**MINI REVIEW**

# Trends in the design and use of elastin-like recombinamers as biomaterials

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## Abstract

Elastin-like recombinamers (ELRs), which derive from one of the repetitive domains found in natural elastin, have been intensively studied in the last few years from several points of view. In this mini review, we discuss all the recent works related to the investigation of ELRs, starting with those that define these polypeptides as model intrinsically disordered proteins or regions (IDPs or IDRs) and its relevance for some biomedical applications. Furthermore, we summarize the current knowledge on the development of drug, vaccine and gene delivery systems based on ELRs, while also emphasizing the multiple tissue engineering approaches involving their use. Finally, we show different studies that explore applications in other fields, and several examples that

describe biomaterial blends in which ELRs have a key role. This review aims to give an overview of the recent advances regarding ELRs and to encourage further investigation of their properties and applications.

## Introduction

As described in previous sections of this special issue, elastin is one of the main components of the extracellular matrix (ECM), and it is involved in conferring elasticity to a variety of organs and tissues, such as lungs, skin and blood vessels, among others, while also contributing to cell signaling [1]. Formed by the lysyl oxidase-mediated cross-linking of lysine residues present in its soluble precursor, tropoelastin, it is composed of highly repetitive and well-conserved domains, including the hydrophobic Val-Pro-Gly-Val-Gly (VPGVG) pentapeptide, first described by Gray *et al*. [2]. Soon after that, Urry's laboratory became interested in the synthesis of this motif to investigate the features of elastin in a feasible way, in order to shed light on the pathophysiology of several diseases in which elastin is directly involved, hence developing the first elastin-like (poly)pentapeptides (ELPs) [3-5]. These ELPs showed an inverse temperature transition, meaning that they remained soluble below the so-called transition temperature ($T_t$), and they aggregated when the temperature was raised above approximately 25ºC. Moreover, physicochemical studies concluded that changes in the fourth amino acid of the pentapeptide led to alterations in the $T_t$, giving flexibility to the ELP design, since the basal monomer became VPGXG, where X (the guest residue) can be any amino acid except proline [6]. However, one of the major limitations at this point was that these primitive ELPs were chemically synthesized, hindering the achievement of long polypeptides. This issue was addressed with the advent of the recombinant DNA technology during the nineteen-eighties, which was easily adapted for the expression of structural protein polymers in heterologous hosts, mainly *Escherichia coli* [7-10] (**Fig.**

**1**). It also increased the versatility of the ELPs, allowing the combination of different structural protein domains [8], or the inclusion of bioactive amino acid sequences [11]. Furthermore, the polymeric and recombinant nature of these ELPs led to a new nomenclature proposed by Rodríguez-Cabello *et al*., elastin-like recombinamers (ELRs), in order to recapitulate both features in a single term [12].

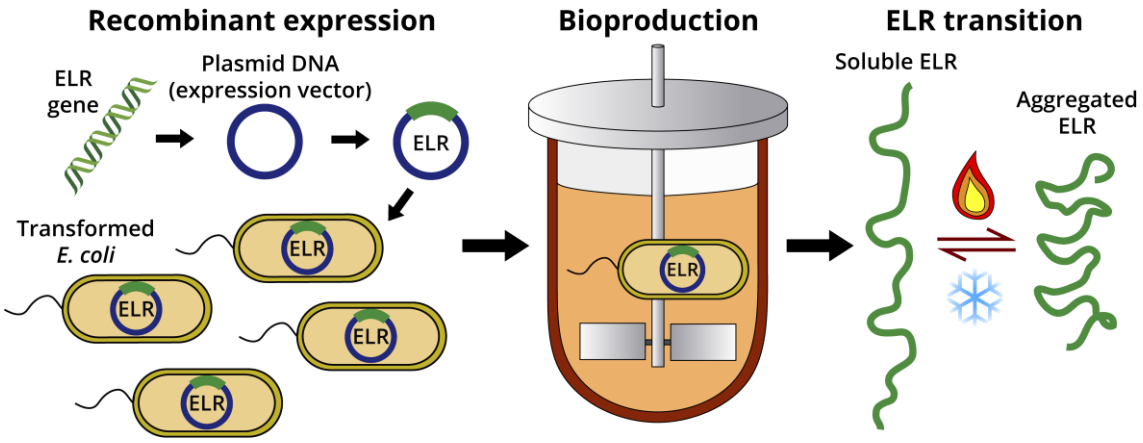


**Fig. 1.** Schematic representation of the recombinant synthesis of ELRs and their reversible transition above the $T_t$.

In the last years, a significant effort has been made to achieve novel ELR designs to extend the fields of application of this biopolymer. In this regard, several approaches for a comprehensive ELR development have been described, including $T_t$ prediction [13] and the study of the effect of proteins fused to the ELRs in its recombinant expression [14] and self-assembly [15]. Moreover, different computational methods have been validated to elucidate the molecular mechanisms involved in the transition from a soluble to an aggregated state (above the $T_t$) of ELRs with different compositions, which may help in the anticipation of the physicochemical properties of novel recombinamers [16-19].

