Tethering QK peptide to enhance angiogenesis in Elastin-like recombinamer (ELR) hydrogels

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Abstract

The development of new capillary networks in engineered constructs is essential for their survival and their integration with the host tissue. It has recently been demonstrated that ELR-based hydrogels encoding different bioactivities are able to

modulate their interaction with the host after injection or implantation, as indicated by an increase in cell adhesion and the ability to trigger vascularization processes. Accordingly, the aim of this study was to increase their angiogenic ability both *in vitro* and *in vivo* using a small VEGF mimetic peptide named QK, which was tethered chemically to ELR-based hydrogels containing cell-adhesion sequences in their backbone, such as REDV and RGD, as well as a proteolytic site (VGVAPG). *In vitro* studies were performed using a co-culture of endothelial and fibroblast cells encapsulated into the ELR-based hydrogels in order to determine cell proliferation after 21 days of culture, as well as the number of cell-cell interactions. It was found that although the presence of this peptide does not influence the morphological and rheological properties of these hydrogels, it has an effect on cell behaviour, inducing an increase in cell proliferation and the formation of endothelial cell clusters. *In vivo* studies demonstrate that the QK peptide enhances the formation of prominent functional capillaries at three weeks post-injection, as confirmed by H&E staining and CD31 immunohistochemistry. The newly formed functional microvasculature ensures perfusion and connection with surrounding tissues. These results show that ELR-QK hydrogels increase capillary network formation and are

therefore attractive candidates for application in tissue regeneration, for example for the treatment of cardiovascular diseases such as myocardial infarction or ischemia.

1. Introduction

Tissue engineering is an interdisciplinary therapeutic field that aims to repair or even replace damaged tissues and organs, thus resulting in the recovery of their integrity and functionality. This discipline meets tissue- and organ-related medical needs by developing engineered constructs that are able to restore or enhance tissue or organ function. [1] Scaffolds for use in vascular tissue engineering, for example hydrogels, simulate most of the required properties for these tissues, thus making them ideal supports for the promotion of tissue regeneration. [2, 3] The presence of a network of vessel-like structures is essential in engineered constructs for the diffusion of nutrients and oxygen, thus providing a pro-angiogenic environment suitable for cell survival and tissue growth. [4] Neovascularization is obtained as a result of marked cell infiltration into the hydrogels, which can be facilitated by the presence of adhesion sequences that increase the quantity of cells, specifically endothelial cells in this case, along with proteolytic target sequences. These latter sequences are particularly important as they allow enzymatic degradation of the scaffold, thus favouring an adequate space to support extensive angiogenesis. [5]

Elastin-like recombinamer (ELR)-based hydrogels are the most recent engineered constructs to have found a use in biomedicine and biotechnology as a result of their potential angiogenic properties. [6] [7] The presence of distinct bioactivities encoded into their backbone results in an increase in cell adhesion, thus contributing to an enhanced

release of angiogenic factors and subsequent endothelial cell organization. [8] Moreover, these systems are characterized by other important properties, such as excellent biocompatibility, non-immunogenicity, optimal biomechanical properties and an ability to respond to different stimuli. [9] They also mimic the elastic properties of natural tissues due to the presence of elastin, which is crucial in tissue regeneration since it is one of the main components of the extracellular matrix (ECM). [10] [11]

ELRs are obtained using recombinant DNA technologies that allow a clear-cut, complex and absolute control of the sequences, thus providing an ability to include different bioactive sequences, such as those guiding cell adhesion, differentiation and protease sensitivity. They are characterized by a self-assembly behaviour associated with a conformational re-organization at the molecular level. Thus, whereas the polymer chains are soluble in water below a transition temperature, above this temperature they self-assemble into nano- and micro-aggregates and become insoluble. This process is completely reversible. [12] [13, 14]

In this work, the bioactivity of the ELR-based hydrogels was enhanced using two cell-adhesion sequences, namely RGD (arginine-glycine-aspartate) and REDV (arginine-glutamic-aspartate-valine), which were coded into their amino acid sequences. Various studies have shown that the RGD sequence promotes the attachment of different cell types and is the principal integrin-binding domain present within ECM proteins such as fibronectin, vitronectin, fibrinogen, osteopontin, and bone sialoprotein. [8] [15] [16-18] In contrast, the REDV sequence, which is derived from fibronectin, binds to endothelial cells selectively via integrin a4b1 and is well known to promote endothelial cell adhesion and migration when immobilized to a wide variety of biomaterials. [19] [20]

Furthermore, the proteolytic site VGVAPG (valine-glycine-valine-alanine-proline-glycine), which is coded into one of the ELRs employed in this work, is known for its

chemotactic activity with respect to various cell types, such as monocytes or fibroblasts, and it also upregulates metalloproteinases and is sensitive to proteolysis by elastolytic enzymes. [21]

Various approaches are currently used to achieve better vascularization in scaffolds, such as implanting scaffolds with endothelial cells, the use of growth factors and the provision of a vascularized tissue. [22] [23] [24] Vascular endothelial growth factor (VEGF) is the main regulator of neovascularization and, specifically, $VEGF_{165}$ is known to stimulate vascularization both *in vitro* and *in vivo*. [25] Several studies have demonstrated that the use of growth factor proteins has several inherent disadvantages, such as immunogenicity, lower stability and loss of bioactivity, therefore the use of shorter bioactive peptide sequences derived from a growth factor protein that confer the same bioactivity and cellular response is becoming increasingly common. [26]

A vascular mimetic peptide known as QK has been widely studied as a promising candidate for the promotion and control of angiogenesis in tissue-engineering constructs. Indeed, it has been shown to possess a similar biological activity to VEGF as regards its ability to induce capillary formation and organization. In this regard, D'Andrea et al. designed and synthesized QK peptide and demonstrated that this peptide has a stable helical conformation in aqueous solution and that an alpha helix conformation to bind the ligand-receptor site is necessary for its functionality. QK is a synthetic 15-amino acid peptide based on the 17-25 alpha region of $VEGF_{165}$ that binds to the main VEGF receptors, such as VEGFR-1 and VEGFR-2, and is required for the cell signalling pathway step in VEGF-R modulated angiogenesis. [27] [28] [29]

Although initially used only as a locally delivered soluble factor, QK peptide has demonstrated promising therapeutic outcomes. Furthermore, it has been immobilized to hydrogels and been shown to retain its bioactivity under all conditions tested. [30, 31]

[32] This peptide provides different advantages, especially a smaller size, ease of use in chemical reactions, lesser likelihood of triggering an immune response, and an ability to be more easily modified to include variations that allow the tuning of a biomimetic matrix system.

