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Biocompatibility of two model elastin-like recombinamer-based hydrogels formed through physical or chemical crosslinking for various applications in tissue engineering and regenerative medicine

Running title: Biocompatibility of ELR-based hydrogels for TERM

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Abstract

Biocompatibility studies, especially innate immunity induction, *in vitro* and *in vivo* cytotoxicity, and fibrosis, are often lacking for many novel biomaterials including recombinant protein-based ones, such as elastin-like recombinamers (ELRs), and has not been extensively explored in the scientific literature, in contrast to traditional biomaterials. Herein we present the results from a set of experiments designed to elucidate the preliminary biocompatibility of two types of ELRs that are able to form extracellular matrix-like hydrogels through either physical or chemical crosslinking, both of which are intended for different applications in tissue engineering and regenerative medicine (TERM). Initially we present *in vitro* cytocompatibility results obtained upon culturing HUVECs on ELR substrates, showing optimal proliferation up to 9 days. Regarding *in vivo* cytocompatibility, luciferase-expressing hMSCs were viable for at least 4 weeks in terms of bioluminescence emission when embedded in ELR hydrogels and injected subcutaneously into immunosuppressed mice. Furthermore, both types of ELR-based hydrogels were injected subcutaneously in immunocompetent mice and serum TNFα, IL-

1β, IL-4, IL-6 and IL-10 concentrations were measured by ELISA, confirming the lack of inflammatory response, as also observed upon macroscopic and histological evaluation. All these findings suggest that both types of ELRs possess broad biocompatibility, thus making them very promising for TERM-related applications.

Keywords

Biocompatibility; cytocompatibility; elastin-like recombinamers; Catalyst-Free Click Gels; silk-elastin multiblock co-recombinamers; tissue engineering; regenerative medicine

1. Introduction

There is an increasing interest in developing bioactive materials with applications in tissue engineering and regenerative medicine (TERM). Many of these materials are designed as multi-purpose biomaterials that can be used to promote the regeneration of different tissues or organs and, as such, initially lack a specific target. Despite this, general preliminary biocompatibility studies are often overlooked. Various families of biomaterials have traditionally been employed in TERM applications, with metals (Frydman and Simonian, 2014), ceramics (Denry and Kelly, 2014), polymers (Hu *et al.*, 2010, Pan and Ding, 2012) or even protein-based materials having been widely studied (Saini *et al.*, 2015). However, all these materials possess some limitations in terms of biocompatibility, such as the absence of functionalization that may improve their interaction with the host, although some progress has been made as a result of the incorporation of bioactive sequences and polypeptides into synthetic polymers (Krishna and Kiick, 2010, Meyers and Grinstaff, 2012). Furthermore, the release of wear debris from these materials has been shown to induce the recruitment of inflammatory cells, principally macrophages and giant cells, which substantially hinders their biocompatibility (Goodman, 2005, Nich and Goodman, 2014).

Novel biomaterials with a different nature and origin are currently being designed to overcome these issues (Veiseh *et al.*, 2015). Among them, we can identify recombinant protein-based biomaterials, which promise a significant improvement in the functionality thereof that has already been demonstrated in many cases (Girotti *et al.*, 2015). Although these materials have been evaluated in terms of physicochemical and mechanical properties, and in some cases *in vitro* cytotoxicity, in contrast to the traditional biomaterials described above their biocompatibility has not been extensively studied.

While a deeper understanding of their biocompatibility is required for specific applications, as suggested by the definition of this term by Williams (Williams, 2008), it is important to determine the degree of biocompatibility of these biomaterials by way of wide-ranging preliminary studies as their implementation in the biomedical field depends highly on this feature rather than just on their advanced functionality.