In this review, our aim is to give an overview of the most 'trendy' strategies described in the literature regarding the design and use of ELRs as biomaterials in different fields.

First, we will discuss works describing how ELRs can be considered model intrinsically disordered proteins (IDPs) and how they can help in the general study of this class of polypeptides. Furthermore, we will comment the diverse applications inferred from the control of their ordered-disordered state. Moreover, while several publications have already reviewed the use of ELRs in drug delivery [20, 21] and in tissue engineering and regenerative medicine (TERM) [1, 22-24], herein we will point out the major advances in the multi-purpose design and use of ELRs in these fields in the past few years. Finally, we will describe other designs and applications that do not completely fit into the aforementioned sections.

**ELRs as model intrinsically disordered proteins**

Traditionally, protein functionality was associated with the ability of polypeptide chains to fold into 3D structures, following the classical structure-function paradigm [25]. However, during the last decades, the increasing number of structural studies have shed light on the functional key role of intrinsically disordered regions (IDRs) and proteins (IDPs), which represent 40% of the eukaryotic proteome [26]. Furthermore, they are characterized by low complexity sequences, mainly hydrophilic, high content in proline and they are usually found in an unfolded state [27]. However, their flexibility enables them to interact with several different targets and to respond to different stimulus, folding into diverse dynamic molecular assemblies [28, 29], which may undergo phase transitions [30]. Thus, IDRs have been found to play a crucial role in cellular communication and gene regulation [31]. Moreover, mutations in their sequences are involved in the development of several human pathologies [32].

From the structural point of view, some protein polymers, such as ELRs or resilin-like polypeptides, fit into the description of IDRs and IDPs, i.e. they are made of the repetition

of low-complexity sequences with high content in proline and glycine [33], and they undergo phase transitions under selective solution conditions, such as specific temperature, pressure or pH [34]. Therefore, since a few years ago, they have been considered as model IDRs or IDPs, and studied accordingly [35, 36]. In addition, their recombinant nature and the feasibility to tune their sequence make them perfect candidates to mimic complex IDRs and study their function and behavior in solution [36, 37]. For instance, depending on the chemical nature of the guest residue of the elastin-like pentapeptide (VPGXG), we can control the propensity of the sequence to be disordered or, on the contrary, to collapse, thus shedding light on the phase separation process of IDRs.

Regarding the evaluation of the physicochemical properties of ELRs, Zhang *et al*. provided an experimental structural model for the study of the early stages of the phase separation behavior [38]. A 40-pentapeptide ELR suitable for nuclear resonance measurements was developed based on a polyVPGVG that contained a different guest residue every seven pentapeptides (Lys, Thr, Ala, Ile, Ser and Leu). Therefore, it was possible to analyze the influence of every single non-valyl amino acid as guest residue (all the aforementioned except for Ile) on the phase transition. By complementing the chemical characterization with computational simulations, they demonstrated that a monomeric ELR behaved as random coils with a small compaction, even if the structure was folded up to 90% into β-turns. Consistently with previous results [39, 40], they suggested that oligomer coacervation is triggered by the temperature-dependent hydrophobic collapse and that, despite the formation of transient β-turns during this process, the intrinsic disorder of the monomers is maintained in this aggregated state.

Furthermore, many IDRs are involved in the formation of subcellular protein compartments due to their phase separation behavior [41], which implies that ELRs may

be used as a model to form and study membraneless organelles. In this way, ELR collapse and phase separation allowed to develop subcellular compartments in mammal cells, controlling the clathrin-mediated endocytosis with an ELR fused to clathrin light chain [42], and to produce membraneless organelle-like structures in bacteria with a modular design based on an amphiphilic ELR [43]. This ELR was also used to produce artificial subcellular compartments based on nanovesicles that were able to encapsulate cellular reactions and processes such as transcription and translation [44]. The protein membranes of the vesicles were non-permeable to small molecules, allowing the specific separation of both processes. Moreover, their dynamic behavior and their ability to grow and generate protocellular compartments was demonstrated. In addition, even the amphiphilic ELR itself was translated inside the vesicles, and the monomers were incorporated within the vesicular membrane upon synthesis.

*Applications derived from the order-disorder balance in ELRs*

The control of disorder in ELRs may play a significant role for their use in biomedical applications. For instance, several studies have highlighted the contribution of scaffolds made of disordered proteins in the biomineralization process [45-47]. In this regard, ELRs have demonstrated their promising potential for the development of a great variety of structures that control mineralization due to their flexibility to self-assemble into diverse conformations. Hence, nanoparticles [48], 3D matrices [49, 50], and even nanotopographical hybrid surfaces that enable enzyme-directed mineralization [51] have been achieved, all of them mimicking the mechanical properties of native hard tissues. However, it is important to emphasize how the balance between order and disorder in ELR scaffolds influences the mineralization process. Li *et al*. developed bone fibrils made of ELRs that mimic collagen ones and demonstrated that their ordered structure is crucial for intrafibrillar mineralization [52]. In fact, the incorporation of short charged sequences

within the ELR monomers seemed to prevent their folding into ordered $\beta$-spiral structures, predetermining, in this way, the effectiveness of the mineralization via a polymer-induced liquid-precursor (PILP) process, which implied that the decrease in the order of the fibrillar microstructures involved a reduction of the mineral density. Similarly, Elsharkawy *et al*. produced mineralized ELR-based membranes with potential applicability for enamel regeneration [53]. The membranes provided a functional acid-resistant scaffold for the nucleation and growth of hydroxyapatite. In addition, it was possible to control and tune the formation of hierarchical mineralized structures and their mechanical properties through the regulation of the crosslinking degree of the ELR molecules within the membranes, which influenced the order-disorder balance.

