Elastin-like recombinamer based devices releasing Kv1.3 blockers for the prevention
of intimal hyperplasia: an *in vitro* and *in vivo* study

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Running title: *PAP-1 releasing ELR-hydrogels against intimal hyperplasia*

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ABSTRACT

Coronary artery disease (CAD) is the most common cardiovascular disorder. Vascular surgery strategies for coronary revascularization (either percutaneous or open) show a high rate of failure because of restenosis of the vessel, due to phenotypic switch of vascular smooth muscle cells (VSMCs) leading to proliferation and migration. We have previously reported that the inhibition of Kv1.3 channel function with selective blockers represents an effective strategy for the prevention of restenosis in human vessels used for coronary angioplasty procedures. However, delivery systems for controlled release of these drugs have not been investigated. Here we tested the efficacy of several formulations of elastin like recombinamers (ELRs) hydrogels to deliver the Kv1.3 blocker PAP-1 in various restenosis models. The dose and time course of PAP-1 release from ELRs click hydrogels was able to inhibit human VSMC proliferation *in vitro* as well as remodeling of human vessels in organ culture and restenosis in *in vivo* models. We conclude that this combination of active compound and advanced delivery method could improve the outcomes of vascular surgery in patients.

Key words: vascular smooth muscle cells; intimal hyperplasia; elastin-like recombinamers; hydrogels; Kv1.3 channels; local drug delivery,

1. INTRODUCTION

Cardiovascular disease is the leading cause of death and disability in developed countries. More than 4 million Europeans die of cardiovascular disease every year, (51% of all deaths among women and 42% among men) and many more require hospitalization or life-long treatments. While mortality rates tend to decrease, the burden of morbidity trends upward since the early 2000s [1]. The development of catheter-based endovascular interventions, with or without stent implantation has represented a huge therapeutical improvement. A limitation of these procedures is restenosis of the target vessel. Mechanical or chemical injury promotes dedifferentiation of the vascular smooth muscle cells (VSMC) of the vessel wall, triggering a proliferative, migratory and secretory phenotype directed to neointima formation [2]. This process of phenotypic modulation (PM) represents the arterial repair mechanisms devised for healing, but an excessive, uncontrolled response leads to an intimal hyperplastic (IH) lesion that renarrows the lumen. Drug-eluting stents (DES) for local release of anti-proliferative agents (i.e., paclitaxel, rapamycin) decrease restenosis, but they also block endothelial cell growth, delaying healing and creating late in-stent thrombosis, which is often fatal [3]. Anti-coagulant therapies cannot completely prevent stent thrombosis, and they are associated with high costs and bleeding problems. Moreover, compromised stents cannot be replaced, so that a secondary, invasive revascularization such as bypass surgery is often required.

Despite the advancements of endovascular interventions (e.g. stent and/or balloon placement), the coronary artery bypass graft (CABG) is the most frequently performed surgical intervention for relieving consequences associated with ruptured or occlusive coronary artery atherosclerotic plaques [4]. Surgery uses undiseased vessels (saphenous vein, internal mammary artery, or radial artery) for diversion of blood from the aorta to the coronary artery, thereby revascularizing cardiac tissue. Long term efficacy of bypass grafts remains restricted due to IH development after surgery and restenosis of the conduits [5]. Though arterial grafts exhibit superior patency rate, shapenous vein is most commonly used as it allows for making of multiple grafts. However, up to 50% suffer failure within 10 years [6]. Several anti-restenotic strategies have been explored, but an efficient system is not yet available in the clinical routine. While the superiority of local drug delivery system was demonstrated (reviewed in [7]), the overall outcome of the clinical trials is poor. These observations call for the development of anti-restenotic therapies, via innovation in both drugs and delivery methods.

