH-group (*Method A*, Scheme 1). By using this approach, an RGD-containing construct was prepared to improve 3D matrices-endothelialization [45]. Albumin (HSA) was selected to increase the hemo- and biocompatibility of matrices, and because it is exposed and tightly held in the external layer of fibers, HSA was first conjugated with the bifunctional linker by NH_2 of lysine residues, thereby affording the HSA-MAL protein. Cyclic pentapeptide c(RGDfC) was selected instead of RGD for its higher resistance to proteases. The peptide was covalently grafted to afford HSA-c(RGDfC) **9** (*Method A1* and Figure 1). A Tecoflex EG80-A polyurethane (PU) matrix was generated as a base on which a surface layer was produced from Tec-HSA/HSA-c(RGDfC) solution. The modification of the matrix by introducing conjugate **9** does not cause changes in elasticity and durability of matrices compared to those of Tec-HSA matrix.

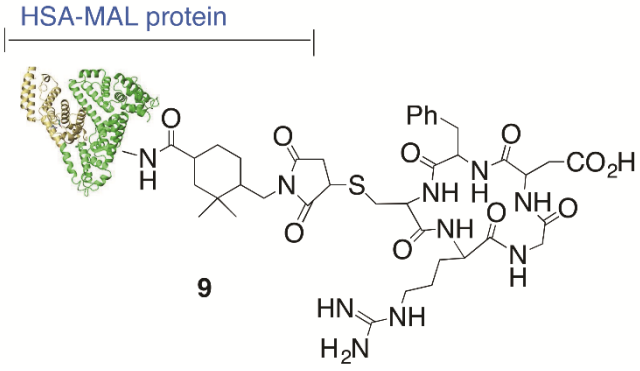


Figure 1. HSA-c(RGDfC) construct **9**.

According to *Method A2,* poly(ε-caprolactone) (PCL) is modified with *p*-MALphenylisocyanate to form PCL-MAL. Then, the SH of the cysteine-containing peptide is reacted with the MAL moiety to yield peptide-PCL conjugate. The authors report [34] an ESP protocol that uses a dual-syringe pump setup containing solutions of two different peptides to create contrasting peptide gradients. Dual-peptide gradients can be used to provide multiple spatial chemical signals, such as binding different biomolecules in contrasting gradients within the scaffold to guide cell behavior. This strategy is suitable for incorporating more than two solutions for more complex scaffold design.

The biological function of the scaffold can be improved by incorporating ECM biomolecules. Nevertheless, their hierarchical distribution mimicking native tissue remains challenging. To overcome this drawback, the abovementioned strategy was applied to the design and synthesis of constructs **10** (Figure 2) targeting GAGs, [glycosaminoglycans, *i.e.*, hyaluronic acid (HA) and chondroitin sulfate (CS)] [19].

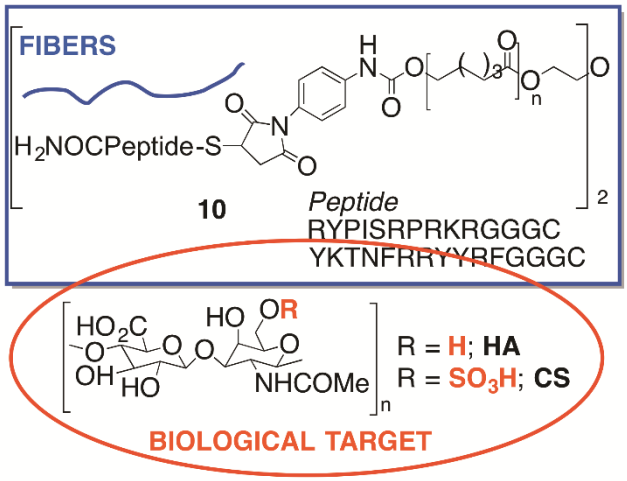


Figure 2. GAG-binding peptide-polymer conjugates **10**

Conjugates **10** were constructed to contain two different sequences: the first one to stabilize HA/proteoglycan aggrecan interactions, and the second one to bind CS to block the inhibition of neurite outgrowth. This approach,

when combined with sequential ESP techniques, yielded single and dual contrasting gradients of peptide concentrations and thus directing the spatial organization of GAGs through the thickness of the scaffold. Increasing the concentration of **10** directly correlated with an increase in GAGs.

A special approach to prepare MAL-PLLA polymers **4** was described in section 2.1 (*Method A3)*. **4** was used to link the peptide, obtained by automated SPPS, affording bioconjugate **5** (Scheme 1) [20]. ESP of a homogeneous two-component solution mixture comprising poly(L-lactide-co-glycolide) (PLGA), as a commodity fiber-forming polymer, and bioconjugate **5** leads to nanofiber meshes. The identification and quantification of the peptide can be performed by measuring the nitrogen concentration, as both polymers do not contain nitrogen.

Moving to acrylate as a Michael acceptor of cysteine-SH, PCL[37][46] can be used. The REDV peptide has been widely studied for its ability to initiate cell-specific binding to vascular endothelial cells (VECs) while avoiding the adhesion of smooth muscle cells (SMCs) and platelets. To enhance VEC-selective adhesion, two kinds of REDV-terminated PCL (Figure 3) spun membranes were prepared [37].

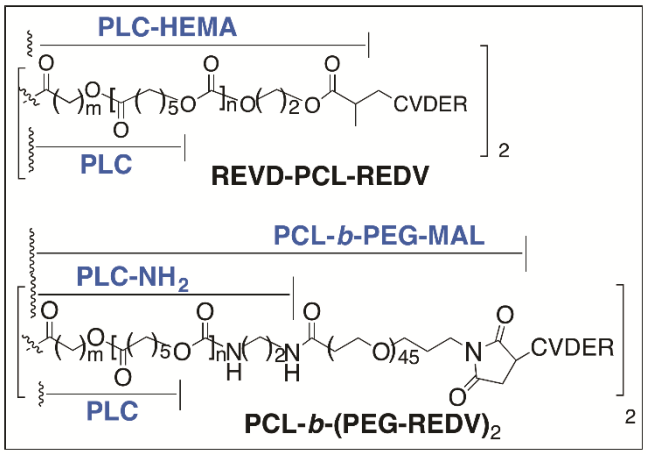


Figure 3. PCL-REDV peptides.

In the first case (*Methods A4*), the PCL terminal OH-groups reacted with hydroxyethyl methacrylate (HEMA). PCL-HEMA polymer was formed and reacted with REDVC-SH (UV light, 365 nm, DMPA) affording copolymer REDV-PCL-REDV. In the other case (*Methods A1*), PCL was condensed with ethylendiamine affording PCL-NH_2 polymer, which was then reacted with MAL-PEG-NHS to give the intermediate PCL-*b*-PEG-MAL. Its reaction with REDVC-SH afforded PCL-*b*-$(PEG\text{-}REDV)_2$.

Bone noncollagenous ECMs mediate nucleation, growth, and stabilization of calcium phosphate (CaP) nanocrystals on collagen fibers, and specific glutamic acid (Glu) sequences in these proteins regulate nucleation and growth of CaP crystals. The effect of CaP deposition on aligned nanofibre surface-modified with a EEGGC peptide (GLU) on osteogenic differentiation of rat marrow stromal cells was investigated in combination with the layer-by-layer (LbL) ESP technique [46]. PLLA was synthesized by ring-opening polymerization of LA using diethylene glycol initiator (DEG) and TOC catalyst. Polymer OH-groups were acylated (acryloyl chloride) to afford **6b** (*Method A4*) and then conjugated with GLU to give PLLA-GLU. PLGA and PLA-GLU were spun

forming-aligned nanofibers (GLU-NF) containing more than 80% of peptide localized onto surface, promoting CAP crystal nucleation.

Resin synthesis of peptide-conjugates is the preferred method to link the polymer to the *N*-terminal end of a peptide (*Method C1*). SPPS (Cl-trityl resin) of the acODLA (acetyl-oligo-D-LA)-IKVAV conjugate was performed by building first IKVAV sequence and then linking acODLA [47]. PLLA nanofibrous and PLLA/acODLA-IKVAV sheets were fabricated by ESP at different temperatures; this was because PLLA becomes amorphous at 60 °C in aqueous conditions. Consequently, the hydrophilic IKVAV sequences migrate to the PLLA surface due to their attraction to water. The peptide retention at the outermost surface of fibers was detected using the FITC staining method, taking advantage of the reaction of exposed lysine-NH_2.

The abovementioned protocol was applied to build an artificial nerve conduit that was modified with a conjugate of acODLA and the neurite outgrowth, thereby promoting peptide AG73 to improve nerve regeneration [48]. In this case, the PAL-PEG resin was selected to build acODLA-AG73 conjugate **11** (Figure 4).

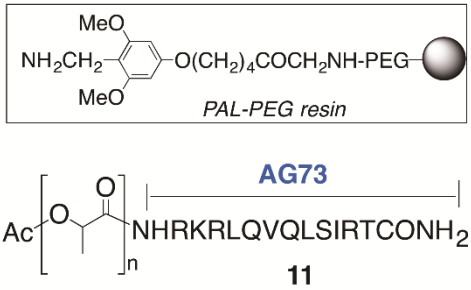


Figure 4. acODLA-AG73 conjugate **11**.

For the inner layer of the conduit, a positively charged PLLA/**11** solution was prepared. For the outer later, a PLLA/PEG solution was used and then electrospun. PLLA/**11** conduit was removed from the target and then transplanted at a gap of 10 mm in the rat sciatic nerve. After 6 months, electrophysiology tests revealed better functional reinnervation than silicone tube or unmodified PLLA nanofibrous conduit.

Ureas and carbamates are an alternative approach to link *N*-terminus peptides to polymers. Orientated biodegradable PCL microfibers for an artificial nerve were prepared and functionalized with GRGDS peptides (from ECMs) to produce guidance structures for Schwann cell migration and axonal regeneration [49]. Two different fiber types were tested, which consisted of high-molecular-weight (HMW) PCL mixed with star-shaped NCO-poly(ethylene glycol)-stat-poly(propylene glycol) (sPEG) with or without the addition of low-molecular-weight (LMW) PCL diol. Covalent binding between the sPEG isocyanate groups and NH_2- (*Method C2*) or OH-groups of peptide or PCL diol yielded urea and carbamate moieties, respectively (Figure 5). By using a mixture of 27% sPEG in PCL/PCL diol, better quality fibers with parallel orientation were obtained with respect to the use of PLC/sPEG mixture.