The order-disorder equilibrium is also important to adjust the mechanical properties of injectable hydrogel scaffolds intended for tissue regeneration. In a recent work, Roberts *et al*. developed a molecular design based on alternating ordered polyalanine motifs with intrinsically disordered ELR domains to produce injectable porous scaffolds [54]. In this work, the authors showed that the interactions between the polyalanine α-helixes enabled the formation of kinetically stable 3D polypeptide networks, improving their mechanical properties in a similar way to the introduction of other ordered domains that form stable links/bonds and stabilize non-covalently cross-linked hydrogels, such as leucine zippers [55]. Moreover, they studied the modular distribution of the α-helix structures and demonstrated that their introduction within the ELR backbone strongly affects phase separation and hydrogel porosity, observing thermal hysteresis in the phase transition behavior. Therefore, the fine-tuning of the ratio between both domains and their composition provided a way to control the aggregation temperature and the mechanical properties of the ELR-based scaffolds.

The modular design and functional versatility of ELRs open up a range of possibilities for the development of covalent coatings for indwelling biomedical devices. In this way, their biocompatibility can be improved through the addition of different biofunctionalities, in order to avoid the failure of the biomaterial upon implantation, mainly by rejection (foreign body response) or infection (**Fig. 2**). In this sense, ELR coatings provide an ECM-like environment that helps to elude unspecific protein adsorption, and that can be tuned to increase the cytocompatibility of the devices, for example, through the inclusion of cell adhesion sequences [56, 57]. Furthermore, it could be possible to produce ELR patterns with diverse biofunctional domains to control the adhesion of different types of cells on different areas of the coating [58, 59]. Moreover, biomimetic ELR coatings for biomedical devices can be combined with antimicrobial peptides (AMPs), which are immunomodulatory short cationic peptides with broad-spectrum antimicrobial activity and constitute one of the most promising alternatives to overcome bacteria resistance to conventional antibiotics [60]. This strategy has recently been described for the development of ELRs with covalently linked AMPs to produce anti-biofilm coatings for titanium implants, enhancing osteogenic differentiation and preventing the colonization of the devices by pathogenic bacteria [61]. In addition, ELRs provide a scalable method for the production of novel antimicrobial materials by incorporating AMPs into their backbone through recombinant DNA technology, thus overcoming the expensive chemical production of AMPs, which hampers their large-scale production. In this regard, ELRs have demonstrated their potential for recombinant production and non-chromatographic purification of a broad range of proteins [62], which may include AMPs, and they can also boost the antimicrobial effect of these peptides when used in the formation of anti-biofilm coatings. In a still unpublished work, we have found that the low-fouling properties of the ELRs, which prevent unspecific protein

adsorption, converge synergistically with the antimicrobial properties of an AMP when both are recombinantly co-produced (Acosta *et al.*, in press). Lastly, ELRs have been used to control the supramolecular assembly of AMPs to enhance their effectiveness. For instance, an AMP-ELR able to self-organize into different structures has been described, allowing the formation of nanoparticles that allow a controlled delivery [63] or films that inhibit bacterial and fungal infection during wound healing [64].