PM of VSMCs associates with an increased functional expression of Kv1.3 channels [8]. Kv1.3 channel blockade decreased VSMC proliferation and migration and prevented IH in human vessels and animal models [9–11]. These data indicated that Kv1.3 selective blockers such as margatoxin (MgTx) or PAP-1 (5-(4-phenoxybutoxy)psoralen) [12] could be used for the treatment of vascular diseases associated with PM of VSMC. However, for these drugs to reach to the clinics, we need to explore delivery systems and/or biocompatible scaffold materials suitable for local application of the blockers with a sustained release at effective concentrations.

Elastin-like recombinamers (ELRs) are recombinant elastin-mimetic polymers inspired in natural elastin that have been widely investigated in a broad range of applications from tissue engineering to nanovaccines [13] or drug delivery systems [14,15]. The recombinant DNA technology allows to bioproduce them with totally controlled composition. The term “recombinamer” highlights their oligomeric nature and their production as recombinant proteins that resemble elastin behavior, avoiding the drawbacks of conventional chemical synthesis [16]. Moreover, these tailored polymers exhibit great biocompatibility, mechanical

properties similar to those of natural elastin, self-assembly capability and a fully reversible thermosensitive behavior. ELRs catalyst-free click hydrogels can be produced under physiological conditions, and are attractive candidates for the fabrication of coated vascular stents by injection molding [17,18] or layer-by-layer dip-coating techniques [19], with good durability, as previously demonstrated in an ELR coated angioplasty balloon [20]. These coatings represent a good strategy for the development of a next generation of DES, and in fact drug loaded ELRs have been used as advanced delivery systems with promising results in diseases such as rheumatoid arthritis [21], cancer [22] or ischemia [23], showing also versatility in the embedded drug to release.

Combining all these previous evidences, we hypothesize that the use of an ELR-hydrogel incorporating the Kv1.3 blocker PAP-1 could represent a more efficient strategy for the prevention of intimal hyperplasia. ELRs biopolymers containing PAP-1 will be used as grid-coating or as lyophilized hydrogel beads and the time-course of PAP-1 release will be explored in human cultured VSMCs or in vessel rings in organ culture. In addition, the effect of injectable ELR-hydrogels containing PAP-1 for the prevention of IH in an animal model of vascular injury will be studied.

2. MATERIALS AND METHODS

2.1. Synthesis, expression and purification of the ELRs.

The ELRs used in this work were biosynthesized in a 15-L bioreactor and purified with several cycles of temperature-dependent reversible precipitations, by centrifugation below and above their transition temperature (Tt) [14]. The ELRs were subsequently dialyzed and lyophilized. Two different ELRs, VKV and IK, were obtained. The amino acid sequences of these polymers was:

VKV: MESLLP VG VPGVG [VPGKG(VPGVG)₅]₂₃ VPGKG (VPGVG)₄

IK: MESLLP [(VPGIG)₂(VPGKG)(VPGIG)₂]₂₄V

The purity and chemical characterization of these ELRs were verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF), amino acid composition analysis, differential scanning calorimetry (DSC) and nuclear magnetic resonance (NMR) [14]. NMR and MALDI-ToF were performed at the Laboratory of Instrumental Techniques (LTI), a core facility of the University of Valladolid.

2.2. Chemical modification of the ELRs.

ELRs were chemically modified by transformation of the ε-amine group from lysine to bear cyclooctine and azide reactive groups, respectively, as reported previously [17,24]. The modified biopolymers VKV-cyclooctine renamed VKV-CO and IK-azide renamed IK-N3 were characterized by MALDI-ToF, NMR and Fourier transform infrared spectroscopy (FTIR-ATR). MALDI-ToF and ¹H-NMR (in DMSO-d₆) allowed us to assess the degree of lysine modification (see Supplementary information).