The current study aims to demonstrate enhancement of the angiogenic activity of ELR-based hydrogels bearing a VEGF-mimetic peptide (QK peptide) both *in vitro* and *in vivo*, thus providing an alternative to the use of growth factor proteins in angiogenic signalling. Moreover, the inclusion of bioactive sequences such as RGD and REDV favours cell adhesion and the proteolytic site VGVAPG favours a customised cell biodegradation, which is an essential pre-requisite for hydrogels in tissue-engineering applications.

Cell behaviour was initially studied *in vitro* employing a co-culture of human umbilical vein endothelial cells (HUVEC) and human foreskin fibroblasts (HFF1), comparing the biological activity of hydrogels with a tethered QK peptide (ELR-QK) with that of hydrogels with soluble VEGF (ELR-VEGF) and with control hydrogels, in this case a factor-free ELR hydrogel (ELR).

Subsequent *in vivo* studies were performed in mice by injecting cell-free ELR-based hydrogels (factor-free ELR and ELR-QK hydrogel) intramuscularly into a hind limb region in order to determine if the presence of this pro-angiogenic peptide enhances the formation of new functional capillaries within the hydrogels, which were detected by immunofluorescence staining with CD31.

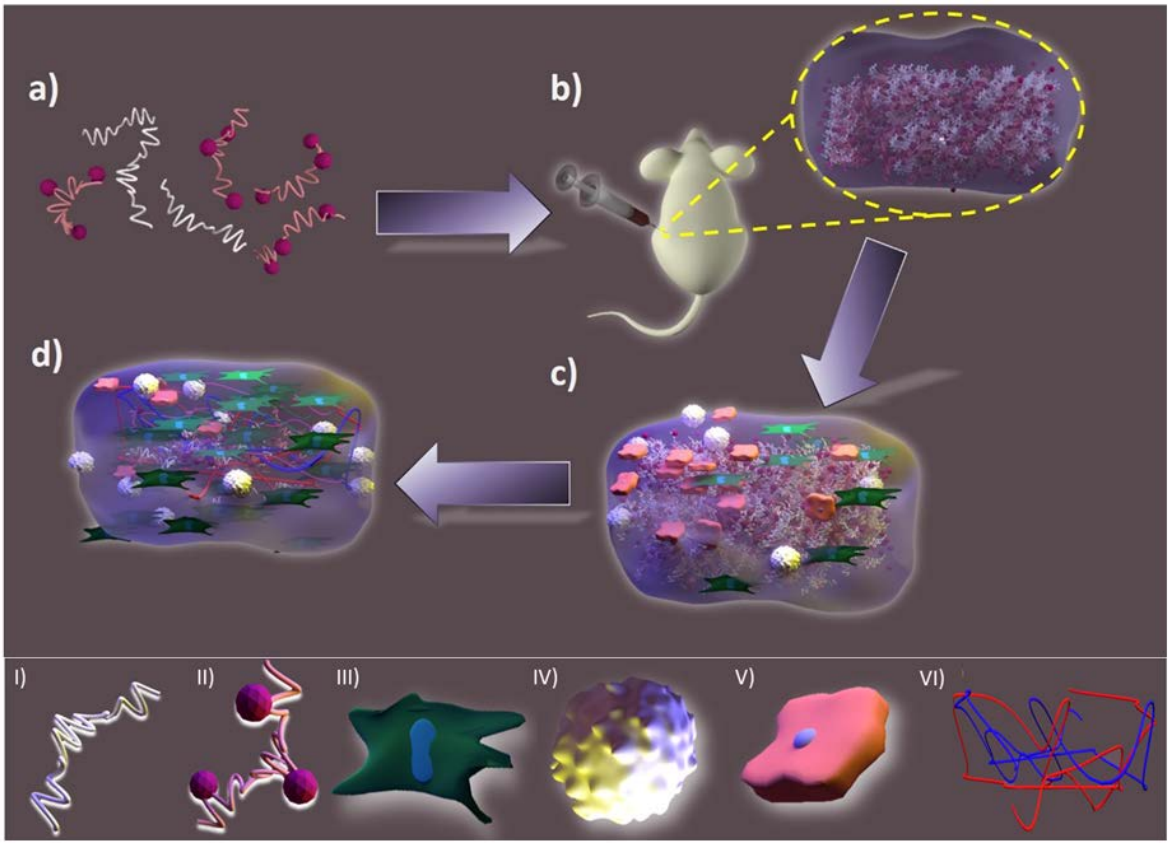

Figure 1. Schematic representation of a) composition of ELR-based hydrogel (REDV-ELR + RGD-QK ELR) b) ELR-QK hydrogel injected in mice c) ELR-QK hydrogel invaded by different cell type d) formation of new capillaries into the hydrogel

I) REDV-ELR II) RGD-QK ELR III) fibroblast cells IV) immune cells V) HUVEC cells VI) capillaries

2. Materials and Methods

2.1 ELR bio-production, modification and characterization

The ELRs used in this work have been previously described elsewhere. [33] Briefly, they were produced using a 15-L bioreactor and purified using a process known as Inverse Transition Cycling (ITC), which involves several cycles of precipitation (heating) and resuspension (cooling) of the supernatant. The final product obtained was dialysed against deionized water with several changes and freeze-dried prior to storage. The ELRs obtained were characterized by SDS-PAGE electrophoresis, ^{1}H NMR and FTIR spectroscopy and DSC. Two different ELRs, namely HRGD6 and REDV, were employed in this study. Chemical modification of these ELRs was achieved using click chemistry and their degree of modification confirmed by NMR and FTIR spectroscopy, respectively. (Figure S5-S6, Supporting Information)

2.2 QK peptide and recombinant human $VEGF_{165}$

QK peptide (KLTWQELYQLKYKGI) was purchased from BACHEM (Switzerland) as a white powder. This peptide presents an azide group in the amino terminal region (5-Azido-pentanoyl-Lys-Lys-Leu-Thr-Trp-Gln-Glu-Leu-Tyr-Gln-Leu-Lys-Tyr-Lys-Gly-Ile-OH), which can be reacted with RGD-ELR previously modified with a cyclootyne group via a click reaction. The resulting recombinamer, known as RGD-QK, was characterized by NMR and FTIR spectroscopy, DSC and MALDI-TOF, thus confirming that QK peptide was correctly bound to the recombinamer (Figure S1-S4, Supporting Information). Recombinant Human Vascular Endothelial Growth Factor ($VEGF_{165}$) was purchased from Lonza (Madrid) in a soluble form.