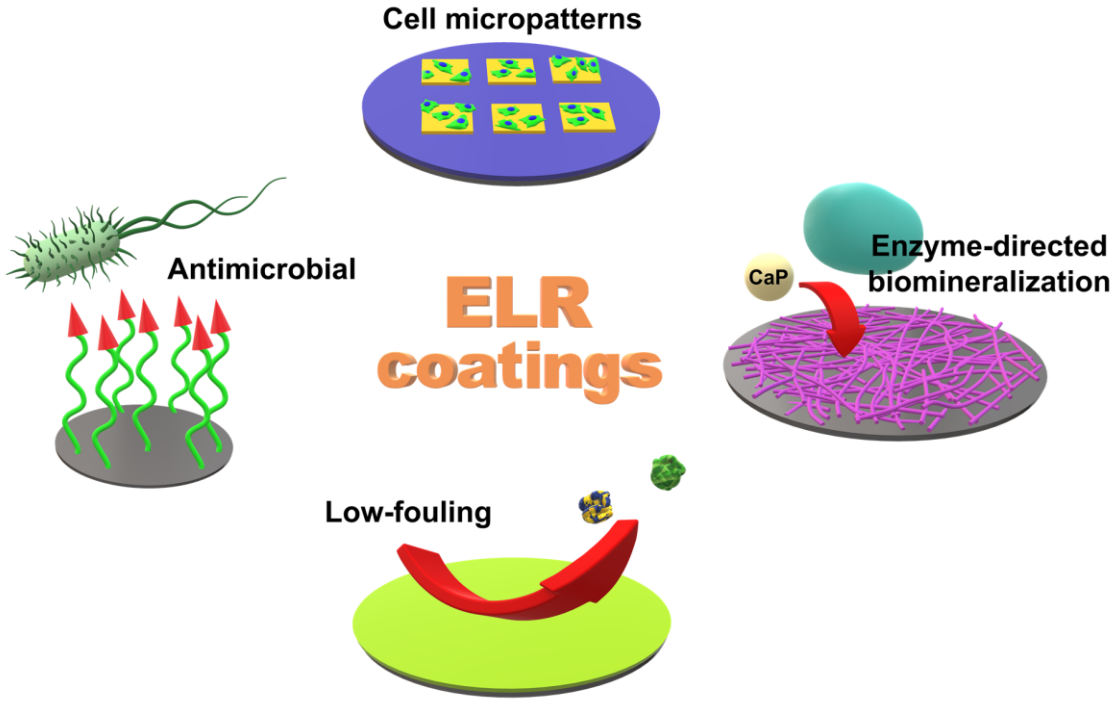


**Fig. 2.** Schematic illustration of different biomaterial surfaces functionalized with ELR coatings and their properties.

**Drug, vaccine and gene delivery systems based on ELRs**

ELRs have been used in the last decades for the development of drug delivery systems (DDS) by following different approaches, mainly the recombinant conjugation of pharmacological polypeptides and the use of drugs as cargo of nanoparticles (NPs) and/or

hydrogels. Moreover, within this section we will also consider immunomodulatory ELRs that act as vaccines, and the use of these recombinamers as gene delivery vectors.

*ELRs in drug delivery*

As aforementioned, recombinant DNA technology permits the genetic fusion of peptides or proteins to the ELRs, giving the opportunity of improving their functionality to almost an infinite extent. By these means, interferon alpha (IFN-α) was fused to an ELR, which not only simplified its purification by inverse transition cycling (ITC), but also improved the pharmacokinetics and tumor accumulation of the drug by increasing its circulating half-life [65]. Similarly, stromal cell-derived growth factor-1 (SDF1), which promotes vascularization, hence enhancing and accelerating re-epithelialization of skin wounds, was fused to an ELR, resulting in a faster wound healing in a diabetic mice model in comparison with the SDF1 by itself [66]. Another example of pharmacologically active ELRs implied the fusion of the fibroblast growth factor 21 (FGF21), a metabolic regulator that enhances insulin sensitivity, thus being a potential treatment for type 2 diabetes [67]. This fusion improved the solubility of the protein drug in *E. coli*, hence avoiding further refolding steps needed when inclusion bodies are formed during expression of FGF21 in this host. Moreover, the delivery of the ELR-FGF21 increased the half-life of the growth factor, so fewer injections were needed for a sustained pharmacological effect. An alternative to the fusion protein drugs is the use of peptides, whose activity does not usually require a complex folding, so they can be easily expressed in prokaryotic hosts such as *E. coli*. In this regard, the so-called 'mini cry' peptide, derived from the intra-vitreal αB crystallin protein, which was found to protect retinal pigment epithelium (RPE) cells from oxidative stress-induced cell death, was genetically conjugated to an ELR to improve the retention of the bioactive peptide from 0.4 to 3.0 days [68]. According to this result, the ELR-mini cry was proposed as a potential DDS to prevent RPE atrophy and

progressive age-related macular degeneration. To further increase the circulation time in plasma of ELRs and ELR particles, novel zwitterionic polypeptides (ZIPP) were designed by introducing cationic and anionic amino acids within the same elastin-like pentapeptide [69]. The results showed that the combination of lysine and glutamic acid improved pharmacokinetics, an effect that was successfully leveraged to obtain a more sustainable delivery of glucagon-like peptide-1, used for the treatment of type 2 diabetes, when fused to the ZIPP.

Another different but classical approach, which is also more versatile because other than protein drugs can be delivered, is the loading of nanoparticles or hydrogels with pharmacologically active compounds. In this last regard, it has been reported the formation of elastin-like (EL) and silk-elastin-like (SEL) hydrogels loaded with timolol maleate (TM), a drug used in the treatment of glaucoma to reduce the intraocular pressure [70]. The retention of TM was improved without eye irritation, an effect that was even more marked for SEL hydrogels, due to the higher stability obtained by the cross-linking of silk-like domains [71]. Therefore, both EL and SEL hydrogels were proposed as delivery systems in ophthalmic applications. As concerns the use of ELR particles as DDS, some design improvements have been achieved in the past few years to overcome different limitations, such as low plasma half-life, similarly to the aforementioned ZIPPs. For instance, an albumin-binding domain (ABD) was fused to an ELR, and the well-known antitumor drug doxorubicin (DOX) was conjugated to the resulting ABD-ELR [72]. The whole system was found to self-assemble into spherical micelles, and the delivery of DOX with the ABD-containing particles resulted in a 3-fold increase in plasma half-life compared to the naked ELR micelles. Moreover, the ABD-ELR-DOX nanoparticles demonstrated higher uptake by the tumor, hence reducing toxicity in other organs, like liver or spleen, and achieving a therapeutic effect with lower DOX doses.