These fibers served as an excellent substrate for orienting cell migration and axonal elongation. On the other hand, to induce cellular movement over long distances, an increasing gradient of attractant molecules may be needed.

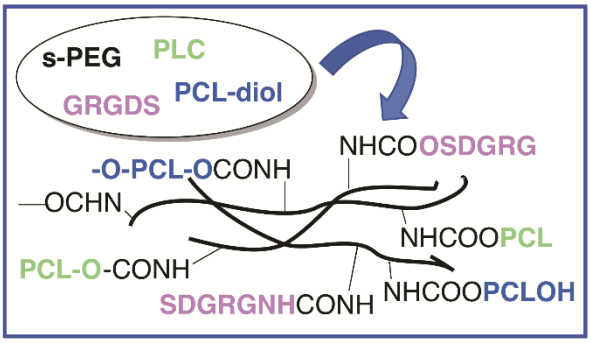


Figure 5. sPEG polyfunctionalized fibers.

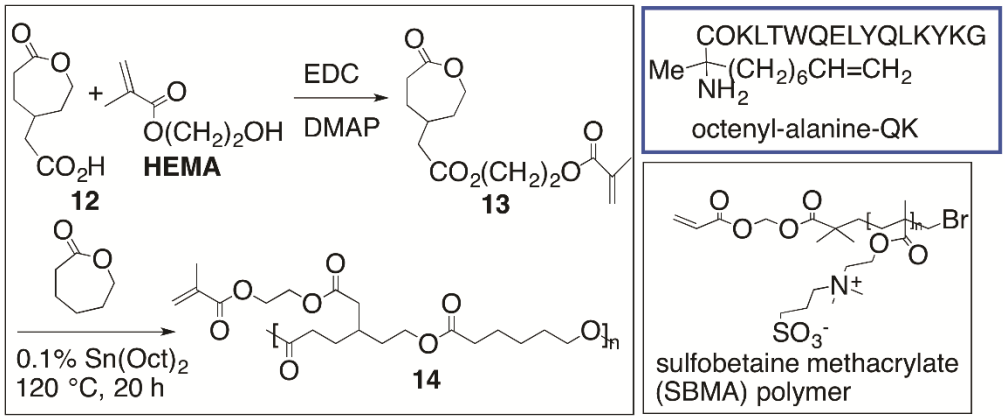
Carbamate function can be used to conjugate the peptide EPLQLKM (E7) to star-shaped poly(L-lactide-co-ε-caprolactone) (PLCL) copolymer [23]. In a study on the application of bone marrow-derived mesenchymal stem cells (BMMSCs) in cell therapy for the regeneration of injured/infarcted tissues, the E7-polymer conjugate was produced, and the potential of this construct to enhance stem cell adhesion/proliferation *in vitro* was evaluated. Eight-arm star-shaped PLCL copolymers were selected because they have higher numbers of end groups than their linear counterparts. Linear and star-shaped PLCL copolymers were synthesized by ring opening polymerization (ROP) from LA and ε-caprolactone (CL; 1-dodecanol and TOC initiators). After activation of PLCL OH-groups (CDI) and reaction with E7-*N*-terminus (Scheme 3; *Method C3*), construct **7** was generated. PLCL/PLCL-E7 meshes obtained by ESP were homogeneous and uniform in size as compared to PLCL fibers in the control and can be assembled into different shapes and structures as required for various tissue engineering applications. The adhesion and proliferation of the hMSCs on both fiber meshes were analyzed and showed a good performance of these new constructs.

C-terminus-grafted peptides by amide formation using amino-functionalized polymers are reported. Biofunctionalized PLGA fibers were prepared to investigate their potential for cardiac tissue engineering application [50]. As laminin signaling is relevant to changes in autonomic regulation occurring during cardiac development or disease, two adhesive peptides derived from laminin were selected. According to *Method D1*, L-polylysine (PLL) was used to conjugate *N*-acetyl-G**RGD**SPGYG and *N*-acetyl-G**YIGSR**GYG *via* carbodiimide reaction to yield PLL-g-peptides. PLGA-EFs with or without PLL-g-peptides were prepared. Altough not to the same extent, the presence of the two peptides significantly enhanced the adhesion of cardiomyocytes that is improved with respect to flat substrates.

PANI [51], a conductive polymer that is easy to synthesize and has tunable properties, is often used *via* NH_2-donor as a matrix for immobilization of proteins and peptides. The chemical and electrochemical polymerization of aniline in the presence of a peptide allowed its grafting (Scheme 4, *Method D2*) [52]. In the first case, aniline and peptide (1:25-1:30 ratio) were polymerized [MeOH, $(NH_4)_2S_2O_8$ catalyst] to afford an LMW polymer with

more than 70% peptide content. Electrochemical synthesis was performed using aniline/peptide mixture (1:125–1:15.6 molar ratio; MeOH, 0.25 M HCl) to afford PANI units containing 10% peptide.

Photo- or radical-crosslink reactions are also often used (*Method G*). Accordingly, a biodegradable acrylic-based crosslinkable PCL polymer was developed and used as a multi-head ESP station to fabricate hybrid scaffolds for promoting quick vascularization and to prevent foreign body rejection. Thus, growth factors can be incorporated for favouring cell proliferation and avoiding catalyst contamination or thermal degradation, as in the case of catalyst or thermal cross-linking [36]**.** Functionalized CL monomer **12** was synthesized and further modified with HEMA to provide vinyl functionality. ROP between scaffold **13** and CL (2:8 ratio; TOC catalyst) yielded copolymer **14** (Scheme 8). The presence of acrylic function in **14** is the key element for covalent grafting by photo-irradiation (*Method G1*). The zwitterionic sulfobetaine methacrylate (SBMA) polymer with antifouling properties and a vascular endothelial growth factor (VEGF)-mimicking peptide help to improve biological functions and enable efficient cell attachment and proliferation. The peptide sequence (QK) was prepared and then functionalized with octenyl-alanine. Functionalized PCL, poly-SBMA, and vinyl-QK (90:8:2, respectively) were spun onto a single matrix by using a hybrid ESP machine equipped with multiple syringe pumps on an x-y stage. Crosslinking was conducted (365 nm, UV light) with and without a photoinitiator (Irgacure-2959) to yield the functionalized construct.



Scheme 8. Synthesis of PCL-based copolymers **14**.

The development of antifouling, protein repellent-based materials is relevant to reduce nonspecific binding and promote simultaneous endothelialization. Non-fouling CGRGD-grafted polymers were synthesized according to different procedures [53]. Acrylate-PEG-NHS was first reacted with NH_2RGD peptide (1:1.2 ratio, *Method C1*). According to *Method G2* (Figure 6), the final acrylate-PEG-peptide **15** was copolymerized (AIBN, acetone/DMF) with hexyl- (HMA), methyl- (MMA), and poly(ethylene glycol)-methacrylates (PEGMA). In the second method, CGRGDS was incorporated by chain transfer in a single step in the presence of the above methacrylates to form polymer **16** (Figure 6). The NHS-chemistry is one of the best peptide-linking methods that does not lower polymer MW and increases the availability of the peptide. It was found that the electrospun materials from **15**

mantain protein resistance and cell adhesion properties, thus suggesting their potential in vascular graft (VG) applications.

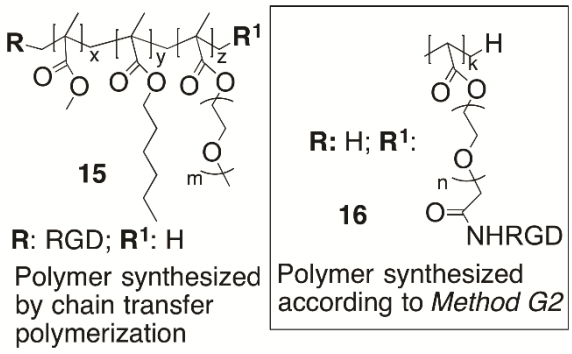


Figure 6. Polymers conjugates **15** and **16** from complex matrices.

A strategy based on the use of AA is applied for the preparation of polyuretanes (PU) elastomers whose chemistry and applications are extensively reviewed by Shah et al. [35]. L-Tyrosine (Tyr), a nonessential AA, can be exploited for polymer synthesis for its importance as a precursor of several neurotransmitters.

2.3 Peptides grafted after electrospinning

Surface modification through covalent linking allows uniform distribution of peptides throughout the biomaterial and is more efficient than simple coating in terms of retaining of molecules and decreasing leakage