Herein we aim to investigate the cyto- and biocompatibility of two recently developed families of hydrogels based on elastin-like recombinamers (ELRs) (Rodríguez-Cabello *et al.*, 2009), both of which are designed as a multi-purpose approach for different TERM applications. ELRs are genetically engineered protein-based materials whose composition is inspired by the primary sequence found in natural elastin, specifically the VPGXG pentapeptide, where X can be any amino acid except L-proline. Moreover, they show thermosensitivity, as characterized by a reversible phase transition associated with a temperature known as the transition temperature (Tt). In an aqueous medium, the ELR chains remain soluble below their Tt, while above that temperature ELRs self-assemble hydrophobically and adopt a regular, non-random structure stabilized by the presence of type II β-turns (Urry, 1993). The first of these families of hydrogels is based on ELR-catalyst-free click gels (ELR-CFCGs) (Gonzalez de Torre *et al.*, 2014), and the second type is based on a silk-elastin-based injectable multiblock co-recombinamer (SELR) that spontaneously forms stable physical nanofibrillar hydrogels under physiological conditions as a result of the fusion of short silk-like peptides taken from the sequence of silk fibroin from the silkworm *Bombyx mori* to the ELR gene (Fernandez-Colino *et al.*, 2014). This SELR has been genetically modified in this work to include the Arg-Gly-Asp (RGD) cell-adhesion sequence (Ruoslahti and Pierschbacher, 1986) in order to improve its bioactivity and to more closely mimic the extracellular matrix (ECM) environment;

this motif has already been included in the ELR used to form ELR-CFCGs. Both families of ELRs have been physicochemically characterized previously (Fernandez-Colino *et al.*, 2014, Gonzalez de Torre *et al.*, 2014), although herein we present the characterization of the novel RGD-containing SELR. Furthermore, ELR-CFCGs have demonstrated certain properties that point to a potentially good biocompatibility, especially their very low *in vitro* thrombogenicity (Gonzalez de Torre *et al.*, 2015). However, apart from those scarce and *ad hoc* tests, the cyto- and biocompatibility of these two representative families of hydrogels have not been tested *in vitro* or *in vivo* to date.

In this study we present results from *in vitro* cell-proliferation assays and from the *in vivo* viability of luciferase-expressing cells at different time-points by bioluminescence detection (Jang *et al.*, 2010, Kim *et al.*, 2015) when embedded inside either ELR-CFCGs or SELR-based hydrogels that were subsequently injected subcutaneously into mice. Moreover, we performed an *in vivo* inflammatory evaluation that involved measuring the concentration of an array of cytokines (TNFα, IL-1β, IL-4, IL-6 and IL-10), all of which are produced and secreted during inflammation (Turner *et al.*, 2014, Zhang and An, 2007), by enzyme-linked immunosorbent assay (ELISA). Finally, macroscopic and histological results after long-term (1, 3 and 6 months) subdermal injection of both types of hydrogels in mice are presented. These experiments are intended to demonstrate the preliminary biocompatibility of two types of ELR hydrogels formed through either physical or chemical crosslinking, which is of paramount importance due to their huge potential in cutting-edge fields such as tissue-engineering and regenerative medicine.

2. Materials and methods

2.1. 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.

Collection of the hMSCs specified below was approved by the Ethical Committee of the University Hospital of Salamanca and was also in accordance with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all subjects included in the study.

2.2. ELR biosynthesis, characterization and modification

The genetic construction of the ELRs used in this work, which were bioproduced and supplied by Technical Proteins Nanobiotechnology, S.L. (TPNBT, S.L., Spain), was performed as described elsewhere (Rodriguez-Cabello *et al.*, 2012). Briefly, they were biosynthesized in a 15-L bioreactor and purified using several cooling and heating purification cycles (Inverse Temperature Cycling) taking advantage of the ability of these recombinamers to precipitate above their transition temperature. Further centrifugation steps led to a highly pure product, which was dialyzed against ultra-pure water, filtered through 0.22 μm filters (Nalgene) to obtain a sterile solution, and freeze-dried prior to storage. This process allowed the production of different ELRs, namely the previously described HRGD6 (TP71254, TPNBT, S.L., Spain) (Costa *et al.*, 2011), which was subsequently modified for chemical crosslinking, and $(EIS)_2$-RGD6 (TP20736, TPNBT, S.L., Spain), which is also known as a silk-elastin-like recombinamer (SELR). This latter recombinamer is able to establish physical interactions between isoleucine-containing hydrophobic domains that are further stabilized by the inclusion of two silk-like motifs per molecule, thus allowing the formation of hydrogels that remain unalterable over time,

as described in a previous study (Fernandez-Colino *et al.*, 2014). Both ELRs contain six RGD cell-adhesion sequences per molecule to permit cell attachment and proliferation. They were found to contain less than two endotoxin units (EU)/mg of ELR, as determined using the limulus amebocyte lysate assay with the Endosafe®-PTS system (Charles River Laboratories).