Another issue when targeting tumors is the low uptake of drug-loaded nanoparticles by cells, thus limiting the activity of chemotherapeutic agents. In order to address this limitation, van Oppen *et al*. described the fusion of an octa-arginine peptide (R8) to an amphiphilic ELR, which promoted the uptake of the micelles by the cells *in vitro* [73]. This result sets the basis for the use of this R8-ELR for the delivery of antitumor drugs, although this work lacks evidence of *in vivo* performance. On the other hand, retention of delivery systems in mucus is a major challenge that was recently overcome by genetic conjugation of a hydrogel-forming ELR with a mucoadhesive peptide able to bind to transferrin receptors in the epithelial cell layer [74]. This DDS can be potentially used towards different *mucosae*, as demonstrated by *in vitro* and *in vivo* results.

*ELR-based vaccines*

Vaccination has also been explored as an opportunity to extend the applications of ELRs in the biomedical field. Specifically, García-Arévalo *et al*. described the fusion of an antigenic sequence from a major membrane protein of *Mycobacterium tuberculosis* to an amphiphilic ELR able to form stable nanoparticles [75]. *In vivo* immune-challenge experiments in mice showed the induction of a response similar to the one found upon vaccination, also highlighting the adjuvant effect of the ELR by itself. Likewise, Ingrole *et al*. designed an ELR fused to the M2e peptide, derived from the highly conserved extracellular domain of the influenza virus transmembrane protein [76]. This construction induced the production of a higher amount of M2e-specific serum antibodies in mice in comparison with the single M2e, thus enhancing the immunogenicity of the antigen. Another strategy has been recently reported, involving the fusion of an ELR to cytotoxic T lymphocyte (CTL) epitopes, which induce the activation of T cells and can be used for the treatment of many different diseases, including cancer. This fusion polypeptide allowed the binding of CTL epitopes to dendritic cells (DCs) directly *in vivo*, avoiding

the steps required for the extraction and CTL-loading of DCs *ex vivo* [77]. By also fusing a matrix metalloproteinase (MMP)-sensitive peptide and an albumin-binding domain, the accumulation of the carrier in lymph nodes was increased by 4-fold and the immune response towards the CTL was improved by 1.5-fold.

*ELR-mediated gene delivery*

Regarding gene delivery, few examples have arisen involving the use of ELRs as transfection agents to substitute other chemical methods and viral vectors, which raise some safety concerns. In a first work, Piña *et al*. described the use of a cationic ELR fused to the LAEL fusogenic peptide, which improves cellular uptake, to form polyplexes with plasmid DNA (pDNA) [78], resulting in an enhanced transfection efficiency compared to the pDNA by itself. In a subsequent study, they conjugated the ELR to an aptamer (short oligonucleotides that can bind molecules with high affinity and selectivity) towards MUC1, a membrane glycoprotein overexpressed in epithelial tumors, such as breast cancer [79]. Therefore, this strategy permitted the specific targeting of breast cancer cells for transfection with toxic agents able to kill tumor cells. Another recent example showed the generation of induced pluripotent stem cells by delivering the four factors described by Yamanaka for such purpose, i.e. Oct-4, Klf4, c-myc and Sox2, with an ELR-based transfection method, thus avoiding viral vectors [80]. Nevertheless, the aforementioned examples need to overcome some issues, such as lower transfection efficiency and higher cost than the widely used polyethylenimine (PEI).

## ELRs as biomaterials for tissue engineering and regenerative medicine (TERM)

### *ELR hydrogels*

In the last years, ELRs have been used as biomaterials for the development of hydrogels formed through different cross-linking methods. Specifically, our group has described the covalent cross-linking of ELRs by catalyst-free (i.e. copper-free) click chemistry [81], termed 1,3-dipolar cycloaddition, which is basically an azide-alkyne cycloaddition, taking advantage of the modification of amine groups present in the side chains of lysines. These hydrogels are very promising, since they have shown good *in vitro* and *in vivo* biocompatibility [82]. Macroporous scaffolds have been also achieved with this strategy, expanding the use of these 'click' hydrogels to applications where a highly porous structure is needed [83]. Similarly, other covalent cross-linking methods have been developed, using residues other than lysine for the cross-linking, such as tyrosine or cysteine [84, 85]. On the other hand, non-covalently cross-linked ELR hydrogels have also been developed [55, 71, 86]. All these hydrogels are good candidates as implants for enhanced tissue regeneration, considering the properties of the ELRs, and some of them have already been used for this purpose, as described below.

### *ELRs for enhancing implant vascularization*

One of the major challenges concerning hydrogels and other tissue-engineered constructs is that they need to be vascularized to achieve a successful grafting upon implantation. The lack of vascularization implies an inability to provide adequate nutrient transport during the initial phase after implantation, due to a lack of blood flow between the construct and the host tissue, thus precluding the survival of implanted and host cells inside the scaffold [87, 88]. Therefore, novel strategies for enhancing vascularization in engineered templates are essential to ensure their clinical translation [89].