2.4 Mechanical and morphological properties of ELR-based hydrogels

The chemically modified ELRs were crosslinked for subsequent formation of the hydrogels via a click reaction. Thus, the recombinamers, with a concentration of 50 mg/mL, were dissolved in PBS 1X and mixed at 4 °C. Specific molds were used to form the hydrogels, and the reaction mixture was maintained at 4 °C for 20 minutes to allow crosslinking. The VEGF hydrogels (ELR-VEGF) were prepared in an identical manner, with the sole difference that the human recombinant $VEGF_{165}$ was added to the endothelial cell growth medium at a concentration of 10 ng/mL, as per the literature. [34] The elastic modulus of the hydrogels was measured using a TA Instruments AR2000 stress-controlled rheometer equipped with a 12 mm diameter load plate, at 1% strain and 37 °C. The hydrogels were kept in PBS to avoid sample shrinking below the diameter of the plate upon heating. Oscillatory experiments were performed within the linear viscoelasticity region, where storage (G') and loss (G'') moduli are independent of the stress magnitude. Three different hydrogels were tested (n = 3).

Scanning electron microscopy (SEM) was used to investigate their morphology. Thus, hydrogels with a concentration of 50 mg/mL were dropped into liquid nitrogen, physically fractured, and subsequently freeze-dried. Micrographs were obtained using a scanning electron microscope (FEI Quanta 200 FEG) in low vacuum mode at 3 keV. Morphological details were evaluated quantitatively using the ZEN (Blue Edition, 2012) software package (Carl Zeiss Microscopy).

2.4 Cell cultures

Human umbilical vein endothelial cells (HUVEC) were purchased from Lonza (Madrid, Spain) and were cultured in EGM-2 medium (M200500, Life Technologies S.A, Madrid, Spain) supplemented with EGM-2 Single Quot Kit Suppl. & Growth Factor (Lonza, Madrid, Spain) at 37 °C and 5% CO_2. Human foreskin fibroblast (HFF1) were purchased from Life Technologies S.A. (Madrid, Spain) and were cultured in DMEM medium

supplemented with 15% FBS and 100 U/100 mg/mL penicillin/streptomycin at 37 °C and 10% CO_2. Cells between passages 2 and 4 were used in all experiments.

2.5 *In vitro* 3D proliferation tests and histological analysis

For *in vitro* studies, recombinamers with a concentration of 50 mg/mL were dissolved in endothelial cell growth medium (basal medium) and mixed at 4 °C. $10*10^6$/mL of HUVEC and $3*10^6$/mL of HFF1 cells were added to previously prepared ELR hydrogels. The hydrogels were formed in 96-well plates, with 45 µL of each recombinamer being mixed and kept at 4 °C for 20 minutes to allow crosslinking prior to subsequent addition of basal medium for the QK-hydrogels and with recombinant human $VEGF_{165}$ for the VEGF-hydrogels. The 96-well plate containing the hydrogels was incubated at 37 °C and 5% CO_2 for 21 days. All measurements were carried out in triplicate. Media were replaced every day for ELR-VEGF hydrogels in order to maintain a constant concentration, and every two days for ELR-QK hydrogels.

After incubation for 21 days, the hydrogels were fixed in 10% formalin for 4 hours, then dehydrated by immersion in ethanol solutions of increasing concentration, and then in xylene solution. Finally, they were immersed in paraffin for 3 hours and stored at -20 °C overnight. All samples were sliced on a microtome and stained with Haematoxylin-Eosin following a general protocol. [35]

2.6 Ethical approval

Experimental procedures involving the use of animals were approved by the Animal Care and Use Committee of the University of Valladolid in accordance with Directive 2010/63/EU of the European Union and Spanish Royal Decree RD 53/2013.

2.7 *In vivo* studies

Swiss C57 mice (male) were used for intramuscular injection of the hydrogels. They were anesthetized by inhalation of Isoflurane at 3%. The ELRs were mixed in an eppendorf flask immediately prior to injection, and were injected cell-free using a 1 mL syringe with a 20G needle. Animals were sacrificed at 21 days post-injection.

2.8 Histological analysis of *in vivo* studies

Hydrogels were extracted from mice and submitted to histological analysis. Specimens were fixed in 10% formalin at 4 °C for 24 h and subsequently dehydrated by immersion in ethanol solutions from 70% to 100%, finishing with two changes in xylene solution. The hydrogels were subsequently embedded in paraffin for 6 h and cut with a microtome. The resulting specimens were placed on slides and deparaffinised, with subsequent immersion in xylene, ethanol solutions of decreasing concentration and, finally, in distilled water. Samples were evaluated by H&E staining to get a general overview of cellular invasion.

2.9 Immunohistochemistry

Specimens were immunostained using a goat polyclonal primary PECAM antibody (Santa Cruz Biotechnology, USA) at a concentration of 1 µg/mL in 1% blocking serum (donkey serum) and with a goat anti-mouse fluorescein-conjugated secondary antibody (Santa Cruz Biotechnology, USA). During each step, the samples were washed three times with PBS 0.1 M for three minutes each. A vectashield HardSet Antifade Mounting Medium with DAPI (Vector Laboratories, USA) was used to stain cell nuclei. All images were acquired using a fluorescent microscope.

2.10 Statistical Analysis

Results are reported as means ± standard error of the mean (n = 3). Data were evaluated statistically using a one-way analysis of variance, applying the Holm–Sidak method. A *p*

value of less than 0.05 was considered statistically significant. n.s.d indicates no significant differences.

3. Results

3.1 Morphological and rheological properties

Variation of the linear viscoelastic range due to the presence of growth factors was measured rheologically in hydrogels at a concentration of 50 mg/mL. Three samples, namely factor-free hydrogel (ELR), ELR-VEGF, and ELR-QK hydrogels were tested in this study. The strain sweep was evaluated in the range 0.6–10 Pa and was found to remain independent of the strain amplitude up to values of about 7-8%. Figure 2 shows the trend in complex modulus for all hydrogels at a frequency of 1 Hz, with 1% strain and at a temperature of 37 °C. No significant differences were found between the samples, all of which exhibit a similar complex modulus of around 1100 Pa.