2.3. Hydrogel formation.

Gels were formed by catalyst free click reactions between an azide group and an activated alkyne as cyclooctine group from both modified ELRs chains. Solutions of VKV-CO and IK-N3 were prepared in ultrapurified water at 100 mg/ml and kept at 4°C for at least 24 h. In PAP-1 loaded gels, the necessary quantity of 26 mM of PAP-1 in DMSO will be added to both solutions to get a final concentration of 1mM. 1) Covered grids (Square 50 Mesh Tabbed Grids Nickel, Agar Scientific AGG2905N) used as mimetic DES [19] were obtained by dip-coating

alternating the solutions of IK-N3 and VKV-CO, kept at 4°C in two separate containers, sequentially immersing the device first in the solution with IK-N3 and then in the VKV-CO solution [19]. This step was repeated 10 times with 5 min gaps between each layer to allow the cross-linking of the azide and the cyclooctine groups of adjacent layers, and macroscopically assessed with a Leica DMS 1000 digital microscope system (Figure 1 A, B). 2) Hydrogel beads were obtained mixing equal quantity of the ELR solutions (VKV-CO: 20 µL, IK-N3: 20 µL) at 4 °C, and immediately placed a quantity of the mixture (30 µL) on a flat surface at 37°C (Figure 1C). After 5 min at 37°C, the gel beads were completely formed.

2.4. In vitro PAP release studies.

Release experiments were performed using the sample-drawing procedure. The covered grids were placed in a 2 mL vial and 100 µL of tempered aqueous delivery medium was added and, at predetermined time intervals (1, 2, 7, 14, 21, 30, 60, 90 days), the solution was completely removed to measure PAP-1 concentration and new tempered solution was added. This study was conducted over 90 days. With the aim of determining the real amount of PAP-1 contained in the covered grids, two of them were placed in a 2 mL vial with 100 µL of DMSO and were subjected to the same procedure as the real aqueous release. In this case, the delivery time was extended to 121 days in order to ensure that all PAP-1 had been released.

The concentration of PAP-1 in the released medium was determined by Ultra Performance Liquid Chromatography (UPLC, Waters Acquity UPLC system and BEH C18 (1.7 µm, 2.1 x 50 mm) column with a Nova-Pak C18 (2.1 x 10 mm) cartridge) in the LTI Research Facilities. The mobile phase is Acetonitrile:H₂O with 0.1 % formic acid and 0.6 mL/min flow rate. The medium was evaporated in the Speed Vac (Thermo Scientific, Savant SpeedVac Kit OP230V), and 0.5 mL of methanol was added and sonicated for 10 min for the total dissolution of PAP-1. These experiments were performed in triplicate.

To explore the release kinetics of PAP-1 *in vitro*, release profiles were analysed using two mathematical models. The Lindner and Lippold equation explains the drug release through a polymeric matrix as a diffusion process. M_t is delivered drug at a given time (t), M_∞ is total drug load, so that M_t/M_∞ represents the fraction of solute released. The parameter 'b' represents the burst effect and k_1 is the Fickian diffusion constant.

$$\frac{M_t}{M_\infty} = k_1 \cdot t^n + b \quad (1)$$

The biexponential Peppas–Sahlin equation [25] determines the contribution to the fraction of drug delivered at time t (M_t/M_∞) of both the Fick diffusion process and relaxation of the polymer chains irrespective of the geometry of the release system.

$$\frac{M_t}{M_\infty} = k_1 \cdot t^n + k_2 \cdot t^{2n} \quad (2)$$

where n is the Fick diffusion exponent for a release system of any geometrical shape, k_1 is the Fickian diffusion constant and k_2 represents the polymer chain relaxation constant.

2.5. Physical properties gel characterization

Swelling ratio and % of porosity were quantified as previously described [17]. Hydrated gels kept in ultrapure deionized water at 37°C for 1 day, were dropped into liquid nitrogen, physically fractured and immersed in liquid nitrogen again before finally being freeze-dried.

The mechanical properties of the hydrogels were studied using rheological test in a strain-controlled AR-2000ex rheometer (TA Instruments) equipped with a Peltier plate temperature control. A stainless steel plate with a diameter of 12 mm and a gap of about 1000 microns

between the plates was used. Measurements were carried out at 37°C, with the sample temperature being controlled with the Peltier device.