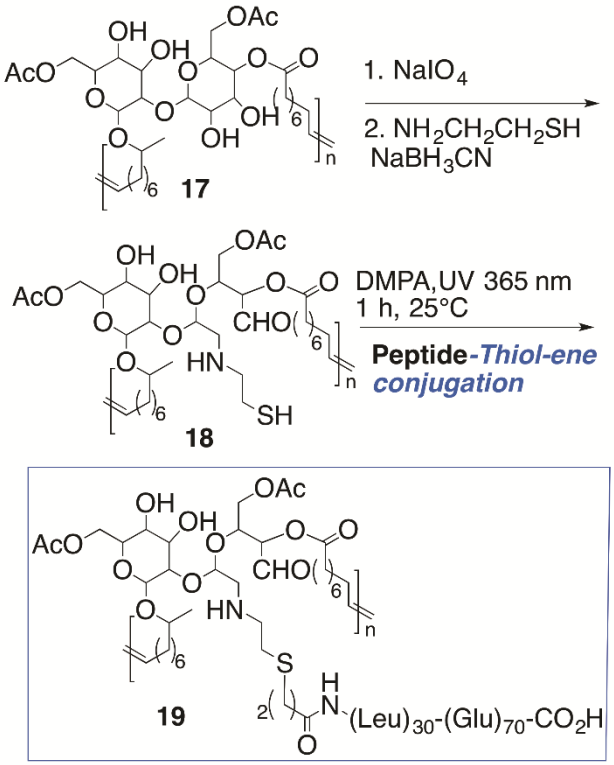
According to *Method A1* (Scheme 1), electrospun scaffolds are derivatized with an amine group [1,6-hexamethylenediamine (HDA) or poly-allylamine] and then functionalized with sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) or 6-MAL caproic acid. The obtained scaffolds are used for several applications described here. PCL fibers, linked to BMMSCs affinity E7-peptide, were used to construct a "MSC-homing device" and to evaluate the effect of recruiting MSCs both *in vitro* and *in vivo* [54]. E7-conjugated PCL mesh was found to be excellent for the adhesion, spreading, and proliferation of MSCs. Similarly, L7 (LTHPRWP), a synovium-derived mesenchymal stem cell (SMSC)-affinity peptide, and RGD as a positive control, were conjugated onto PCL-surface [55]. The resulting fibers had a good property for hSMSCs attachment and adhesion. Lattice-like patterned PLLA nanofibrous scaffolds [56] functionalized with peptide E7 to absorb native MSCs from bone marrow were fabricated and incorporated into autologous tendon. After implantation (3 months), the regenerated ligament-bone insertion exhibited abundant ECM and fibrocartilage growth and better tensile strength than control, thus demonstrating the great potential of this construct in accelerating ligament-bone healing process after anterior cruciate ligament reconstruction. Coaxial ESP is used for codelivery systems for peptide E7 combined with *i)* bone morphogenetic protein-2 (BMP2)-derived peptide [57] or *ii)* recombinant human TGF-β1 [58]. In the first case, microfiber scaffold was constructed using a polyvinylpyrrolidone (PVP)/bovine serum albumin (BSA)/BMP2-derived peptide (core solution) and a

PCL/collagen I (shell solution.) In the second case, PVP/BSA/rhTGF-β1 composite solution (core fluid) and PCL solution (sheath fluid) were used. A dual drug release system developed using both scaffolds was further functionalized with E7. Overall, the scaffolds simultaneously improved all the essential elements (scaffold, seed cells, and bioactive factors) required for bone/cartilage tissue engineering, thus providing a promising strategy for tissue regeneration.

An innovative LbL assembly method for creating 3D gradient peptide structures was realized to modulate cellular response at the nanoscale and induce faster bone formation [28]. EFs based on PLGA and nanohydroxyapatite (nHAp) were modified using PAAHCl and poly(sodium 4-styrenesulfonate) as polyelectrolytes. Different peptides were grafted into the discrete nanolayers by reaction of the MAL group with the SH of the cysteine-containing peptides. According to the dissolution of the multilayered coating, cells interact with the different peptides for: *i*) enhancing adhesion, spreading, and proliferation (KRSR; top layer), *ii*) guiding osteoblast differentiation (NSPVNSKIPKACCVPTELSA; middle layer), and *iii*) improving mineralization of the matrix (FHRRIKA; bottom layer).

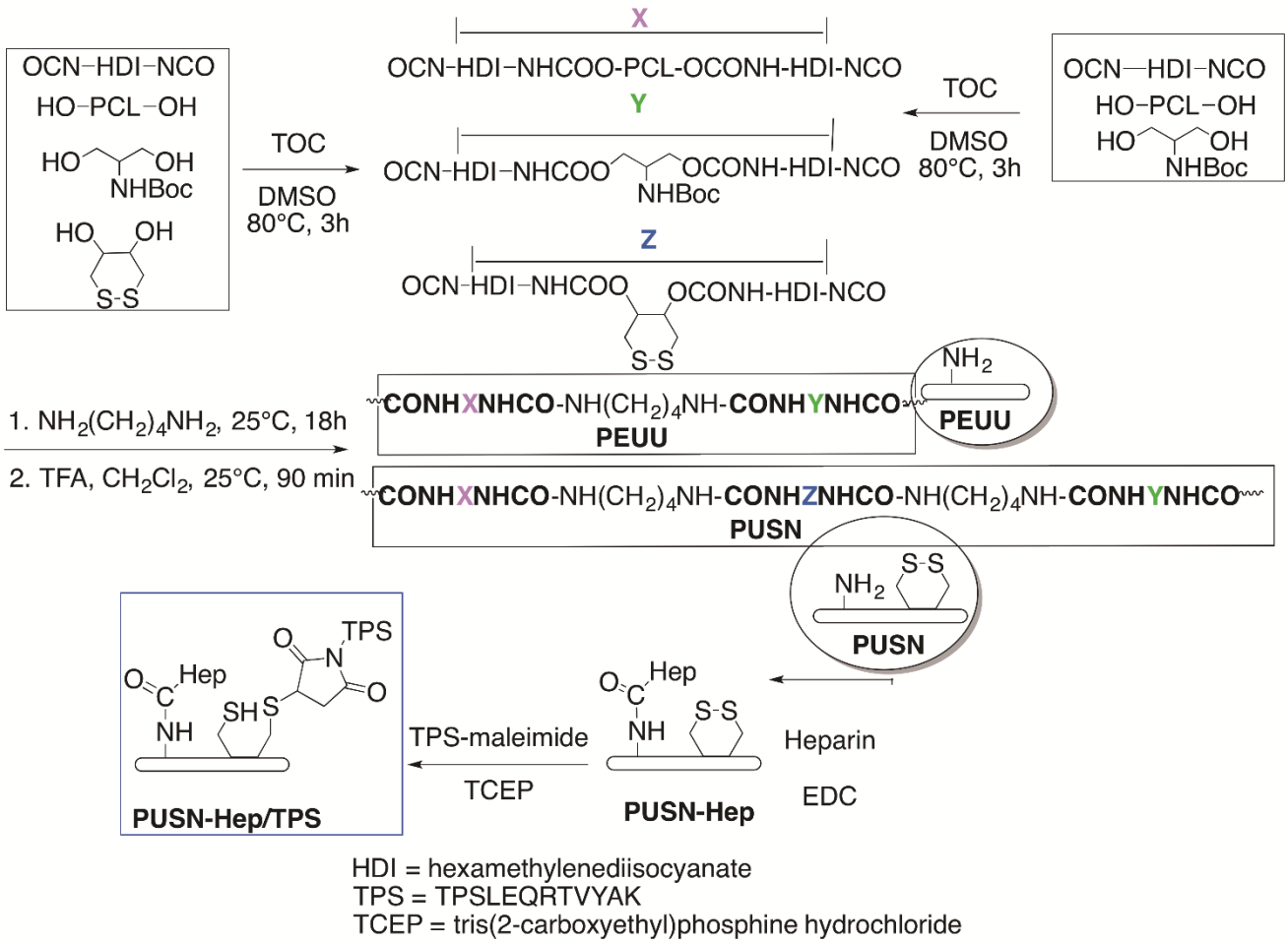
Silk Fibroin (SF) membranes without bead formation were obtained [59], and their CO_2H groups (EDC/NHS activation) were reacted with an N-(2-aminoethyl)MAL linker to graft the model antimicrobial peptide (AMP) KR12 modified with a cysteine to the *N*-terminus as a key element for its immobilization. This method allowed to precisely control peptide immobilization density.

Thiol-ene reaction is a well-known method to covalently graft alkene functionalized peptides [60] to the thiol functionalized mats (*Method B1*). Precisely (Scheme 9), periodate oxidation of **17** afforded the intermediate dialdehyde, which underwent reductive amination with cysteamine to give **18**. Its reaction with the pentenyl-peptide (DMPA, UV 365 nm) afforded **19**. To determine the surface functionalization with peptide, the N/S ratio was evaluated; it was observed that approximately 1 out of 2 SH groups undergoes the thiol-ene reaction with an alkene-terminated peptide.



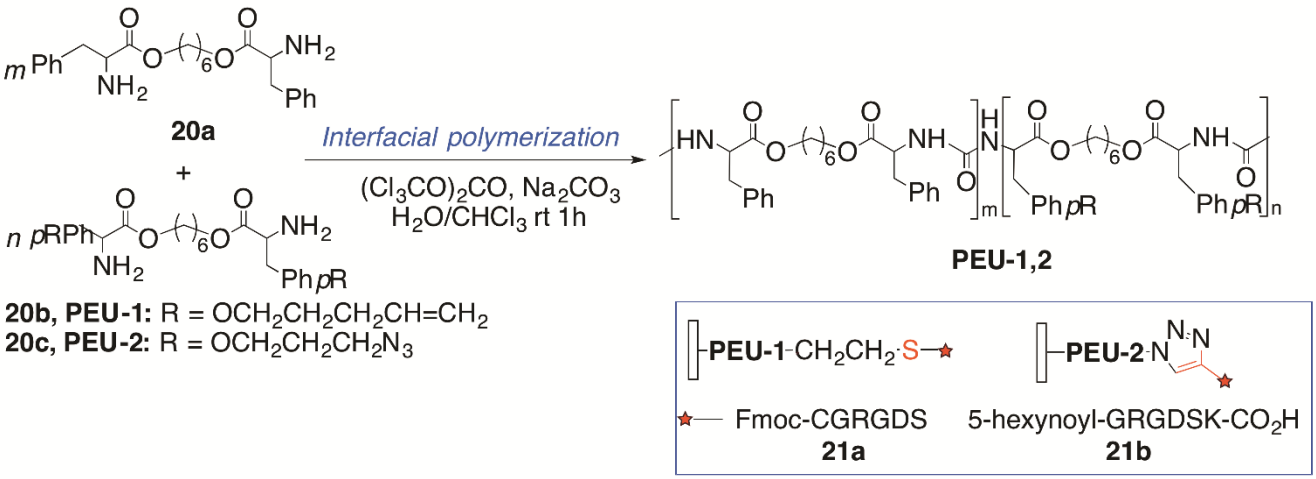
Scheme 9. Preparation of construct **19**.

A blood-contacting device based on PUSN scaffolds and derived from intermediates **X**, **Y**, and **Z** was prepared (Scheme 10) and functionalized with both antithrombogenic heparin (Hep) and TPS peptide for recruiting EPC (endothelial progenitor cells) [61]. PUSN-EFs were first treated with Hep to afford PUSN-Hep. *N*-terminus TPS-MAL was then immobilized (*Method B1*) to yield PUSN-Hep/TPS. Dual-modified PUSN scaffolds showed satisfactory performance based on the synergistic effect of the two functionalizations.