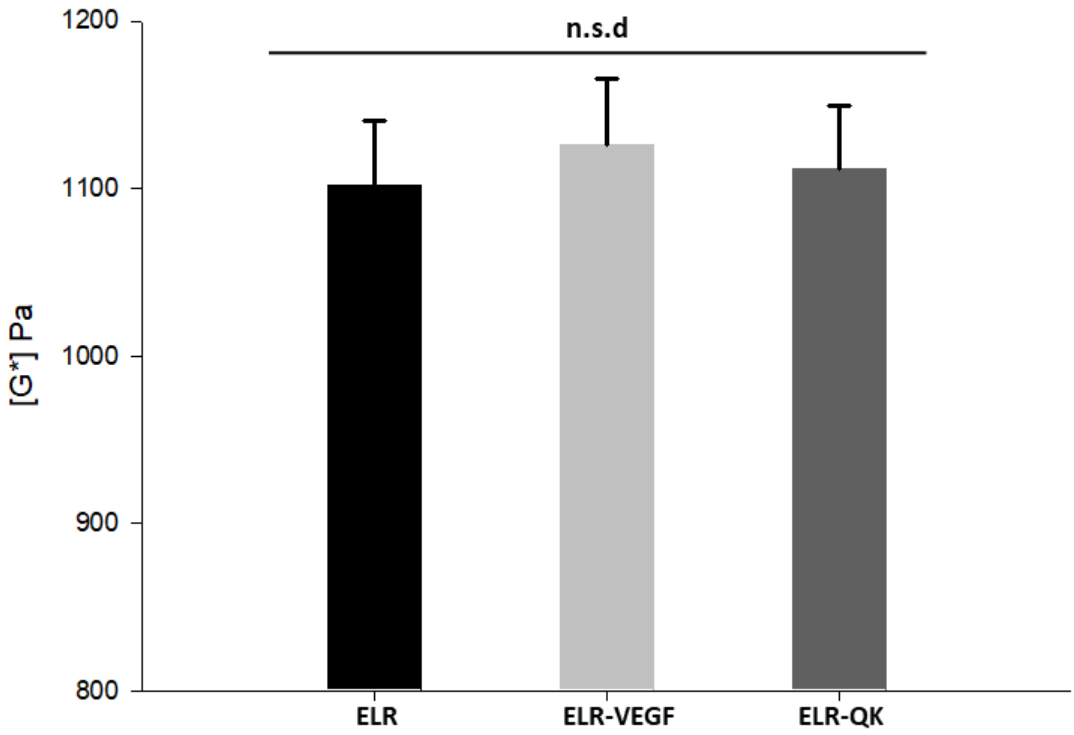

Figure 2. Graphical representation of the complex elastic modulus [G*] for: factor-free ELR hydrogel (ELR); ELR hydrogel with soluble VEGF protein (ELR-VEGF) and ELR hydrogel with QK peptide tethered chemically (ELR-QK). Statistical analysis was carried out by performing an analysis of variance using the Holm–Sidak method. *p<0.05; n.s.d no significant differences.

SEM micrographs for the factor-free hydrogel (ELR) (a), ELR-VEGF (b) and ELR-QK (c) hydrogels can be found in Figure 3. These structures exhibit high porosity and well-interconnected pore networks with a pore size of between 12 and 15 μm. Similar experimental results have been reported in a previous study of ELR-based catalyst-free click hydrogels. [33]

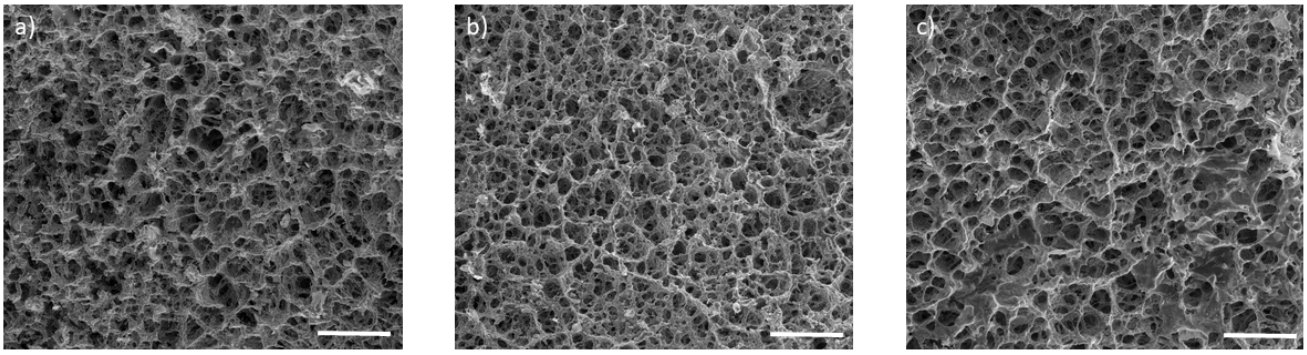

Figure 3. Representative SEM images for freeze-dried cross-sectional sections of: a) factor-free ELR hydrogel; b) ELR-VEGF and c) ELR-QK hydrogel. Scale bar: 200μm.

3.2 *In vitro* 3D cell proliferation assay

In accordance with the literature, the pro-angiogenic activity of QK peptide was evaluated *in vitro* employing endothelial cells and fibroblasts encapsulated into the hydrogels in a proportion of 3:1, respectively. [36] Figure 4 shows H&E staining images for factor-free hydrogel (ELR) (a1), ELR-VEGF (a2) and ELR-QK hydrogel (a3) after culture for 21 days. These images demonstrate that all hydrogels exhibit good cytocompatibility at three weeks given the number of cells present. It is also clear that they exhibit different trends in terms of cell proliferation, with the factor-free ELR hydrogel containing a lower number of cells than for the ELR-VEGF and ELR-QK hydrogels. These latter two samples exhibit similar trends in terms of cell proliferation, with only a minor difference between the ELR-QK and ELR-VEGF hydrogels. The number of cells/cm^2 was quantified for each sample by applying Image J to the H&E staining images. Specifically,

the number of cells is 245±22 for factor-free ELR hydrogel, 382±35 for ELR-VEGF and 375±29 for ELR-QK hydrogels, as shown graphically in Figure 5. As can be seen, there is no significant difference between ELR-VEGF and ELR-QK hydrogels in terms of cell proliferation, whereas the factor-free ELR hydrogel contains a much lower number of cells at three weeks.

Moreover, the ELR-VEGF and ELR-QK hydrogels exhibit various regions containing adherens junctions (indicated with a black arrow in Figure 4-b2 and Figure 4-b3, respectively). These junctions are required for correct organization of the new capillaries formed.

CD31 immunofluorescence staining was also performed to confirm the presence of endothelial cell junctions as this protein has been reported to be able to mediate cell-cell adhesion. [37] The images shown in Figure 6 (a1-a2-a3) indicate that all hydrogels exhibit marked CD31 expression, albeit with significant differences between them. Thus, CD31 expression in ELR-VEGF (Figure 6-a2) and ELR-QK hydrogels (Figure 6-a3) is significantly higher than in factor-free ELR hydrogels (Figure 6-a1). A similar behaviour, namely that the number of cells which form clusters is higher in VEGF-ELR and QK-ELR than in factor free ELR hydrogels, the latter of which present a lower number of fused cells, was observed in samples stained with H&E.