Particularly, ELR-based hydrogels, whose application in tissue engineering and regenerative medicine has significantly increased in the past few years, are characterized by potential angiogenic properties, which is an essential step for their integration with the host tissue when implanted or injected. Recently, Marsano and co-workers have studied the angiogenic activity of ELR-based hydrogels that included cell adhesion sequences such as the Arg-Gly-Asp (RGD) tri-peptide, which promotes the attachment of different cell types [90], and Arg-Glu-Asp-Val (REDV), a selective cell adhesion motif for endothelial cells [91]. These hydrogels also comprised proteolytic sites, namely the Val-Gly-Val-Ala-Pro-Gly (VGVAPG) sequence, sensitive to elastolytic enzymes [92]. Overall, this study demonstrated the control of angiogenesis by modulating the presence of bioactive sequences within ELR-based hydrogels, as they favor the integration with the host tissue, as well as the beginning of the vascularization process [93]. Similarly, Alagoz *et al*. coated poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) scaffolds with ELRs containing REDV sequence, aiming for bone regeneration. This work shows that the presence of REDV motifs improves endothelial cell adhesion and growth *in vitro*, with a consequent enhancement of vascularization, which is an important event when facing bone regeneration [94]. As described above, Roberts *et al*. developed a complex system that consists in the combination of stimuli-responsive disordered ELRs and structurally stable polyalanine helices [54]. When injected subcutaneously in C57BL/6 mice, this system formed a stable porous scaffold that was invaded by a high quantity of cells. This was identified as a mild inflammatory response at the first timepoints, but it was followed by a uniform vascularization, as observed by the formation of capillaries and some large vessels after 21 days post-injection. Hence, their ability to promote angiogenesis makes them optimal candidates for diverse applications in regenerative medicine.

An alternative approach to enhance vascularization in tissue engineering constructs relies on the use of growth factors that induce vascular organization and remodeling. Among them, the vascular endothelial growth factor (VEGF) plays a key role in the majority of angiogenic processes, namely by promoting endothelial cell proliferation and migration [95]. However, the use of VEGF is limited by the need of recombinant expression in eukaryotic cells, mainly mammalian, for its production, and by its low stability in solution. Thus, to overcome these limitations, new growth factor-mimetic peptides have been identified, which can be easily synthesized and chemically tethered to the constructs. For instance, Cai *et al*. conjugated the so-called QK peptide (a VEGF-mimetic peptide) within ELR hydrogels, without affecting their mechanical properties [96]. Different concentrations of this peptide, i.e. 10 nM, 1 μM and 100 μM, were studied using human umbilical vein endothelial cells (HUVECs) *in vitro*, demonstrating that, at low concentrations, the QK peptide promoted better cell adhesion and proliferation than higher concentrations, which inhibited their outgrowth. In a subsequent experiment, the QK peptide was tethered to ELR molecules at a concentration of 1 μM and they were injected intramuscularly in a hind limb zone in mice to form hydrogels *in situ* [97]. The results showed that the QK peptide enhanced the *de novo* formation of functional capillaries within the ELR construct, favoring cell survival and tissue growth *in vivo*. In summary, these studies provide an alternative approach to the use of growth factor proteins, such as VEGF, in angiogenesis signalling.

Another important feature of engineered constructs that also affects vascularization is their biodegradation, which is known to regulate many cellular behaviors. Biomaterials with a controlled and predictable biodegradation are in high demand, and they should simultaneously provide mechanical support, biological signals and resist physiological loads during the early stages of implantation. Madl *et al.* demonstrated that the formation

of vascular-like structures depends on the number of cleavage sites in a polymer sequence, as well as on the changes of the construct architecture upon biodegradation [98]. In addition, protease-mediated degradation of biomaterials plays an important role in the development of 3D systems intended for tissue engineering, as it provides a more precise control on cell infiltration (**Fig. 3**). Recently, Straley *et al*. demonstrated that the kinetics and sensitivity to proteolytic cleavage of urokinase plasminogen activator (uPA) epitopes are different depending on the amino acid sequence [99]. For instance, the Gly-Thr-Ala-Arg (GTAR) sequence shows a high sensitivity to proteolytic cleavage, giving a fast response, while the Asp-Arg-Ile-Arg (DRIR) sequence confers a low cleavage efficiency, resulting in slower degradation kinetics. In another related work, Flora *et al*. evaluated the spatiotemporal control of cell infiltration in a 3D hydrogel that consists of a sandwich-like three-layer disc made of two different protease-sensitive ELRs that included the aforementioned proteolytic sequences (Flora *et al*., in press). This 3D construct was implanted subcutaneously in mice and the spatiotemporal progression of cell invasion was studied for twelve weeks. It was observed that cell infiltration progressed through an inside-to-outside pattern, meaning that the central layer of the 3D system, which is formed by the GTAR-ELR (characterized by a fast degradation rate), was first colonized, degraded and vascularized. Subsequently, the external layers made of DRIR-ELR (slow degradation rate) were invaded and degraded mainly by the cells that migrated from the inner part of the construct. This study offers new opportunities for the generation of tunable biodegradable systems that closely mimic complex biological structures, such as organoids or organs, and that can be implemented in tissue engineering and regenerative medicine.