The characterization techniques used for the novel $(EIS)_2$-RGD6 included sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) for purity and molecular weight evaluation compared to the theoretical value of 121 012 Da; HPLC to calculate the amino acid composition; differential scanning calorimetry (DSC) to determine the transition temperature; and nuclear magnetic resonance (NMR) to provide recombinamer fingerprint data.

Chemical modification of the HRGD6 elastin-like recombinamer was achieved by transformation of the ε-amine group in the side chain of the lysine residues to bear cyclooctyne and azide groups, as reported previously (Gonzalez de Torre *et al.*, 2014), thus giving rise to HRGD6-N_3 (TP71254, TPNBT, S.L., Spain) and HRGD6-cyclooctyne (TP70254, TPNBT, S.L., Spain) recombinamers, which were characterized by NMR and FTIR to assess the degree of modification.

2.3. Gel formation

Since chemical crosslinking of the azide- and cyclooctyne-modified recombinamers takes place without the need for a catalyst, such as copper, the hydrogels obtained upon combining the two components are termed catalyst-free click gels (CFCG). The two recombinamers were dissolved separately in a water-based solvent (ultra-pure water,

MilliQ, Millipore), PBS or culture medium) at the final concentration and kept at 4 °C for at least 24 hours. ELR-CFCGs were obtained by mixing the solutions at 4 °C. Gels were formed after 15 minutes.

For the SELR, physically crosslinked hydrogels were obtained by dissolving the polymer in a water-based solvent at the desired concentration for 24 h at 4 °C. Gels were then formed by casting the cold solution into the appropriate molds, depending on the expected applications.

2.4. Cell cultures

Commercially available human umbilical vein endothelial cells (HUVEC, ATCC CRL-1730) at passage 2-5 were used. Medium 200 (Gibco) supplemented with the antibiotics gentamicin/amphotericin (1%, Gibco) and low serum growth supplement (LSGS, Gibco) were utilized to maintain cell culture at 37 °C and 5% CO_2, with medium replacement every two days until 80% confluence, at which point the medium was replaced every day. When required, cells were detached using a solution of 0.05% Trypsin-EDTA (Gibco), centrifuged and re-suspended at the desired concentration.

The human mesenchymal stromal cells (hMSCs) used for subcutaneous injection of the cell-ELR mixture were obtained from the bone marrow of healthy donors at the Hospital Universitario de Salamanca (Salamanca, Spain). First, mononucleated cells (MNCs) still not identified as MSCs were isolated through a density gradient using Ficoll (Sigma-Aldrich), seeded in culture flasks at a concentration of 10^6 MNC/cm^2 and cultured at 37 °C, 5% CO_2 until 80-90% confluence was reached, with medium replacement every 3-4 days. At that stage the cells were trypsinized and seeded into new flasks at 5 000 cells/cm^2. After passage 3, the cells were confirmed as hMSCs according to the

International Society for Cellular Therapy (ISCT) minimum criteria (Dominici *et al.*, 2006). hMSCs were expanded in DMEM supplemented with 1% penicillin/streptomycin and 10% FBS (Gibco). Further modification of these cells was achieved by lentiviral transduction of the pLV-CMV-Luc2-IRES-GFP, with the modification being confirmed by flow cytometry. These transduced genes led to the expression of luciferase and GFP, thus allowing the hMSCs to be tracked non-invasively *in vivo*.