Despite these differences, it can be seen that factor-free ELR hydrogels (Figure 6-a1) still contain CD31 expression cells due to the presence of bioactive sequences such as RGD and REDV, which enhance cell adhesion and endothelial cell organization *in vitro*. Nevertheless, the difference between this latter sample and ELR-VEGF and ELR-QK hydrogels lies in the number of endothelial cell clusters formed. Thus, in ELR-VEGF (Figure 6-a2) and ELR-QK (Figure 6-a3) hydrogels, endothelial cells form a large number of cell aggregates or clusters in unorganized assemblies of more than five cells, whereas

factor-free ELR hydrogels are characterized mainly by containing single cells within the hydrogel.

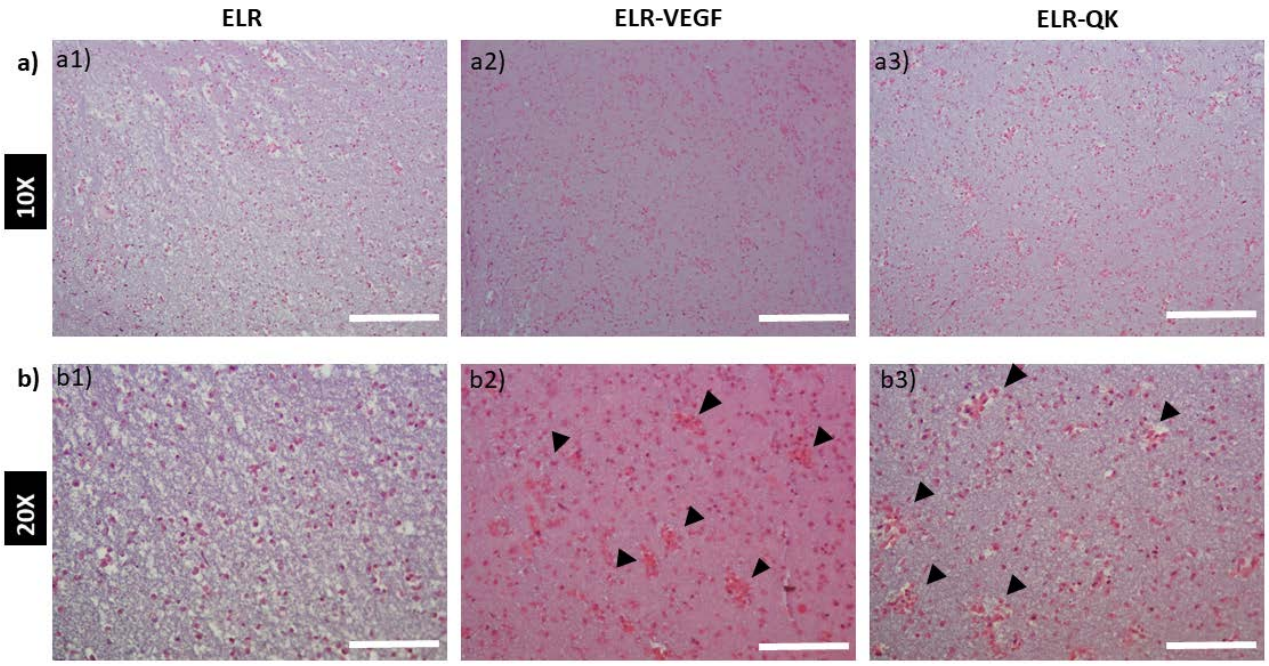

Figure 4. H&E staining with 10X magnification of: a1) factor-free ELR hydrogel (ELR); (a2) ELR-VEGF; and (a3) ELR-QK hydrogel after co-culturing HUVEC and HFF1 cells for 21 days. b) 20X Magnification of: b1) factor-free ELR hydrogel (ELR); b2) ELR-VEGF; and b3) ELR-QK hydrogels. Black arrows indicate the presence of endothelial cells clusters. Scale bar: 500µm.

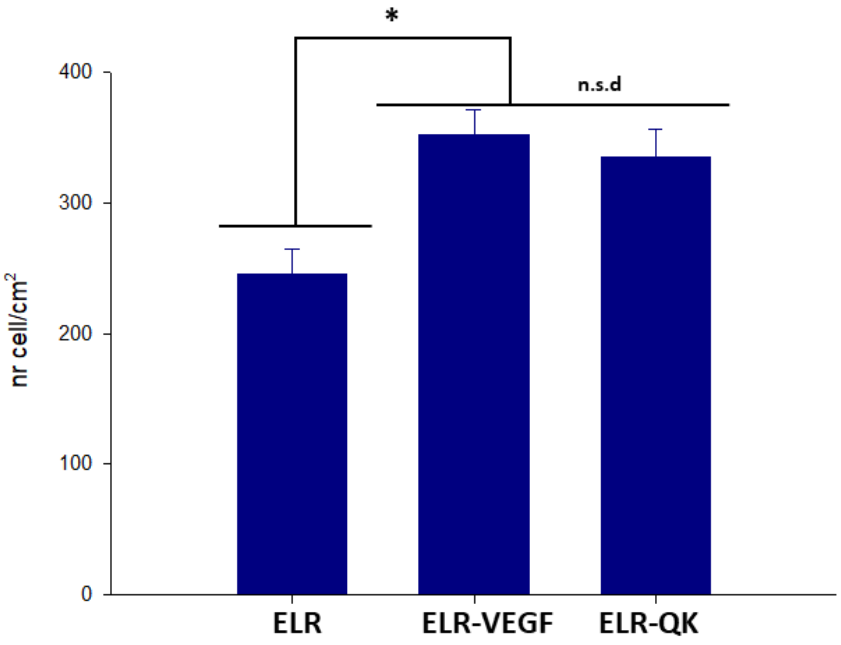

Figure 5. Graphical representation of number of cells/cm^2, as counted using Image J. Data are reported as mean ± SD (n=3). Statistical analysis was carried out by performing an analysis of variance using the Holm–Sidak method. *p<0.05; n.s.d., no significant difference.

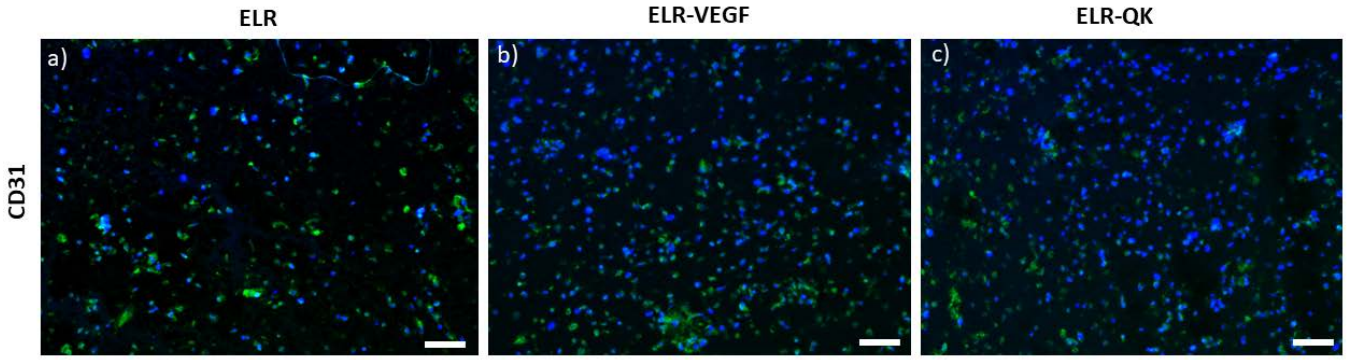

Figure 6. CD31 immunostaining of: a) factor-free ELR hydrogel (ELR); (b) ELR-VEGF; and (a3) ELR-QK hydrogel. Blue: DAPI staining. Green: CD31 expression. Scale bar: 50µm.