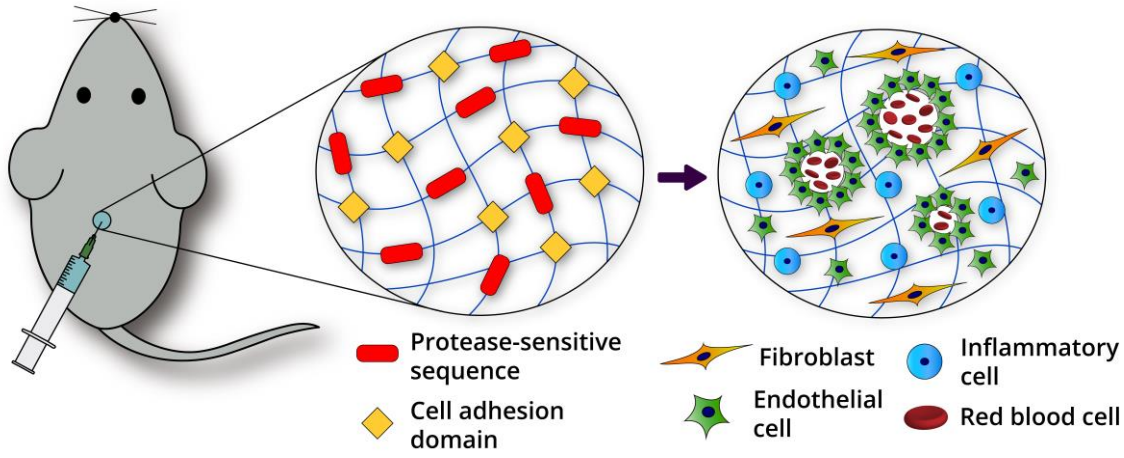


**Fig. 3.** Graphical representation of ELR-based hydrogels with protease-sensitive and cell adhesion domains injected subcutaneously in mice. Due to their bioactivity, cells would be able to infiltrate over time, even forming vessel-like structures, which is indicative of angiogenesis.

*TERM applications of ELR hydrogels*

ELR-based hydrogels have been used in cardiovascular applications, taking advantage of their features, such as stable mechanical properties under physiological pressure and flow conditions, elasticity, biocompatibility and hemocompatibility, which make them good candidates in this field [100]. For instance, Gonzalez de Torre *et al*. covered metal stents with ELR-catalyst-free click gels that presented different bioactive sequences, promoting endothelialisation (formation of native endothelium) in less than 2 weeks *in vitro* [101]. When exposed to blood flow in dynamic conditions, minimal platelet adhesion and fibrinogen adsorption were detected on the surface of the ELR-covered stents, showing high hemocompatibility. Thus, this method represents an effective approach to obtain bio-stents that prevent the formation of an atherosclerotic plaque. Moreover, different ELRs have been used to fabricate vascular grafts. Specifically, Mahara *et al*. developed a small-calibre blood vessel made of ELR-based hydrogels reinforced with poly(lactic acid)

nanofibers as a new therapeutic strategy for reconstructive surgery [102]. When implanted *in vivo,* the tubular scaffold showed a rapid tissue regeneration with a high patency, maintaining a physiologic blood flow without thrombogenicity. Furthermore, Inostroza-Brito *et al*. fabricated geometrically complex structures, namely tubes, through spatiotemporally controlled self-assembly. For this purpose, peptide amphiphiles (PAs) were employed to guide the assembly of a larger protein, i.e. an ELR, into self-growing tubes. These structures promoted mouse-adipose-derived stem cells (mADSCs) and HUVECs adhesion and proliferation, due to the presence of bioactive RGD motifs coded within the ELR backbone. Therefore, these biomimetic tubes could be implemented in cardiovascular tissue engineering for the achievement of vascular implants [103].

Musculoskeletal tissues have also been proposed as targets for ELR hydrogels-mediated regeneration. In this regard, some *in vitro* tests have suggested the potential application of cartilage with a silk-elastin-like recombinamer [104], and bone with pro-mineralizing ELRs [105, 106]. Moreover, a non-covalently cross-linked bioactive ELR-based hydrogel, in combination with human mesenchymal stem cells, has been used to successfully regenerate an osteochondral defect in rabbits, showing even the formation of hyaline-like cartilage [107]. On the other hand, bone defects have been also treated with bioactive and biodegradable ELR-based hydrogels formed with an ELR fused to the bone morphogenetic protein-2 (BMP-2), which is a very powerful osteogenic factor. In this case, fully repaired defects were found after 3 months, showing the good performance of the hydrogels to promote this regeneration [108].

ELRs have also aimed for the regeneration of neural tissue, although short steps have been taken towards it up until now. In one work, Johnson *et al*. genetically fused two different neurotrophins to an ELR, separately: the nerve growth factor (NGF) and the brain-derived neurotrophic factor (BDNF), which could be potentially used for the

stimulation of neural regeneration both *in vitro* and *in vivo* [109]. In another work, S.C. Heilshorn and co-workers found that the maintenance of stemness in neural progenitor cells needs a scaffold (i.e. a hydrogel) that is able to be biodegraded over time, simulating matrix remodeling [110].