Scheme 10. PEUU and PUSN preparation and PUSN mats functionalization with Hep and TPS peptide.

Phe-based poly(ester urea)s **PEU-1,2** were obtained *via* interfacial polymerization of monomer **20a** with **20b** or **20c** [32]. **21a** was obtained by click reaction (UV light exposure) from **PEU-1**, functionalized with an alkenyl moiety and Fmoc-RGD-thiol (*Method B2*, Scheme 11).

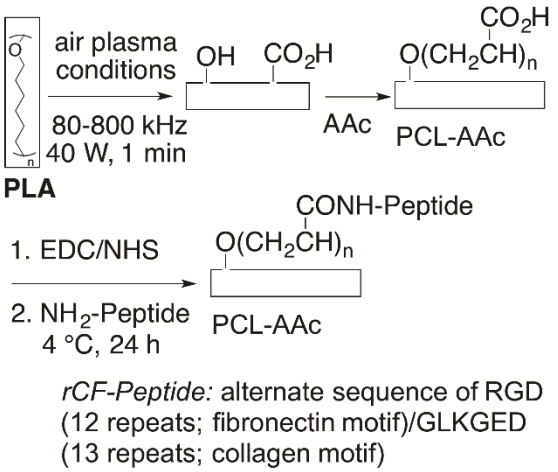


Scheme 11. Interfacial polycondensation affording PEUs with clickable pendants.

A photoclickable peptide microarray platform was developed [62] [*Method B2*] taking advantage of the same thiol-ene reaction between cysteine-functionalized peptides (CRGDS, CRGES, CIKVAV, CYIGSR, CDGEA, and CVAPG) and norbornene functionalized fibers. Combining four-arm PEG norbornene monomers (PEGNB) with PEG dithiol monomers (PEGDT) and a photoinitiator, three-dimensional fibrous hydrogels were generated. Different peptide solutions were prepared in a printing buffer, mixed, and individual spots were deposited on PEG-based fibrous hydrogels. After printing, peptides are "clicked" to the fiber array by UV exposure. This approach adds several improvements in comparison to existing platforms: the ability to integrate orthogonal reactive groups post-functionalization, three-dimensional fibrous geometry, ease of fabrication and printing with potential manufacturing ability, storage stability, low cost, and high-throughput potentiality.

Method C1 is often used to link the *N*-terminal end of a peptide to CO_2H-functionalized mats as listed below. Peptide of collagen IV origin [KK-(GPRGDPG)- F(4-I)] was linked *via* lysine side chain to the carboxyl group of gelatin (GE) in crosslinked PCL/GE fibers [63]. Peptide was functionalized with 4-I-phenylalanine as a tag to track its immobilization. After peptide link, no dramatic changes were observed in the morphology and diameter of these constructs, having a randomly oriented structure with nanoscale diameters. PCL and PCL/GE composite scaffolds modified with perlecan domain IV (PlnDIV) peptide, a specific cell recognition motif targeting prostate cancer cells, were produced [64]. Membranes with smaller fiber diameter showed greater coverage without a significant effect on the geometry of modified scaffolds but without uniform distribution of the biotin-PlnDIV-BSA complex. Functionalization of PLCL with acrylic acid (AAc) was performed (γ-ray irradiation, cobalt 60), and fibrous meshes were prepared [65]. To introduce a cell adhesive ligand, RGD peptide (GGGRGDS) was immobilized onto AAc-PLCL meshes [EDC/NHS, 2-(*N*-morpholino)-ethanesulfonic acid buffer solution). The RGD-AAc-PLCL matrices were characterized by measuring water contact angles. Their value significantly decreased after sequential reaction of the meshes with AAc and RGD peptide.

According to Scheme 12, PCL-AAc scaffold was generated [66] by first treatment of PLA with RF plasma under Ar/O_2 discharge and then with AAc. The rCF peptide grafting significantly enhanced cells and PCL scaffold interaction, thereby improving the adhesion and proliferation of cells.



Scheme 12. PCL functionalization with the rCF peptide.

With the same aim, (CMC)/PCL [67] and polyvinylalchol (PVA)/HA [68] polysaccharide nanofibers-mats were developed to graft both VN-peptide (Ac-KGGPQVTRGDVFTMP) and carboxymethylchitosan *via N*-terminus lysine (NHS/EDC activation), thereby affording fibers favourable for human pluripotent stem cell proliferation. SF mat, composed of porous multilayers with nanofibers in a random network, was functionalized with RGDS and KRSR [69]. Next, 100% of RGDS was grafted within 2 h. Nanofibrous structure did not change, except for a slight increase in fiber diameter. Lower immobilization efficiency of KRSR is likely due to Lys hindrance at *N*-terminus. Poly-(3-hydroxybutyrate) (PHB) and poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), with good biocompatibility and mechanical properties, were used to prepare uniaxially aligned nanofibers using a high speed mandrel [70]. Fibers were then functionalized (EDC/sulfo-NHS) with collagen or peptide sequences GRGDS, YIGSR, or RNIAEIIKDI.

An alternative approach to link a peptide *via* its *N*-terminus consists of the formation of a urea (*Method C2*). A sPEG/PLGA blend was electrospun. Randomly orientated nonwoven stabilized by overlapped fibers were gathered [71] on sPEG-coated silicon wafers with a protein and cell repellent background. After deriving sequences from fibronectin (GRGDS) and collagen IV (GEFYFDLRLKGDK), two of the major ECM constituents were used to modify fibers. Their immobilization leads to contrasting cellular responses, but a synergistic effect of both peptides significantly enhances the numbers of adherent keratinocytes.

C-terminus-linked peptides *via* amide formation using amino-functionalized polymers are also very useful (*Method D1).* Poly(ester-uretane)urea (PEUU)-based polymer was synthesized *via* a two-step solution polymerization from hexamethylenediisocyanate (HDI), PCL, and *N*-serinol (Scheme 10, intermediate **X** and **Y**) followed by conjugation with butanediamine [72]. After deprotection, PEUU-NH_2 polymer was obtained, spun, and functionalized with Ac-GRGD. The nanofiber surface showed a porous 3D structure and a relatively narrow distribution range diameter. The peptide incorporation improved mechanical properties and did not affect the morphology of the constructs having cytocompatibility and possessing lower hemolysis rate with improved inhibition of platelet adhesion. PU-PEG surfaces [73] are prepared by grafting HDI-PEG-HDI onto the PU surface through an allophanate reaction in the presence of dibutyltin dilaurate. PU-PEG-NCO films were hydrolyzed to afford the amine groups useful for linking Hep, GRGDS (G), and YIGSR (Y). *In vitro/vivo* studies showed that this construct is promising for the development of VG.

A directionally aligned chitosan nanofiber hydrogel was prepared [74] by combining ESP and mechanical stretching methods. EFs were then linked with RGI (Ac-RGIDKRHWNSQGG) and KLT (Ac-KLTWQELYQLKYKGIGG), mimicking brain-derived neurotrophic factor (BDNF) and VEGF. The aligned fibers were used as a nerve conduit filler to repair sciatic nerve defects in rats. Nano- or micro-fibrous PCL nonwovens were produced by ESP and coated in a radiofrequency (RF) plasma process to allow the covalent coupling to VEGF by forming stable amide bonds [75]. The variation in plasma process parameters allowed to yield different amounts of CO_2H-groups, and VEGF concentrations allowed to obtain distinct scaffolds with

variable VEGF concentrations. This approach appears to be a versatile method for the production of growth factor-loaded scaffolds at specific concentrations.

Polycarbonate-urethane (PCNU) materials, treated with surface modifying additives (ECP-BFSM: Elastin crosslinking peptide bioactive fluorinated surface modifiers), were electrospun, and fibrous mats with adequate diameter to be used as SMC support scaffolds in vascular applications were generated [76]. Elastin-like polypeptide ELP4, mimicking the structure and function of native tropoelastin and containing the VGVAPG repeat, was linked *via* a lysine side chain of ECP (Figure 7) to afford construct **22.**

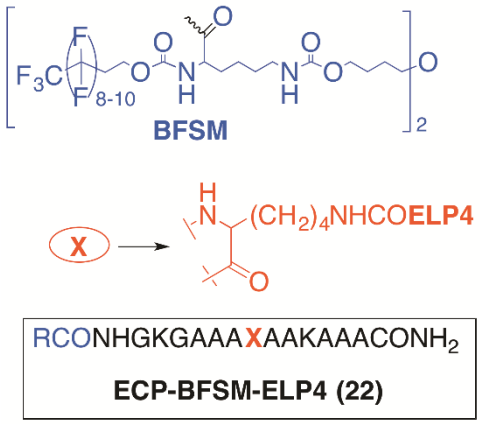


Figure 7. ELP4 linked to ECP-BFSM through a Lys NH_2 lateral chain of ECP.

An original technique was reported [77] to enrich PCL electrospun membranes of amino groups consisting of the deposition of a coating of (3-aminopropyl) triethoxysilane (APTES) by APPJ (atmospheric pressure plasma jet) and Argon. The duration of the deposition process is crucial, leading to different amounts of amino groups. HPV-construct **8** (Scheme 4, *Method D3*) was developed, and the peptide orientation was ensured by reductive amination between the aldehyde-functionalized HVP and the amine-functionalized APTES. Total nitrogen determination was used to monitor the concentration of the amino groups, obtained by APPJ deposition, and of the peptide after grafting. An increase in human osteoblast viability as a function of peptide concentration was demonstrated.

CuAAC and SPAAC are useful methods for functionalizing nanofibers (*Methods E).* Fibers with a smooth morphology were fabricated from poly(styrene-co-vinylbenzylchloride) (PsVBC) macroinitiator [39]. To stabilize the structure of the fiber matrix for later procedures, the fibers were annealed and modified with a low-fouling polymer brush coating, incorporating a trimethylsilyl-protected PEG-alkyne monomer, thereby allowing covalent linking of the azido-functionalized fluorescent RGD peptide (*Method E1*).