2.5. Cell proliferation experiments

HUVEC proliferation when cultured on both ELR-CFCG hydrogels and SELR adsorbed onto the well plate was evaluated at 1, 5 and 9 days after cell seeding. ELR-CFCGs were formed at 75 mg/mL by adding 15 µL of each solution into the well of a 96-well tissue culture plate (TCP, Fisher), while SELR was adsorbed onto the well surface (0.33 cm^2) of 96-well plates by incubation of a 5 mg/mL solution at 4 °C for at least 24 h prior to cell culture. Previously formed hydrogels or absorbed recombinamers were exposed to UV light for 3 h for surface sterilization, and then washed with minimum cell culture medium for at least 2 h, even for non-coated TCPs. 2 500 HUVECs suspended in 100 µL of complete culture medium (25 000 cells/mL) were seeded on each recombinamer and allowed to grow in the appropriate media. A quantitative Calcein-AM assay (Molecular Probes) was performed at each time point according to the manufacturer's instructions, and fluorescence intensity was measured at 530 nm using a plate reader (SpectraMax M2e, Molecular Devices). This fluorescence intensity, which corresponded to live cells, was then used to calculate cell numbers using calibration curves obtained with different known quantities of cells (from 1 000 to 15 000 cells per well) seeded on black 96-well plates with a clear bottom 24 h before the measurement. Cell-free ELR substrates were

used as blank, except for the case of cells cultured on non-coated TCP. Each condition was performed in triplicate and three experiments were performed for each.

2.6. Determination of hMSC bioluminescence *in vivo*

To assess the potential use of these hydrogels as scaffolds for cell therapy, the transduced hMSCs described above were embedded in HRGD6-N_3 and SELR solutions in penicillin/streptomycin-supplemented DMEM at a final concentration of 10^6 cells/mL. A 100 µL aliquot of each suspension was used for subcutaneous injection in nude Swiss nu/nu mice (Charles River Laboratories). For the ELR-CFCGs, HRGD6-N_3 containing hMSCs and HRGD6-cyclooctyne solutions were mixed in a 1:1 ratio (50 µL of each) immediately prior to injection for optimal homogenization. Solutions of hydrogel-forming ELRs were injected at various concentrations in four different points of the same animal (n = 3). The final concentration values used in the case of ELR-CFCGs were 25, 50, 75 and 100 mg/mL, whereas for SELR gels they were 75, 100, 125 and 150 mg/mL. These concentrations will be referred to as 1, 2, 3 and 4, respectively, for each kind of recombinamer. Cells were tracked through space and over time by luminescence measurements (see below). All procedures were performed under sterile conditions in facilities with positive pressure and laminar flow cabinets. Animals were stored in filtered air racks.

Luciferase-expressing hMSCs were tracked using the Xenogen IVIS 50 bioluminescence system (Xenogen Corporation, Caliper Life Science). Briefly, animals were anesthetized with isoflurane and then injected intraperitoneally with 15 µg luciferin/g body weight. They were then introduced into the IVIS chamber 10 minutes post-injection, which was found to be the most appropriate moment for obtaining reproducible results. Images were

analyzed using the Living Image 2.50.1 software (Xenogen Corporation, Caliper Life Science) by determining the number of photons emitted per second from a selected region of interest (ROI). Bioluminescence was measured at day 1 and then weekly over 4 weeks.

2.7. Subcutaneous implantation and ELISA *in vivo*

Three albino Swiss mice (male) per group were used for the subcutaneous injection of 100 μL of each hydrogel-forming ELR solution. In the case of ELR-CFCGs, 50 μL HRGD6-N_3 and 50 μL HRGD6-cyclooctyne cold solutions (75 mg/mL in PBS) were mixed immediately prior to subdermal implantation. The SELR was dissolved at 150 mg/mL in cold PBS. The solutions used to obtain both kinds of gels were injected using a 20G needle and a 1 mL syringe at one side of the spinal cord. Chemically or physically crosslinked hydrogels were formed instantaneously and could be observed as a small bulge under the skin. These same animals were also employed for blood harvesting to obtain data about the acute inflammatory response that these recombinamers could trigger, using PBS and bacterial lipopolysaccharide (LPS, Sigma-Aldrich) at 3 mg/kg (Lehner *et al.*, 2001) (1 endotoxin unit = 100 pg of LPS), as negative and positive controls, respectively. Hence, four groups were used in this experiment to study the inflammatory response by ELISA.