Although several elastin-based materials, composed of tropoelastin or α-elastin, among other versions of elastin, have been designed for wound healing applications, not so many ELRs have been postulated in this regard [24]. Recently, our group obtained oriented electrospun clickable ELR fibers, which have proven good cytocompatibility with fibroblasts and keratinocytes *in vitro*, suggesting their potential application in the formation of artificial skin or to promote skin regeneration [111]. Contemporarily, Fernández-Colino *et al*. achieved similar fibers, which highlights their ease of attainment and their potential application in different tissue engineering fields [112].

**Other designs and applications of ELRs**

In addition to the aforementioned designs and applications of ELRs, other interesting uses have been explored in the last years. For instance, there are different works describing the design of ELRs for antibody precipitation as an alternative to chromatography [113, 114]. Moreover, hydrogels containing ELRs have been developed to study and modulate cell behavior in 3D cultures through the incorporation of different ligand chemistries or by tuning the stiffness of the scaffolds [115, 116]. On the other hand, fluorescent proteins have been fused to ELRs, enabling the study and prediction of self-assembly of diverse ELR constructs when genetically conjugated to large proteins [117], or even being proposed as potential Förster resonance energy transfer (FRET)-paired biosensors [118]. Another novel design proved the use of an ELR as a cytocompatible underwater adhesive intended for biomedical applications by the incorporation of the non-canonical amino

acid 3,4-dihydroxyphenylalanine (DOPA), which mimics the adhesion of mussels to surfaces [119]. Additionally, Yang *et al*. described how the genetic incorporation of the self-assembling peptide RADA-16 led to the obtaining of a hemostatic sponge able to stop the bleeding of wounds in mice [120].

Furthermore, it is also noteworthy the increasing number of works describing the combination of ELRs with other polymers or structural proteins, giving blends or biohybrids that recapitulate the properties of all the components. The first, and probably the most exploited blend, came from the recombinant fusion of ELRs with silk-like domains to give silk-elastin-like recombinamers (SELRs) that are able to form fibers and hydrogels, among other structures, whose stability relies on the β-sheet cross-linking between silk-like motifs [8, 71, 121-123]. Collagen-like peptides have also been explored as partners of ELRs in a recombinant way [124], even achieving nanoplatelets [125]. Furthermore, other non-polypeptide polymers have been combined with ELRs, including the widely used polyethylene glycol (PEG) [126, 127], inorganic bioglass [128], hyaluronic acid (HA) [129], self-assembling peptides [130], and lipids [131]. All these examples highlight the interest of using ELRs in combination with other biomaterials to obtain superior structures, such as hydrogels, that recapitulate the properties of their components, hence getting closer to the complexity of biological tissues and matrices.

**Final considerations**

Elastin-like recombinamers have gained an increasing interest during the last decades, with a great boost in the past few years, as highlighted by data: there are more PUBMED-indexed publications regarding ELRs issued during the last five years than in all the previous years since their discovery. This fact has enormous implications, since it means that ELRs are currently being thoroughly investigated from different points of view,

elucidating their molecular mechanisms of self-assembly and shedding further light on their physicochemical and mechanical properties. Furthermore, it also implies that many groups are designing and using novel or already existing ELRs for still unexplored applications, broadening their scientific impact, and probably getting them closer to be translated into clinics as biomaterials. In this review, we have gathered multiple recent examples that reflect this interest in different fields, from the basic research of the physicochemical properties of ELRs and their consideration as IDPs/IDRs, to the several applications where they have found uses, including drug, vaccine and gene delivery, and TERM. All these examples provide convincing evidences of the high potential of ELRs as biomaterials, and encourage further investigation that may lead to consider them as a benchmark in the biomedical field.

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**Abbreviations**

3,4-dihydroxyphenylalanine (DOPA); albumin-binding domain (ABD); antimicrobial peptide (AMP); bone morphogenetic protein-2 (BMP-2); brain-derived neurotrophic factor (BDNF); cytotoxic T lymphocyte (CTL); dendritic cell (DC); doxorubicin (DOX) drug delivery system (DDS); elastin-like polypentapeptide (ELP); elastin-like recombinamer (ELR); extracellular matrix (ECM); fibroblast growth factor 21 (FGF21); Förster resonance energy transfer (FRET); human umbilical vein endothelial cell (HUVEC); hyaluronic acid (HA); interferon alpha (IFN-α); intrinsically disordered

protein (IDP); intrinsically disordered region (IDR); inverse transition cycling (ITC); matrix metallo-proteinase (MMP); mouse-adipose-derived stem cell (mADSC); nanoparticle (NP); nerve growth factor (NGF); peptide amphiphile (PA); poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV); polyethylene glycol (PEG); polyethylenimine (PEI); polymer-induced liquid-precursor (PILP); retinal pigment epithelium (RPE); silk-elastin-like (SEL); stromal cell-derived growth factor-1 (SDF1); timolol maleate (TM); tissue engineering and regenerative medicine (TERM); transition temperature ($T_t$); urokinase plasminogen activator (uPA); vascular endothelial growth factor (VEGF); zwitterionic polypeptides (ZIPP).

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