EF grafts (PLLA and PCL), showing an elastic modulus similar to the native arteries, were fabricated [78] and reacted with NH_2-PEG-alkyne to afford the alkyne-decorated scaffold. Because non-natural AAs are known to provide proteolytic stability [79], LXW7 (cGRGDdvc), a cyclic octa-peptide containing four unnatural D-AAs, was selected for its strong binding affinity to EPCs/ECs, weak binding to platelets, and no binding to

inflammatory cells. The NH_2-lysine-side chain of LXW7 was functionalized with 5-azidopentanoic acid, and after click reaction, scaffold **23** was obtained (Figure 8).

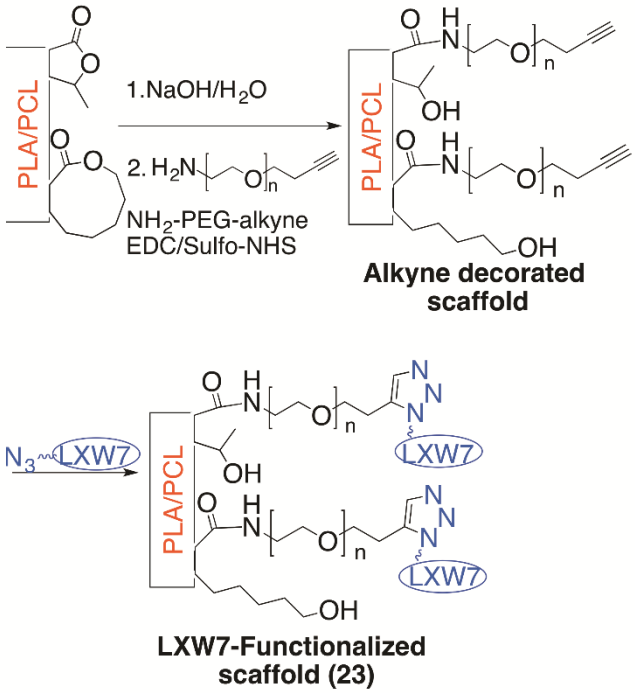
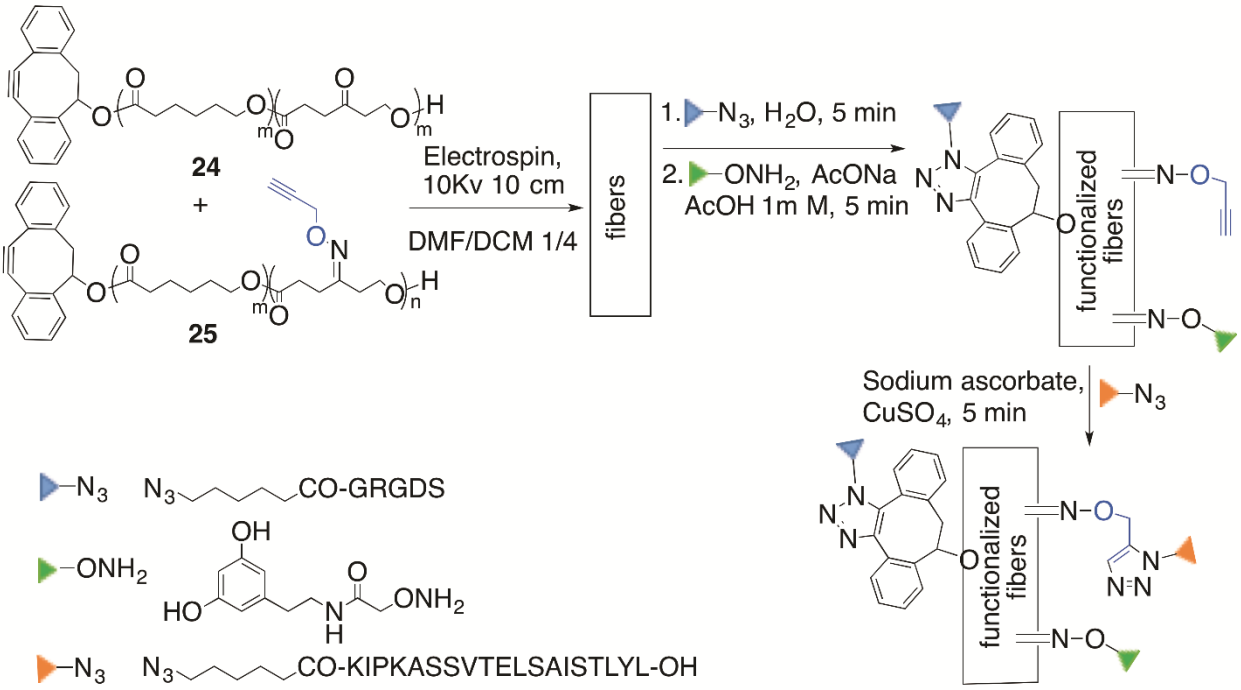


Figure 8. Preparation of the functionalized scaffold **23** *via* CuAAC.

Different chemical reactions are used in combination (CuAAC, SPAAC, and oxime ligation) to generate trifunctionalized nanofiber scaffolds (Scheme 13) [38]. Polymers **24**, functionalized with both an alkyne group (4-dibenzocyclooctyne, DIBCO) and a keto-group, were synthesized and reacted with O-(prop-2-yn-1-yl)hydroxylamine (oxime ligation) to afford **25** (Scheme 13). Subsequently, mixtures of polymers **24** and **25**, containing separate chemical handles (cycloalkyne, keto group, triple bond-functionalized oxime), were co-electrospun into well-defined nanofibers. SPAAC, CuAAC, and oxime ligation were used to selectively functionalize the nanofibers first with fluorescent reporters and then separately with bioactive peptides and DA. These highly functional materials can be used to study the synergistic and concentration-dependent influence of biomolecule combinations on cellular systems.

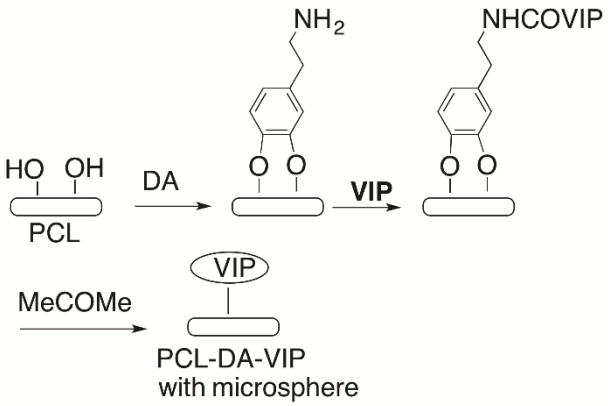


Scheme 13. Derivatized polymers **24** and **25** as tools for highly functionalized fibers.

Several PEUs carrying different clickable pendant groups were prepared (Scheme 11) [32]. The use of **PEU-2**, obtained from **20c** and functionalized with the azido group, in combination with the corresponding functionalized peptide and "click reactions" allows the covalent grafting of the peptide, generating **21b**.

The biological functions of PLLA/PCL-EFs, covalently functionalized with NH_2-PEG-N_3 *via* CO_2H-groups (EDC/Sulfo-NHS), were improved by functionalizing the polymer surface with LLP2A-DIBCO by click chemistry (*Method E2*) [80]. After LLP2A modification, a significant decrease in fiber diameters was observed compared to unmodified fibers. Scaffold contact angle decreased significantly, indicating that the LLP2A increases the hydrophilicity of the surface, which is expected to further enhance its cell cytocompatibility.

According to *Method F*, the coating with DA is often used as it offers convenient surface activity to several functional groups. DA-coated PCL nanosheets were functionalized with a vasoactive intestinal 28-AA peptide (VIP, Scheme 14) to promote angiogenesis and wound healing [81]. VIP adheres to DA because of the formation of an amide bond with the DA amino group (*Method F1*). VIP-loaded microspheres were generated *in situ* by immersion in acetone solution. They were immobilized and distributed homogeneously throughout the inner structure. Encapsulation efficiency and loading capacity were evaluated by HPLC. Interestingly, a prolonged release profile of VIP was shown.



VIP Peptide:
HSDAVFTDNYTRLRKQMAVKKYLDSILN-NH_2

Scheme 14. PCL-DA-VIP nanosheets with *in situ*-generated microspheres.

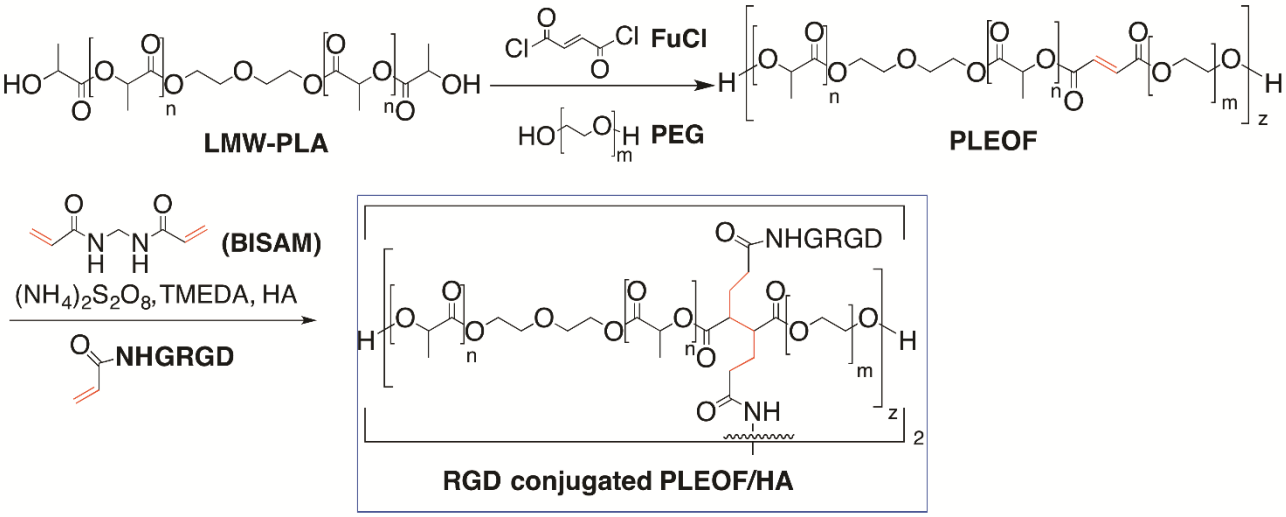
The covalent bond can also be realized between a peptide and DA before coating [82]. To promote attachment and proliferation of human umbilical vein endothelial cells (HUVECs), RGD [GGGGG (PRGD-Y)] was immobilized on electrospun PU meshes by using tyrosinase (Ty, phosphate buffer, 37 ºC). Thus, not only RGD but also other bioactive molecules (proteins, antibodies, etc.) could be conjugated to different surfaces.