Five different cytokines, namely TNFα, IL-1β, IL-4, IL-6 and IL-10 (product reference EMTNFA, EM2IL1B, EMIL4, EM2IL6 and EM2IL10, respectively; Thermo Fisher), were quantitatively studied to evaluate the acute inflammatory response towards ELR hydrogels. Approximately 250 μL of blood was collected in Microvette CB 300 K2E treated capillary tubes (Sarstedt) by tail clipping following by centrifugation at 6 000 rpm for 10 minutes at room temperature to finally obtain a cleared serum, which was

subsequently ultra-frozen at -80 °C until assay. The process was repeated at days 1, 2 and 7 post-injection.

Sera were thawed in ice and undiluted samples were used. ELISAs were performed in duplicate, as recommended in the guidelines for each anti-mouse cytokine kit. Colorimetric results, measured using a plate reader (SpectraMax M2e, Molecular Devices), were translated into protein concentration values with the help of a standard curve obtained using known quantities of the recombinant cytokines included in each kit. The amounts used for the calibration curve varied for each, but they always ranged between the lowest and highest ELISA sensitivity thresholds.

2.8. Long-term hydrogel stability *in vivo*

For the evaluation of long-term stability, ELR-CFCG and SELR hydrogels were injected as cold solutions at 75 and 150 mg/mL in PBS, respectively, into three albino Swiss mice (male) per group, in a similar manner to the ELRs used in the subcutaneous injection for ELISA experiments. Mice were euthanized by cervical dislocation at 1, 3 and 6 months post-injection, as recommended in standard procedures. The hydrogels were then extracted to determine their long-term stability. Macroscopic assessment of the hydrogels and of the surrounding tissues was performed, along with microscopic observations after histological processing of the hydrogels.

2.9. Histological processing

Hydrogels were extracted from the mice and immersed in paraformaldehyde at 4% in PBS using 10-fold the gel volume. Samples were stored at 4 °C for at least 24 h and dehydrated by immersion in ethanol solutions of increasing concentration (75%, 95% and

100%) with a final dehydration step in xylene (Sigma-Aldrich). Finally, hydrogels were embedded in paraffin (Sigma-Aldrich) and cut with a microtome (Leica) to obtain sections with a thickness of 10 μm. These were placed in slides and deparaffinized, with subsequent immersion in xylene, ethanol solutions of decreasing concentration (100%, 95%, and 75%) and, finally, in water.

Hematoxylin-eosin staining was performed following a previously described method.(Fischer *et al.*, 2008) Briefly, slides were dipped in hematoxylin stain for 30 seconds and rinsed in water for 1 minute. A 1% eosin solution was then used for staining during 30 seconds, with shaking, and samples were dehydrated by immersion in ethanol solutions and xylene. Finally, mounting medium was used to cover the sample on the slide with a microscope coverslip. Images were taken using a bright field microscope (Nikon Eclipse 80i) coupled to a color camera (Nikon Digital Sight DS-Fi1) with different magnifications.

2.10. Statistical analysis

Data are reported as mean ± SD (n = 3). Statistical analysis was performed using a one-way analysis of variance using the Holm–Sidak method. A p-value of less than 0.05 was considered to be statistically significant. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; $P > 0.05$ indicates no significant differences (n.s.d.).

3. Results and discussion

3.1. ELR bioproduction and characterization

Both ELRs were provided by TPNBT after characterization for internal batch control using standardized methods. Nonetheless, full characterization of the RGD-containing

SELR (namely, $(EIS)_2$-RGD6) was performed since it was produced for the first time in this study (see Table S2 for the amino acid sequence and molecular weight). The experimental molecular weight was found to be similar to the estimated value (120.4 vs. 121.0 kDa), as confirmed by SDS-PAGE and MALDI-TOF (Figure S1, Supporting Information). Furthermore, the Tt was found to be 16.8 °C in PBS (Figure S2, Supporting Information), which is 2.4 °C higher than the Tt reported for $(EIS)_2$, i.e. the non-RGD-containing SELR, in the same solvent (Fernandez-Colino *et al.*, 2014). This result is consistent with those reported in other studies, which predict an increase in the Tt when hydrophilic (poly)peptides are fused to the ELR sequence (Christensen *et al.*, 2013), which is the case of the RGD motif comprising several polar amino acids. In addition, ^1H NMR data and amino acid analysis showed good agreement with the expected values (Figure S3 and Table S1, Table S3, respectively).