3.3 *In vivo* behaviour of QK peptide

The formation of new vasculature in the ELR hydrogels enhanced by QK peptide was evaluated *in vivo* using mice in which hydrogels were injected into a hind limb region. Two main samples, namely a factor-free ELR hydrogel (ELR) and ELR-QK hydrogel, were used for the *in vivo* analysis. This choice was based on the previous *in vitro* results, which demonstrated that the soluble VEGF and VEGF-mimetic peptides (QK peptide) exhibit similar biological activities.

To get an overview of cell invasion in ELR hydrogels, histological H&E staining was performed; the corresponding images are shown in Figure 7 (factor-free ELR hydrogel (7a) and ELR-QK hydrogel (7b)). ELR-hydrogels are surrounded by a discontinuous line and labelled with the letter H, whereas host tissue is indicated with a letter T. Figures 7c and 7d, which are magnifications of Figures 7a and 7b, respectively, both show good biocompatibility as the hydrogels are well infiltrated by different cell types at three weeks post-injection.

The hydrogels were entirely present up to 21 days, thus indicating early host cell infiltration, although there were clear differences between them. Thus, in ELR-QK hydrogels (Figure 7b), the cells reached the center of the hydrogel after three weeks, whereas the factor-free ELR hydrogel (Figure 7a) presented different areas that are almost intact, thus indicating a lack of cellular infiltration. This becomes clearer in Figure 8a, which is a magnification of a non-infiltrated area.

Upon initial inspection, this difference suggests that QK peptide facilitates the recruitment of cells and tissue integration and increases the rate of cell migration.

A closer examination of the ELR-QK sample stained with H&E (Figure 7d) indicated the formation of new capillaries with a uniform distribution inside the hydrogel. These new capillaries are indicated in Figure 7d with a black arrow, and the magnification in Figure 8b shows the presence of red blood cells forming the lumen of blood vessels surrounded by endothelial cells. [X] The size of the new capillaries formed varies between 25- and 50 μm. The total number of capillaries present in the hydrogel (>30) is significantly higher than for the factor-free ELR hydrogel. Indeed, new capillaries are completely absent from the factor-free ELR hydrogel (Figure 7a). This does not mean that they cannot be formed, however, simply that more time is needed for both complete colonization and for the organization of endothelial cells.

To further confirm new capillary formation in the scaffolds, CD31 protein expression was also determined by immunohistochemical staining, which allowed us to confirm the presence of endothelial cells junction. (Figure 9) As shown in Figure 9e-9f, the new capillaries (indicated by the presence of green florescence) are located in the ELR-QK hydrogel, which contrasts with the situation for the factor-free ELR hydrogel, Figure 9b-9c.

QK peptide therefore stimulates angiogenesis and enhances cellular migration, and therefore subsequent capillary structure formation, *in vivo*. These results agree with those obtained *in vitro* upon triggering the auto-phosphorylation of $VEGFR_2$ intracellular domains, which leads to the induction of HUVEC proliferation, survival and migration. [38]