Method F2 was used for different applications. pDA was coated under slightly alkaline aqueous solution on the surface of aligned PCL nanofibers after ESP, followed by covalent immobilization of the BFP-1 peptide (KGGQGFSYPYKAVFSTQ) onto a pDA-coated nanofiber surface [83]. PLLA fiber sheets were prepared and homogeneously coated with DA [84]. Then, fibers were immersed in OP (DWIVAGSGDWIVA) solution for linking the *N*-terminus OP to the catechol/quinone groups of pDA structure to be retained on the surface. Surface chemical characterization demonstrated that the coating time regulates the amount of pDA, and the immobilization efficiency and retention of OP on the surface depend on this concentration. This is an interesting result considering that OP is a relatively short peptide, which is difficult to retain within the materials, a limitation for long-term delivery.

Many other studies were published that used DA/pDA coating for peptide immobilization, but without specifying the groups involved in the covalent bond formation (**Table 2,** [85–88]).

According to *Method G1*, an interesting approach to fabricate peptide-tethered nanofiber microspheres (NMs) by combining ESP, electrospraying, and surface conjugation techniques was developed [89]. First, BMP2- and VEGF-mimicking QK peptides were modified at *N*-terminus with octenyl alanine to form BMP2-OCTAL and QK-OCTAL. Then, peptides were linked to PCL and GelMA nanofibers, containing an acrylate group as a pendant, through photocrosslinking (365 nm, Irgacure 2959, PBS) between the double bonds. By varying the processing parameters during the fabrication process, the composition and size of NMs can be tuned. BMP2-OCTAL peptide-tethered NMs significantly promote osteogenic differentiation of BMSCs. Moreover, HUVECs seeded on VEGF-mimicking peptide QK-OCTAL-tethered NMs significantly upregulated vascular-specific proteins, leading to microvascularization.

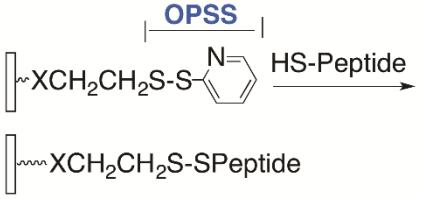
By applying *Method G2*, layers of electrospun PLLA fiber mesh coated with a poly(lactide-co-ethylene oxide fumarate) (PLEOF) hydrogel precursor solution were stacked and pressed. After crosslinking, a laminated fiber-reinforced composite was produced. To obtain an osteoconductive matrix for BMS cells, HA nanocrystals were added to the precursor solution. PLEOF/HA hydrogel was functionalized with Ac-GRGD to promote focal point adhesion of BMS cells (Scheme 15) [90].



Scheme 15. Synthesis and crosslinking of PLEOF affording RGD-conjugated fibers.

In many cases, specific protocols were applied, as described below. PCL-PIBMD (b-poly(isobutyl-morpholine-2,5-dione) was electrospun with SF to prepare PCL-PIBMD/SF nanofibers that were aminated by the copolymerization of DA and HDA [91]. They were further reacted with orthopyridyl disulfide (OPSS)-PEG-NHS through active ester chemistry. Finally, cell-adhesive peptide sequences were conjugated to OPSS-modified surface (Scheme 16). This biofunctionalization is an effective method to improve the endothelialization and long-term performance of synthetic VGs.

Taking advantage of OPSS-linker, a similar strategy was applied for generating cardiovascular biomaterials that can enable rapid endothelialization [92]. In this case, poly (ethylene glycol)-b-poly(L-lactide-co-ε-caprolactone) (PELCL) membranes were hydrolyzed and reacted with 2-(2-pyridinyldithio)ethaneamine hydrochloride (EDC/NHS). Peptides were covalently linked to the scaffold because of SH reaction with the disulfide (Scheme 16).

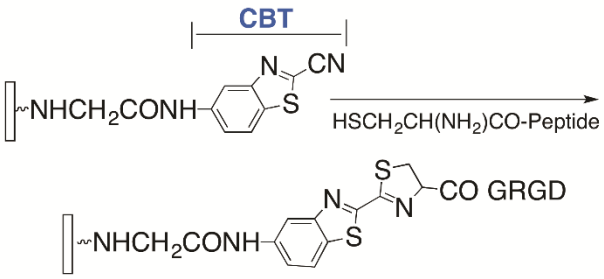


Ref. 91: X = NHCONH; PeptideSH: **CREDVW; CAGW**
Ref. 92: X = CONH
PeptideSH: **VAPGSH** (CVGVAPG); **RDVSH**
QKSH CK(Ac)LTWQELYQLK(Ac)YK(Ac)G)

Scheme 16. OPSS strategy to functionalized different scaffolds.

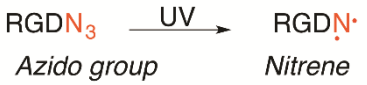
Polypropylene grafted with *L*-Cys was developed with an increased crystallinity. The SH surface functionalization efficiently crosslinks the antimicrobial peptide (AMP) Cys-LC-LL-37 through a disulfide bond [93]. The presence of the AMP dramatically reduced the growth of *S. aureus* and *P. aeruginosa*, thus producing a new material that is safe and alternative to antibiotics for potential use in garments under hospital settings.

A particular and efficient protocol to immobilize RGD on PCL scaffolds was developed [94] based on a water-compatible condensation reaction between 2-cyanobenzothiazole (CBT) and *N*-terminus D-Cys of the RGD peptide (Scheme 17).



Scheme 17. RGD immobilization-based PCL functionalization with 2-cyanobenzothiazole (CBT).

Finally, EFs from solution of P(LLA-CL) or PCL were obtained. N_3RGD sequence containing two arylazides at *C*- and *N*-termini to increase the covalent linking was selected (Figure 9) [95]. The fibers were incubated with peptide and the azides converted into nitrene (UV 254 nm, 30 min) as a tool for grafting. This method, even if not particularly efficient, is simple, versatile, and provides scaffolds with different peptide density, thus ensuring selective grafting of the adhesive peptides.



N_3RGD = *p*N_3Phe-(GRGDSP)$_4$-*p*N_3Ph-NH_2

Figure 9. Photoreactive peptide N_3RGD grafted on the surface through conversion into a nitrene.

Finally, materials containing single functional α-AA covalently linked to polymers were produced, providing materials with combined properties of synthetic polymers and natural polypeptides. The functionalized AA can be used as a tool for further functionalization of the fiber.

Poly(ester amide)s (PEAs) are biodegradable polymers containing ester and amide linkages along the polymer backbone and are synthesized from natural α-AAs. Owing to the carboxylate pendant, the introduction of Asp allows the conjugation of biomolecules at their *N*-terminus as well as a negative charge to cells. The *L*-phenylalanine (Phe)-based di-*p*-toluenesulfonate monomers **26** or **27** and *bis*-*L*-Asp derivative **28** were prepared and reacted with sebacoyl chloride ($ClCO(CH_2)_8COCl$)[96]. Polymers 8-Phe-4-Asp(O-t-Bu)-4 and 8-Phe-8-

Asp(O-t-Bu)-4 were respectively obtained and then deprotected to β-*t*-butyl ester function to give **29** (Figure 10). To optimize 3-D electrospun PEA fibers, different solutions of PEA in a co-solvent ($CHCl_3$: DMSO or DMF) were prepared. It is accepted that the bioactivity of the growth factor TGF-β1 is associated with the octapeptide sequence near the *C*-terminus, indicating a beneficial bioactivity if conjugation is realized through its amino-terminus. As a result, 8-Phe-4-Asp(OH)-4 electrospun mats were conjugated with TGF-β1 using a conventional technique (Figure 10).

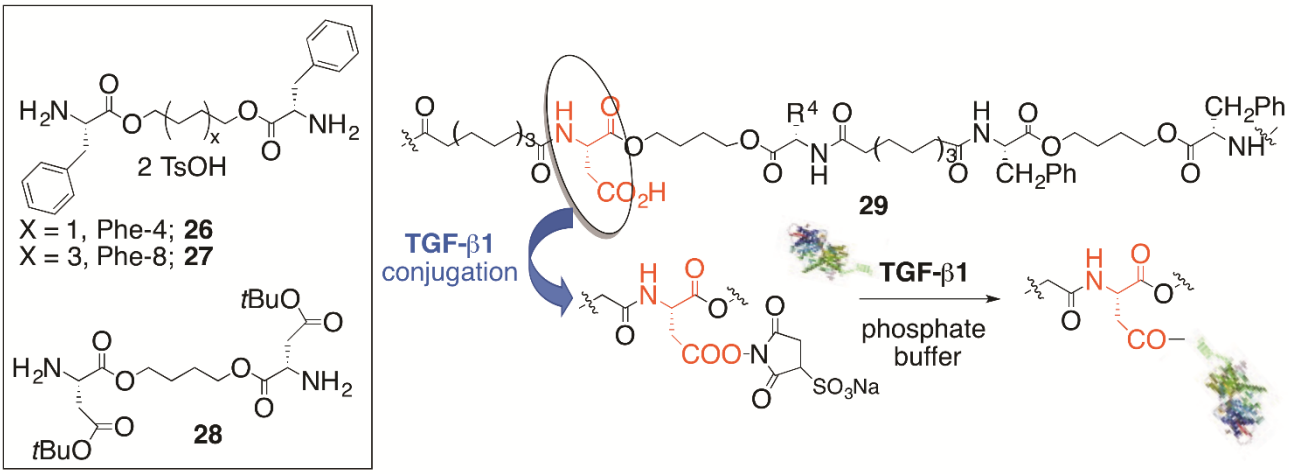


Figure 10. PEA fiber as a tool to conjugate TGF-β1.