3.2. HUVEC proliferation on ELR substrates

In order to shed light on the proliferation of cells on SELR and ELR-CFCG substrates, HUVECs were seeded on previously ELR-coated TCPs. Non-coated TCPs were used as control. This cell line is widely used to test the cytocompatibility and suitability of vascular devices. In this case, we chose HUVECs due to the previously described use of ELR-CFCGs in cardiovascular applications, for example the development of heart valves in combination with fibrin (Weber *et al.*, 2015).

Given the presence of RGD-containing domains along the ELR molecules, a specific interaction between this 12-mer peptide, which includes the RGD tripeptide, and cell membrane integrins was expected, thus conferring a favorable environment for cell spreading and proliferation. For this purpose, TCPs were covered with the hydrogel-

forming ELRs as described above (see Materials and Methods) to form hydrogels (ELR-CFCGs) or to adsorb ELR molecules on the well surface (SELRs) prior to cell seeding.

Cells were found to be adhered to the modified plates for both recombinamers, similarly to the TCP control, thus meaning that they promote cell adhesion. Rapid proliferation was observed for the ELR-CFCGs (Figure 1), with a high cell number at day 4, showing significant differences with SELRs ($P < 0.05$). In contrast, HUVEC proliferation was slower for the SELR and it followed a similar trend to the negative control (non-coated TCP). These dissimilar proliferation rates can be explained by the different charges on the ELRs, which present a negative surface charge on the outer side of the cell membrane that may influence their interaction with cells. In contrast, HRGD6, which is the ELR used for the formation of ELR-CFCGs, possesses a positive charge due to the presence of amine groups in the side chain of the lysine residues included in its primary sequence, thus meaning that a better interaction with cells is more likely. In contrast, the $(EIS)_2$-RGD6 (also referred to as SELR) comprises glutamic acid-substituted elastin-like blocks, which present acid groups that confer a negative charge. This is in good agreement with previous studies, which concluded that a negative charge may hinder cell proliferation (Chen *et al.*, 2009, Liu *et al.*, 2014). Despite this, both types of ELRs showed no significant differences with respect to the non-coated TCP at all times tested. Taken together, these results suggest that both ELRs promote cell proliferation *in vitro*.

3.3. *In vivo* cell tracking and viability of hMSCs in ELR-based hydrogels

To obtain information about the viability of cells embedded in both types of ELR hydrogels at different concentrations after *in vivo* implantation, we used luciferase-expressing hMSCs, a multipotent cell type widely used in regenerative medicine that

often shows poor retention and viability if injected without a scaffold (Martens *et al.*, 2009, Roche *et al.*, 2014). Our aim was to assess the viability of hMSCs inside the ELR hydrogels *in vivo*, thereby simulating the therapeutic conditions. Cells were initially mixed with solutions of each type of ELR at low temperatures (below the Tt). The resulting ELR-cell suspensions were then injected subcutaneously in mice and hydrogels were formed instantaneously, as confirmed by the observation of a protuberance under the skin of the animals.

With regard to the SELRs, bioluminescence signals were observed for hMSCs embedded in hydrogels at SELR concentrations of 125 and 150 mg/mL for three weeks (label 3 and 4 in Figure 2C, respectively), whereas they could be observed up to the fourth week for a concentration of 100 mg/mL (label 2, Figure 2D). For the ELR-CFCGs implanted with cells, bioluminescence was detected for all concentrations tested up to the end of the experiment at week 4 (images E, F, G and H in Figure 2).

The differences between the two groups may be due to the easier migration of cells from SELR-based hydrogels, which are physically crosslinked, thus meaning that the network is stabilized mainly by hydrophobic interactions and H-bonds, whereas ELR-CFCGs form covalent bonds that may hinder cell migration, thus meaning that hMSCs remain in this type of hydrogel for longer.