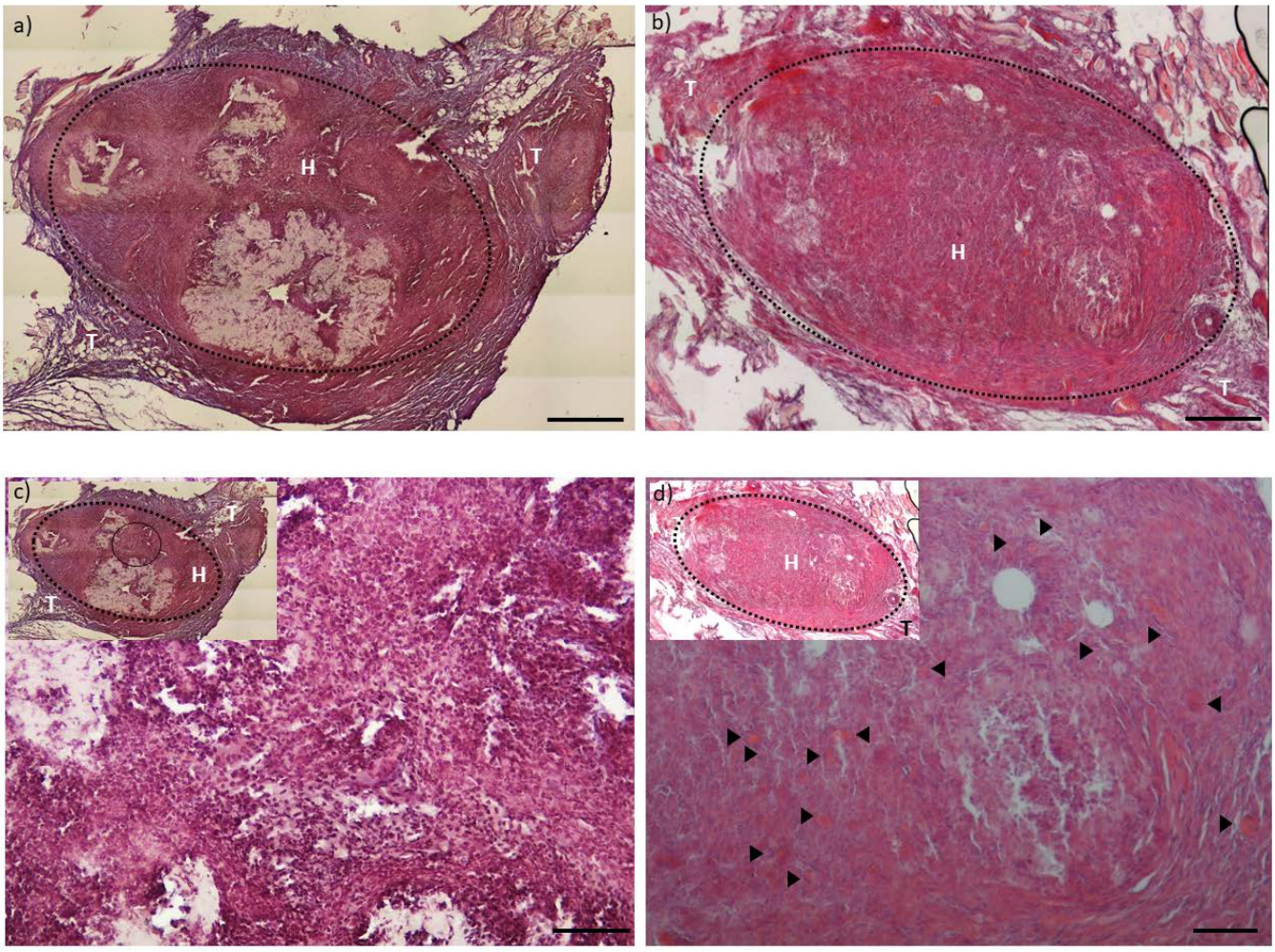

Figure 7. H&E staining images of: a) ELR factor-free hydrogel; and b) ELR-QK hydrogel; c) magnification 20X of image a); d) magnification 20X of image b). The black arrows indicate the presence of capillaries formed. Scale bar: 100µm

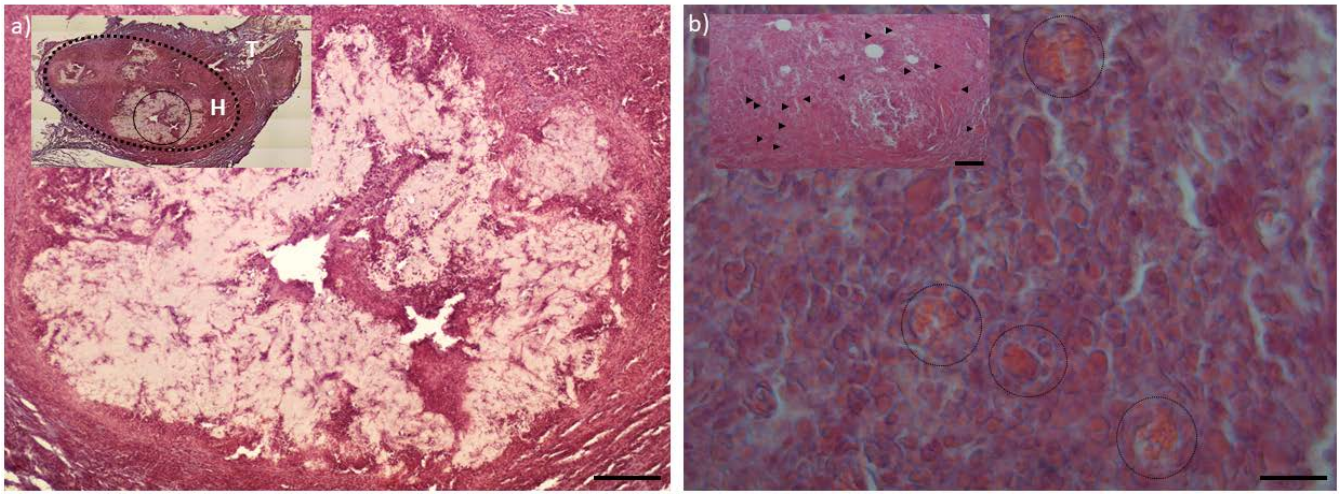

Figure 8. Magnification of H&E staining images of a non-colonized area of ELR factor-free hydrogel, which is indicated with a black circle. b) Magnification of ELR-QK hydrogel image in Figure 7b to better visualize the presence of new capillaries. Scale bar: 100µm.

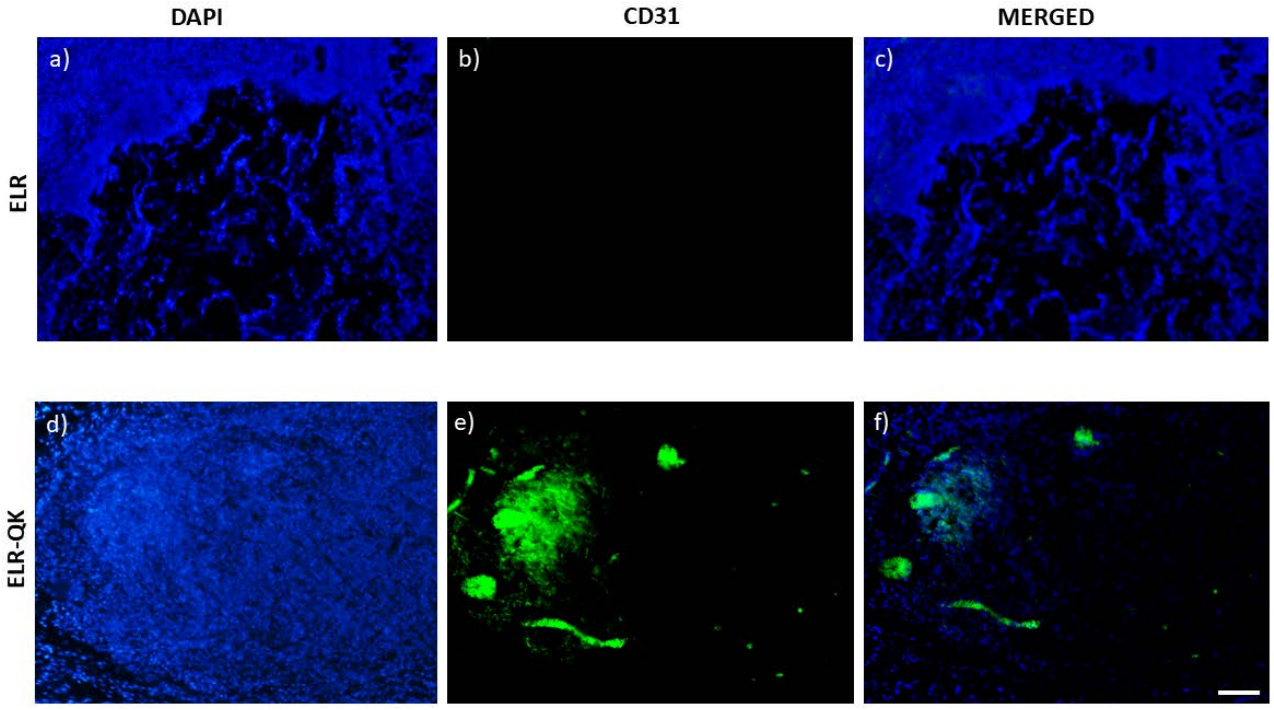

Figure 9. CD31 immunofluorescent staining images of: a-b-c) factor free ELR hydrogel (ELR) d-e-f) ELR-QK hydrogel. DAPI: nucleus staining (9a; 9d). CD31: expression of CD31 protein (9b; 9e). MERGED: merged channels (DAPI+CD31) (9c; 9f). Scale bar for all the images: 50 µm.