Conclusion

In conclusion, peptides were widely used in the last decade to functionalize polymers to generate EFs or to directly functionalize EFs. Covalent linking allows a uniform distribution of the peptides throughout the material. This strategy is reported to be more effective in terms of concentration of adsorbed molecules, thereby avoiding leakage, a typical phenomenon that occurs in blending or physical absorption. Moreover, it improves the performance and provides materials with a better biological response with respect to the polymer itself. The use of suitable orthogonal groups to covalently graft the peptide allows not only its correct orientation on the scaffold surface but also selective functionalization with different biomolecules. Covalent linking methodology before ESP offers advantages in terms of functionalization in a single step, thus avoiding changes in the mechanical properties of the matrices. This strategy, however, is less used with respect to the direct functionalization of the matrices because remarkable amounts of the bioactive molecule are buried in the nanofiber and consequently are not available on the surface. The manufacturing process and the regulatory strategy suffer from inadequate control over surface functionality, particularly if these materials should be advanced to clinical applications. The functionalization after ESP can often take advantage from surface modification

techniques (e.g., DA-, RF-, and APPJ-coating process, see Table 2), thereby broadening its use. The use of different scaffolds functionalized with the same peptide is reported, proving that a wide variety of polymers, the application of particular technologies, and the specific activity of a single or a combination of peptides, provide materials with similar but never identical properties [54] [56–58] [87] [23] [67,68]). The use of peptides in ESP has a great potential mostly in the area of life sciencesa [97], but it can also serve several important purposes in other fields [98–100] ranging from energy harvesting devices [101] to protective clothing [102] or production of functional foods with enhanced functionality and stability [29].

Table 1. Effects/Benefits of peptide conjugation *before* electrospinning and applications

Entry	Functionalized polymer	Peptide	Co-electrospun agent	Advantages of the methodology Effects/Benefits of peptide conjugation	Application	Ref.[a]
1	HAS-MAL	c(RGDf C)	Tecoflex(TEC) EG80-A PU	- increasing of endothelization of electrospun 3D matrices - no changes in elasticity and durability compared with TEC-HAS	HUVECS improved cellular adhesion in a dose dependent manner	[45]
2	PCL-MAL	[b]	PCL	- setup of a specific protocol to design a complex scaffolds with opposing peptide gradient conjugates (see entry 3 for application)	Strategy to drive in opposing gradients biomolecules binding within the scaffold, to guide cell behavior	[34]
3	PCL-MAL	RYPISRPRKRGGGC YKTNFRRYYRFGGC	PCL	- spatial organization of GAG in dynamic gradient scaffolds mimicking the spatial biomolecules arrangement architecture of native tissue	Potential application in articular cartilage due to increase in the amount of GAGs adhesion	[19]
4	MAL-PLLA	CGGRGDS	PLGA	- porous fibers with adjustable diameters - pronounced effect on cell adhesion/migration of fibroblasts as compared to pure PLGA fibers - EFs increase of hydrophilicity mediated by PLLA-b-CGGRGDS content generating meshes more attractive for cell infiltration and more sophisticated for tissue engineering strategies - no indication of toxic adverse effects on cell proliferation	Direct production of cell-loaded scaffolds or biohybrid materials	[20]
5	PCL-$(HEMA)_2$ PCL-b-$(PEG\text{-}MAL)_2$	REDVC	PELCL miR-126	- faster release profiles of miR-126 than PELCL membrane - improved VECs adhesion/proliferation in comparison with the PELCL membrane - downregulation of SPRED-1 gene expression	Tissue engineering of small-diameter blood vessels	[37]
6	AAc-PLA	EEGGC (GLU)	PLGA	- regulation of nucleation extent and growth of CaP crystals on the fibers - increased toughness of the micro-sheets in relation to the formation of CaP crosslinked network of fibers - increased osteogenic differentiation of marrow stromal cells and mineralization	Biomimetic matrix in the regeneration of skeletal tissues	[46]
7	acODLA	IKVAV	PLLA	- setup of a specific protocol based on temperature optimization effect to generate EFs with optimized exposure of peptides (see entry 8 for application) - quantitative method to detect active surface peptides from those embedded in the matrix and comparison with conventional surface analytical methods (XPS, X Ray)	General study: functionalization of matrix as a means to improve bioactivity	[47]
8	acODLA	RKRLQVQLSIRT (AG73)	PLLA	- better functional reinnervation in comparison with silicone tube or unmodified PLLA nanofibrous conduit	Nerve regeneration- Artificial nerve conduit	[48]
9	sPEG	GRGDS	PCL PLC-diol	- improving guidance of SCM and axonal growth with respect to non-PCL functionalized fibers - beneficial effect of PCL-diol additive on parallel orientated fibers - reduction of non-specific protein adsorption	Nerve repair	[49]
10	star-shaped PLCL copolymers	EPLQLKM (E7)	PLCL	- homogeneous fibers, uniform in size in comparison to PLCL fibers control - possibility to be fabricated scaffolds with different shapes as needed for various tissue engineering applications	Tissue engineering: improvement of stem cells adhesion and retention	[23]
11	PLL	*N*-acetyl GRGDSPGYG *N*-Acetyl-GYIGSRGYG	PLGA	- significant enhancement of adhesion of cardiomyocytes with respect to flat substrates.	Cardiac tissue engineering	[50]
12	PANI	FLDQV	gelatin	-set up of two different polymerization protocols to tune % of peptide on EPs surface	Potential extraction and determination of chlorinated toxins from water	[52]
13	AAcPCL copolymerized with PCL	VEGF-mimicking Sequence	SBMA[c]	- development of an optimized synthetic procedure preventing catalyst contamination or thermal degradation of a biological material - enhancement of vascularization - enhancement of biochemical signaling useful for tissue engineering and regenerative transplants	Cell transplantation. Development of a cell delivery scaffold for the long-term function and survival of insulin producing β-cell grafts	[36]
14	Acrylate-PEG-NHS	CGRGD	HMA/MMA/ PEGMA	- identification of NHS-RGD coupling chemistry as better peptide linking method with respect to chain transfer - generation of a specific surface matrix with binding affinity toward selected cells but not proteins - selective interaction of transmembrane integrins promoting cell adhesion - selection of optimized functionalized polymers for vascular graft application	Antifouling, protein-repellent base materials for vascular grafts	[53]

[a]References are reported as cited in Section 2.2. [b]Peptides not defined. For details see ref [34]. [c]Sulfobetaine methacrylate (SBMA)

Table 2. Effects/Benefits of peptide conjugation *after* electrospinning and applications

Entry	Polymer	Linker/ Coating	Peptide	Advantages of the methodology Effects/Benefit of EF's peptide conjugation	Application	Ref[a]
1	PCL	-	E7	- development of a "MSC-homing device" for MSCs recruitment (both *in vitro* and *in vivo*) - enhancement of MSCs adhesion, spreading and proliferation. - increasing of specificity toward MSCs compared with RGD-conjugated PCL electrospun meshes.	Tissue engineeering Regenerative medicine	[54]
2	PCL	-	LTHPRWP (L7) RGD control	- significant enhancement of SMSCs adhesion and well spreading	Tissue engineering: cartilage regeneration	[55]
3	PLLA		E7	- better tensile strength than control - abundant ECM and fibrocartilage growth	Ligament-bone healing process after ACL reconstruction	[56]
4	PVP/BSA/ BMP-2 peptide (core solution) PCL/Collagen (sheath solution) *Coaxial ESP*	-	EPLQLKM (E7)	- development of a dual drug release system for the sustained delivery of the BMP2-derived peptide and E7 - improved adhesion of BMSCs - improved wettability facilitating nutrient transport, necessary for cell growth - enhancement of new bone formation and defect closure in a rat calvarial defect model	Bone regeneration	[57]
5	PVP/BSA/ rh-TGF-□1 (core solution) and PCL (sheath solution) *Coaxial ESP*	-	EPLQLKM (E7)	- improvement of the biocompatibility of scaffolds and promotion of BMSCs adhesion and chondrogenic differentiation - sustained release of encapsulated rhTGF-b1	Cartilage tissue engineering	[58]
6	PLGA/nHA	PAAHCl/poly(sodium 4-styrenesulfonate)	KRSR FHRRIKA NSPVNSKIPKACCVPTELSA	- LbL approach to multilayer nanoencapsulation of biofunctional peptides - enhancement of cellular processes with good viability and significant increase of alkaline phosphatase activity, osteopontin/osteocalcin - favorable *in vivo* response after implantation	Bone regeneration in orthopedic and craniofacial medical devices	[28]
7	SF	-	CKR12	- peptide immobilization density control - antimicrobial activity against pathogenic bacterial strains (*Staphylococcus aureus* and *epidermidis*, *Escherichia coli*, and *Pseudomonas aeruginosa*) - no biofilm formation on the membrane surface - facilitating the proliferation of keratinocytes and fibroblasts - promoting the differentiation of keratinocytes with enhanced cell-cell attachment; - suppression of the LPS-induced TNF-a expression of monocytes (Raw264.7) cultured on the membrane.	Wound dressing material	[59]
8	polyLSL[6′Ac,6″Ac]	-	$(Leu)_{30}$-$(Glu)_{70}$	- development of an effective strategy to covalently bound biomolecules to the surface of an unique bioresorbable and biocompatible polymeric material	Tissue engineering Controlled drug delivery	[60]
9	PUSN	Hep	TPS	- reduced platelet adhesion - high efficiency of EPC proliferation - synergic effect of TPS/Hep	Potential application in small diameter vascular regeneration	[61]
10	PEUs carrying different clickable pendants		GRGDSK CGRGDS	- development of a versatile platform to prepare ECM-like materials with biological functionalities	Tissue engineering	[32]
11	PEGNB	PEGDT	'RGDS, CRGES, CIKVAV, CYIGSR CDGEA, CVAPG	- development and validation of a photoclickable peptide microarray platform	Tissue engineering: new, facile and rapid screening method for probing cellular	[62]