Luciferin-mediated bioluminescence was observed in cells embedded in at least one of the hydrogel concentrations used at every time, thus indicating that hMSCs express luciferase and that cells remaining inside the scaffolds were alive for up to 4 weeks, since the luminescence signal coincides with the protuberances observed under the skin, which correspond to ELR hydrogels. This result strongly highlights the ability of physically or

chemically crosslinked ELR-based hydrogels to sustain cells embedded in them when injected *in vivo*, hence providing an excellent approach for the development of ECM-like scaffolds for cell delivery in different applications in regenerative medicine.

3.4. Evaluation of the inflammatory response mediated by ELR hydrogels *in vivo*

With the aim of elucidating whether ELR hydrogels induced an innate immune response, namely an inflammatory reaction, or not, we injected ELR solutions (below the Tt) subcutaneously into mice to form hydrogels instantaneously. Blood samples were then collected at different timepoints and sera were obtained after centrifugation. These sera were used to measure the concentration of different cytokines, namely the pro-inflammatory TNFα, IL-1β and IL-6, and the anti-inflammatory IL-4 and IL-10, all of which are expressed during inflammation, as discussed above (see Introduction).

The cytokine analyses are presented in Figure 3. The results of these studies showed that LPS triggered an innate immune response in terms of acute inflammation, as expected. On the other hand, lower concentrations of each cytokine were found for both types of ELR-based hydrogels (ELR-CFCG and SELR) than those for the positive control (LPS) ($P < 0.05$), with some exceptions. For instance, the concentration of IL-6 for the LPS group at day 2 did not differ significantly from that for the SELR group, with this also being observed at day 7 for LPS with every other group, including the negative control (PBS). Moreover, the cytokine concentrations for the SELR and ELR-CFCG groups were similar to that for the PBS group in every case (n.s.d.), apart from the IL-6 levels for SELR-based hydrogels at day 1.

The cytokine concentrations for the ELR-CFCG and SELR groups were therefore similar to those observed for the negative control (PBS), thus allowing us to conclude that the injection of ELR-based hydrogels does not trigger an acute inflammatory response. These results support the findings observed in previous studies (Rincón *et al.*, 2006, Urry *et al.*, 1998), although they assessed biocompatibility mainly on the basis of histological findings. Furthermore, we were able to confirm the absence of active bacterial LPS residues (widely known as endotoxins) in any of the ELRs, which addresses one of the main fears regarding the use of recombinant proteins produced in *E. coli*.

3.5. Macroscopic evaluation of the long-term stability of ELR-based hydrogels *in vivo*

Long-term stability after injection of the ELRs was evaluated by extracting the subcutaneous implants at different timepoints (1, 3 and 6 months). Both gels (ELR-CFCGs and SELRs) were easily found attached to the hypodermis in the three mice used in each group at every timepoint (Figure 4). This result highlights that both types of ELR hydrogels are very stable when implanted subdermally *in vivo*, even when the hydrogel network is only stabilised via non-covalent interactions, which is the case of the SELR. This is in good agreement with previous findings for this type of recombinamer *in vitro* (Fernandez-Colino *et al.*, 2014) and suggests that additional biodegradation domains should be included in the amino acid sequence of the ELRs if a temporary implant is needed. However, the good stability over time observed here is relevant for both tissue-engineering applications and for the development of drug-delivery systems since the formation of a stable system could be crucial for achieving the long-lasting effects required in many of these applications.

Furthermore, no encapsulation of ELR hydrogels by fibrotic tissue, or signs of inflammation, redness or swelling of the tissues surrounding the hydrogels, was observed macroscopically, although some vascularization close to the implant area could be detected. The edges of the hydrogels were well defined and no macroscopic debris was found at any time point.

With regard to the morphology of the hydrogels, clear differences were seen in the color and general appearance of the two types of hydrogels. Thus, whereas ELR-CFCGs are white and have a "granular" appearance, the SELRs are more transparent and homogeneous. The explanation for these differences resides in the different composition of the backbone of the ELRs and in the different crosslinking methods used to obtain the hydrogels. In any case, their appearance did not change over time while implanted, as can be seen from Figure 4.