4. Discussion

In recent years, one of the most important efforts undertaken to achieve functional vascularization in scaffolds utilized in tissue engineering has involved modulating angiogenesis by targeting VEGF and its receptors. [22] Indeed, a VEGF mimetic peptide known as QK is commonly used to improve vascularization into 3D scaffolds as this protein has been demonstrated to exhibit pro-angiogenic activity *in vitro* and *in vivo* by binding to VEGFRs, thus initiating VEGF-induced signalling cascades and giving rise to the formation of new capillaries. [39, 40]

In this study, we proposed the use of QK peptide tethered chemically to ELR-based hydrogels via a click reaction to enhance vascularization within engineered constructs, which is also favoured by the presence of two main adhesion sequences, namely the universal cell-adhesion sequence RGD and a specific sequence for endothelial cells (REDV), encoded into their backbone. Moreover, the presence of the VGVAPG sequence codified into the recombinamer chains favours the chemotactic ability of monocytes and facilitates biodegradation of the ELR-based hydrogels by increasing their sensitivity to elastolytic enzymes. [8]

Several previous studies reported in the literature used small peptides such as RGD and the collagen-binding CMP domain bonded chemically to PEG scaffolds to increase cell adhesion and proliferation. [41] The proteinaceous nature of ELRs results in an absolute control over the amino acid sequences, thus allowing codification of the adhesion sequences into their backbone and overcoming the limitations that may be presented by these chemically bonded peptides. [42] In addition, proteolytic biodegradation is an essential feature for promoting cellular infiltration, which subsequently induces an enhanced cellular migration into the scaffold.

It has been shown herein that the presence of QK peptide does not affect the mechanical properties of the ELR-based hydrogels or their morphological features, although it significantly alters cell behaviour, such as proliferation, in the hydrogels. In light of this, we studied its biological activity *in vitro* using a co-culture of HUVEC and HFF1 cells, demonstrating that this peptide enhances cell proliferation, which in turn gives rise to the development of cell clusters and, subsequently, the formation of capillary structures. The use of a co-culture is mandatory before *in vivo* studies to confirm the biocompatibility of the hydrogels as well as their influence on *in vitro* angiogenesis. [43] These cell types were employed since they are the most widely used in angiogenesis assays given that the majority of ECM components produced provide support for capillary-like structure formation.

A quantitative analysis involving *in vitro* assays showed that QK peptide has a similar biological activity to the soluble VEGF used as positive control. Moreover, an estimation of cell numbers in a histological section of ELR-QK, ELR-VEGF and factor-free ELR hydrogels confirmed that the former two samples contained similar numbers of cells and demonstrated significant differences with respect to factor-free ELR hydrogels. These studies allowed us to determine that the presence of QK peptide enhances cell proliferation.

Small peptides have many benefits with respect to large proteins, such as a lack of denaturation, structural stability, ease of synthesis, absence of a need for a secondary conformation to preserve their biological activity, and they tend to be characterized by the absence of immunogenicity and toxicity. [44] Furthermore, the constant delivery of growth factors from a hydrogel to obtain the optimal concentrations and gradients required for precise spatial-temporal control remains a big challenge even though several strategies, such as covalent binding, use of carriers, electrostatic interactions, etc., have

been used. [45] A major limitation of natural growth factors is their short effective half-life, low stability, and rapid deactivation of their specialized properties by enzymes at body temperature. In this regard, our *in vitro* studies confirmed that QK peptide mimics the biological activity of VEGF when tethered chemically, inducing angiogenesis-related behaviour such as cell proliferation, cell-cell contact and the formation of capillary-like structures. The advantage of a chemically tethered peptide is that its concentration is controlled over time instead of a soluble growth factor.

Subsequent *in vivo* studies involved the intramuscular injection of hydrogels into mice. At three weeks post-injection the ELR-QK hydrogels were completely infiltrated by different cell types. Moreover, although inflammatory cells were still present at 21 days post-injection, there was no corresponding chronic inflammatory response. It has been widely demonstrated that inflammatory cells play a crucial role in restoring vascular networks as a result of reconstruction of the ECM, fusion of endothelial cells and increasing collagen synthesis. Macrophages and fibroblasts tend to degrade hydrogel networks and produce type I collagen, thus resulting in the gradual replacement of the hydrogel by regenerated tissue. In addition, the enzymatic degradation of these gels caused by the presence of proteolytic sites enhances the cellular invasion response and consequent formation of new micro-vasculature. [46]

There is a noticeable difference between the factor-free ELR and ELR-QK hydrogels. Firstly, the former was not completely invaded by cells, as demonstrated by the presence of intact regions from which cells were absent. In contrast, the entire ELR-QK hydrogel was invaded by cells and the formation of new functional blood vessels was clearly visible. To confirm the formation of new vasculature, CD31 immunostaining was performed to confirm the presence of this vasculature at the same position as observed with H&E staining.

This study therefore reveals that an angiogenic stimulus is important for the replacement of hydrogels by vascularized tissues and confirms that small peptides, such as QK peptide, behave in a similar manner as their larger VEGF counterpart, in other words by activating the canonical pathway in VEGF signalling. Combination of the *in vivo* data with the *in vitro* findings indicates that the presence of QK peptide enhances the formation of new vasculature in ELR-based hydrogels and could therefore give rise to a new design process for biomimetic cellular environments, thus highlighting their advantages compared to the use of soluble growth factors such as VEGF.

In light of the above, ELR-based hydrogels are promising constructs for future applications in tissue engineering, especially in cardiovascular disease, since they exhibit significant pro-angiogenic activity and allow the inclusion of several functionalities that are required for the tissue-repair process and for the formation of new vasculature.

5. Conclusions

This study has demonstrated that a VEGF mimetic peptide (QK peptide), tethered chemically into ELR-based hydrogels and injected into a hind limb region in mice, enhances the formation of new capillaries within the constructs. It has also been established that QK peptide affects the biological behavior of the cells in a similar manner to VEGF, as seen from the increase in pro-angiogenic activity (endothelial cell proliferation, migration and organization) in the ELR constructs. The *in vivo* findings showed that a functional microvasculature was obtained in QK hydrogels, thus providing a pro-angiogenic environment for cell survival and tissue growth. The approach described herein provides new insights that could be applied to a wide range of cardiovascular diseases requiring a spatial-temporal control of growth factors. It also provides useful

cues that could be utilized in both therapeutic angiogenesis and in other clinical models where the presence of new vasculature is essential.

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