				behavior in 3D tissue microenvironments		
12	PCL/GE	-	KK-(GPRGDPG)- F(4-I)	- increasing hBMSCs adhesion and proliferation - improving cell-matrix interaction	Regenerative medicine	[63]
13	PCL PCL/GE	-	PlnDIV (perlecan domain IV)	- development of successful 3D-pharmacokinetic cancer model - enhancement of adherence/infiltration of metastatic prostate cancer cells - cells cultured on functionalized matrices organized stress fibers increasing proliferation - reduced expression of tight junction protein - increased focal adhesion kinase (FAK) phosphorylation on tyrosine 397	Biomaterials scaffolds for cancer cell studies	[64]
14	AAc-PLCL	γ-ray irradiation, cobalt 60	GGGRGDS	- greater adhesion and proliferation of MC3T3-E1 pre-osteoblastic cells - mature formation of F-actin stress fibers and focal adhesion (co-localized with vinculin) - significant up-regulation of the expression of selected osteogenic genes, Cbfa1, ALP, OCN - higher ALP activity and calcium content	Tissue engineering	[65]
15	PCL	RF plasma of Ar/O_2 discharge and AAc treatment	rCF peptide	- significant increasing of surface hydrophilicity - enhancement of cell adhesion/proliferation	Tissue engineering	[66]
16	PCL	CMC	Vitronectin (VN)	- promotion of hPSCs attachment/proliferation	Bone regeneration and tissue engineering	[67]
17	PVA/HA	-	VN-peptide	- support hiPSCs proliferation - promotion of down-regulated expression of pluripotency	HiPSC culture	[68]
18	SF	-	RGDS, KRSR	- improving cell adhesion, spreading and proliferation of specific cell types.	Tissue engineering	[69]
19	PHB/PHBV	-	GRGDS, YIGSR, RNIAEIIKDI Collagen	- uniform, uniaxially oriented nanofibers - SCs incresed metabolic activity/proliferation - significant up-regulation of neural genes indicating differentiation/maturation of SCs	Nerve regeneration	[70]
20	sPEG/PLGA	-	GRGDS GEFYFDLRLKGDK	- synergistic effects of both peptides increasing the numbers of adherent keratinocytes inducing cell adhesion - lower effect of GRGDS with respect to GEFYFDLRLKGDK	Dermal tissue regeneration	[71]
21	PEUU	-	Ac-GRGD	- improving of fiber mechanical properties - no negative effect on the morphology and the structure of PEUU nanofibers - cytocompatibility, lower hemolysis rate and improved inhibition of platelet adhesion	Vascular tissue engineering	[72]
22	PU	PEG	GRGDS, YIGSR	- significantly higher grafts patency - enhancement of the attachment/proliferation of HUVECs	Small caliber vascular grafts	[73]
23	Chitosane		AcRGI, AcKLT	- directionally aligned fiber promoted by ESP and mechanical stetching methods - orientation of the Schwann cells - proliferation/secretion of neurotrophic factors by Schwann cells - enhance of nerve regeneration and vascular penetration at an early injury stage	Artificial nerve grafts	[74]
24	PCL	Radio frequency (RF) plasma coating process	VEGF	- development of distinct scaffolds presenting high or low VEGF concentrations varying plasma process parameters and initial VEGF solution concentrations - significant enhancement of primary endothelial cell number or immortalized endothelial cells	Tissue engineering	[75]
25	PCNU	ECP-BFSM	ELP4	- enhancement of SMC adhesion - promoting cell alignment	Contractile vascular tissues engineering	[76]

26	PCL	APPJ deposition of coating; APTES	HVP	- setup of a specific protocol based on RF plasma to enrich the scaffolds of NH_2 tuning biomolecules superficial concentration - increase of human osteoblasts viability as a function of peptide concentration directly correlated with scan number of APPJ process	Bone scaffold	[77]
27	PsVBC	PEG-alkyne	Azido-RGD	- enhancement of attachment and adhesion - minimal protein adsorption on the fiber - study on systems that decouple the surface and bulk properties of the resulting fibers, allowing these to be independently processed and optimized for specific applications - study on modification of EFs with a low-fouling polymer brush coating, to allow the conjugation of cell signaling peptides while reducing non-specific interactions - enhancement of cell attachment/adhesion with minimal protein adsorption on the fiber	Versatile system for different tissue engineering application	[39]
28	PLLA/PCL	NH_2PEG alkyne	LXW7	- improved EPC attachmen/proliferation and endothelial differentiation - suppression of platelet attachment - improvement of the patency of small diameter vascular grafts	Small diameter vascular grafts	[78]
29	DIBO-[P(CL-co-OPD)]	-	GRGDS, BMP-2	- set up of a protocol to tether multiple bioactive molecules to the scaffold	Envisioned applications in the study of synergistic effects on cellular activity regulation	[38]
30	PLLA/PCL		LLP2A	- significant decrement diameters compared to unmodified fibers - scaffold contact angle decreased significantly indicating the increasing of the hydrophilicity further enhancing cell cytocompatibility - enhanced CV-MSC adhesion, spreading and viability - strong binding to MSCs of other sources (*i.e.* BM-MSCs) and adipose tissue-derived mesenchymal stem cells (AT-MSCs).	Fetal tissue engineering	[80]
31	PCL	DA coating	VIP	- selection of PCL/DA/VIP microspheres with proper diameters - comparison of VIP adhesion in microspheres with or without DA - enhancement of cell adhesion than the PCL-DA nanosheets - wound healing significantly faster	Wound treatment and vascular tissue engineering Wound healing and Angiogenesis	[81]
32	PU	Tyrosinase	DA conjugated GGGGGPRGDY (RGD peptide)	- Set up of a new facile immobilization methods of biomolecules using tyrosinase - enhancement of HUVECs attachment proliferation and spreading	Vascular tissue engineering	[82]
33	PCL	pDA coating	BFP-1	- improvement of cytocompatibility in terms of cell adhesion, spreading, proliferation - synergic action of aligned nanofibers and surface-grafted BFP-1 peptides to enhance the osteogenic differentiation of stem cells even under non-osteoinductive conditions	Bone tissue engineering and regenerative medicine	[83]
34	PLLA	pDA coating	OP	- significantly enhanced bone formation - well organized bone regeneration with mechanical properties similar to those of the host bone	Bone formation	[84]
35	PLCL/GE	DA coating	LTF-PRIVFVLG (ACH 11) CAG	- suppression of the denaturation of absorbed fibrinogen, hindering coagulation factor Xa activation, inhibition of platelet adhesion and aggregation - selective enhancement of endothelial cells adhesion, proliferation and release of nitric oxide	Small-caliber vascular grafts for the treatment of cardiovascular diseases	[85]
36	SF/HA	DA coating	HHC-36	- significant enhancement of the hydrophilicity of the implant surface and decreasing of the contact angle - no significant effect on the average fiber diameter nanofibers - ability to enhance osteinductivity and mineralization	Improving osseointegration preventing infections	[86]

				- eradication of Gram-negative and Gram positive bacteria (up to 21 days)		
37	SF	DA coating	E7	- improvement of hydrophilicity - facilitated cell proliferation and adhesion - boosted osteogenic differentiation of BMSCs	Bone tissue engineering	[87]
38	nHA/PLA/GEL 3D scaffold	pDA-assisted coating	BMP2-derived peptide	- collaborative effect of nHA and BMP-2 peptides in osteoinduction both *in vitro* and *in vivo* - ALP activity significantly exceeded that of PLA/GEL and nHA/PLA/GEL 3D scaffolds without peptide - more pores with a uniform distribution that preferentially promoted cell migration and differentiation.	Bone tissue engineering	[88]
39	PCL-gelatin-GelMa	-	IPKASSVPTELSAISTLYL (BMP-2-OCTAL) KLTWQELYQLKYKGI (QK-OCTAL)	- set up of a protocol to fabricate peptide-tethered NMs by combining electrospinning/electrospray in surface conjugation techniques - tuning of composition and size of fibers by varying the processing parameters during the fabrication - promoting osteogenic differentiation of BMSCs - up-regulation of vascular-specific proteins, leading to microvascularization	Osteogenesis/Angiogenenci s flexible platform technology to tether a wide variety of peptides/ biomolecules to NMs for directing cellular response through a minimally invasive procedure	[89]
40	PLLA	PLEOF/ HA	AcGRGD	- promoting focal point adhesion of BMS cells - higher calcium content - significant higher expression of osteopontin/osteocalcin	Bone regeneration	[90]
41	PCL-PIBMD/SF	DA/HDA	CREDVW, CAGW	- improvement of the hemocompatibility - enhancement of long-term patency and endothelialization	Small-caliber vascular graft	[91]
42	PELCL	-	REDV, VAPG, QK	- improved hydrophilicity - proper mechanical properties - acceleration of VECs proliferation - less protein (BSA and BFg) adsorption capacity and lower platelet adhesion ability	Vascular grafts	[92]
43	PP-g-SH	-	CLCLL-37	- higher bactericidal activity with respect to PP-g-SH alone - no cytotoxicity	Hospital garment	[93]
44	PCL	-	CGGRGD	- significant enhancement of cell attachment/proliferation - faster cells spreading	Tissue engineering	[94]
45	P(LLA-CL) or PCL	-	N_3RGD	- enhancement of the adhesion with a dose-dependent effect - control of the proliferation of HUVEC	Tissue engineering, vascular grafts	[95]
46	PEA	-	TGF-b1	- 3D PEA scaffold optimization - increased in SmαA/calponin expression from HCASMCs cultured on 3-D fibrous scaffolds compared to 2-D films - HCASMC attachment, spreading and proliferation - adopted a proliferative phenotype (absence of smooth muscle α-actin expression)	Viable biomaterials for investigation in vascular tissue engineering	[96]

[a]References are reported as cited in Section 2.3.

Statement of Significance

The primary aim of this review is to provide an overview of the main use of peptides in electrospinning. The reviewed literature primarily emphasizes the technical aspects and general principles of electrospinning or the use of plant- or animal-derived proteins or synthetic polymers for generating elecrospun scaffolds. A special focus is given to applications, largely from specific biomedical fields (tissue engineering, drug delivery, and biomedical materials). The combination of peptides and polymers is a very interesting tool to improve the performance of the material. Different strategies are applied to integrate peptides: coating, blending, and covalent grafting are mostly used. The present review focuses on the methodologies for covalently linking peptides to polymers in order to functionalize the fibers. The review covers studies published from 2010 to date.

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Declaration of competing interests

None.

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