3.6. Histological evaluation of ELR-based hydrogels injected subcutaneously

Histological analysis was performed on both SELR hydrogels and ELR-CFCGs extracted at six months post-implantation, with hematoxylin-eosin staining used to add information to the macroscopic evaluation of the behavior of ELR-based hydrogels when implanted *in vivo*. This study revealed no presence of macrophages or giant cells that could indicate a chronic inflammatory process (Figure 5). Furthermore, a few layers of connective tissue were observed surrounding the ELR-based gels, thus indicating a lack of encapsulation or a fibrotic response. Moreover, these connective tissue layers were irrigated by blood vessels in both types of hydrogels (Figure 5D and 5H, black arrows).

The infiltration of cells inside the scaffold was also observed in the case of the SELR hydrogels, whereas this was not so obvious for ELR-CFCGs due to the differences in

staining, probably because of the composition (lysine (ELR-CFCG) instead of glutamic acid (SELR) residues) and the granular structure, which results in a darker staining of the hydrogel in some areas. Nevertheless, many cells were found at the edges of the chemically crosslinked hydrogels, i.e. ELR-CFCGs, and, to a lesser extent, in their inner structure, as shown in Figure 5 (blue arrow). This cell colonization is probably enhanced by the presence of the RGD amino acid sequence, which allows cell attachment (as confirmed by HUVEC proliferation experiments) and thus provides an ECM-mimetic scaffold that can be invaded by endogenous cells. Moreover, due to their high stability over time and their optimal biocompatibility, both types of ELR-based hydrogels have been shown to be suitable for long-term testing to investigate possible chronic inflammation reactions or cytotoxic effects induced by drugs or other agents via the incorporation thereof into the scaffolds.

It should be noted that the different concentrations of the ELR solution used to form both types of hydrogels have a crucial effect on the final structure of the gels. This difference can be explained by the diverse mechanism of gelification: more and larger ELR molecules (thus a higher concentration) are needed to establish a physically crosslinked stable network, as is the case of SELR hydrogels, while a lower concentration is sufficient for chemically crosslinked ELR-CFCGs if sufficient anchoring points are present within the ELR molecules. As such, SELRs with a concentration of 150 mg/mL produced a denser and more continuous structure than ELR-CFCGs with a concentration of only 75 mg/mL, which showed a granular structure, as can be seen from the histological pictures (Figure 5), thus confirming the previously observed macroscopic findings (Figure 4).

4. Conclusions

We have presented the results obtained upon combining different methods with the aim of assessing the preliminary wide-ranging biocompatibility of two recently developed types of ELR-based hydrogels formed via either chemical (ELR-CFCGs) or physical (SELR hydrogels) crosslinking. HUVEC proliferation on ELR substrates evaluated over 9 days was found to be optimal, i.e. similar to that for the non-coated TCP control. Cell viability inside ELR-CFCGs and SELR hydrogels was also confirmed since bioluminescence could be detected from luciferase-expressing hMSCs for up to 4 weeks. Furthermore, we found an absence of an early acute immune response, namely inflammation, as confirmed by the values obtained for the concentration of relevant cytokines (TNFα, IL-1β, IL-4, IL-6 and IL-10) in mice blood samples after subcutaneous injection of ELR-based hydrogels, which were similar to the concentrations obtained for the negative control (PBS). This was further corroborated by the macroscopic and histological evaluation of ELR-based hydrogels and their surroundings after long-term subcutaneous injection, which revealed an excellent stability up to 6 months, and a very low immune response in terms of foreign body response. Specifically, histological analysis revealed the growth of a thin layer of newly formed connective tissue around the hydrogels, even showing the presence of small blood vessels. Moreover, an invasion of the hydrogels by host cells was observed, with this being most clearly seen in the case of SELR hydrogels.

In summary, this work confirms the preliminary biocompatibility of two types of multi-purpose ELR-based hydrogels and is thus the first step towards the use of both types of ELRs in different applications in the field of tissue engineering and regenerative medicine.

Conflict of interest

The authors declare no competing financial interest.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's website.

Supporting figures and tables. Different figures and tables are shown regarding the full characterization of the SELR used in this work, namely SDS-PAGE analysis, MALDI-TOF spectrum, DSC spectra, ^{1}H NMR spectra and table summarizing results, amino acid composition.