The dynamic shear modulus of the hydrogel was characterized using previously a strain and frequency sweep to determine the linear region of viscoelasticity. Then, a stress relaxation test was carried out to evaluate the mechanical behavior of the hydrogels. These experiments were performed within the linear viscoelasticity region, where storage (G') and loss (G'') moduli are independent of the stress magnitude. A final time of 300 s was selected to determine long-term hydrogel behavior.

Rheological measurements provide G' and G'' values and complex viscosity magnitude as a function of strain or frequency at a fixed temperature in $n=3$ different hydrogels.

2.6. Human samples

Human mammary arteries (hMA) and human saphenous veins (hSV) from COLMAH (COlección de Muestras Arteriales Humanas) collection (https://www.redheracles.net/plataformas/en_coleccion-muestras-arteriales-humanas.html) were obtained from donors undergoing heart bypass surgery at the Clinic Hospital of Valladolid, with the written informed consent obtained from each participant. The sample collection and the study were approved by the Hospital Ethics according to the standards of the Helsinki Declaration. The vessels were collected in culture medium (DMEM/F-12 with HEPES Buffer) and used within 24 hours for organ culture or for VSMCs cultures

2.7. Primary VSMCs cultures

VSMCs were obtained from explants of hMA and hSV and maintained as previously described [26]. Once VSMCs reached confluence, they were trypsinized and seeded again at 30-50% confluence in SMC P-STIM medium (MEM with 10% FBS, 5 μ g/ml insulin, 1 ng/ml bFGF, 5 ng/ml EGF, 100 U/ml penicillin, 100 U/ml streptomycin, 0.25 μ g/ml fungizone and 2 mM L-glutamine). VSMCs in passages 3-9 were used for proliferation

2.8 Proliferation assays.

The percentage of cells at the S phase was quantified using EdU (5-ethynyl-2'-deoxyuridine) incorporation for 6h with a commercial kit (Click-iT® EdU Imaging Cell Proliferation Assay, Invitrogen) as described [11]. Proliferation was induced by addition of PDGF (20 ng/ml), alone or in the presence of PAP-1 (100nM) or ELR-containing-PAP- during 24h. 20.000 to 30.000 cells were seeded onto fibronectin coated 12 mm diameter cover slips placed at the bottom of a 4-well plate (15,6 mm diameter, Nunc®), and covered grids or hydrogel beads were placed in the periphery of the plate where they remain floating, so that no mechanical damage is induced to the attached cells. Triplicate samples were used and controls were included in all experiments. The percentage of EdU positive cells (EdU+) from the total cell number stained with Hoechst, was obtained from the average of 4 randomly selected images per coverslip, captured using the 4X objective in a Nikon Eclipse 90i microscope. ImageJ (Fiji) software was used to analyze the images in a blind manner.

2.9. Organ culture of human saphenous veins.

Sequential 5mm-wide rings of hSV were kept for 2 weeks at 37°C in 5% CO₂ humidified atmosphere as previously described [11]. Each ring was placed in a well of a 4-well plate. 20% Fetal Bovine Serum (FBS) was added to induce intimal hyperplasia (positive control), while FBS-free medium was used as negative control. Experimental conditions include the presence of the Kv1.3 blocker PAP-1 in the 20%FBS medium or in the different ELR formats (grids or beads) as indicated. Media were changed every 3 days.

2.10. In vivo model of lesion.

Carotid artery ligation was carried out in Blood Pressure High (BPH) mice (Jackson Laboratories) to induce intimal hyperplasia (IH). The disruption of blood flow in the common carotid artery by ligation near the bifurcation, leads to lumen decrease [27] due to the combination of two mechanisms: neointima formation in response to flow changes, and constriction of the vessel diameter, which initially represents a vasoactive response of the endothelium but at 2-4 weeks becomes a fixed change in the vessel wall structure. While this model does not resemble the situation of endovascular surgeries, as thrombus formation is not observed because the endothelium is not injured, it can recapitulate the remodeling observed in CABG, mainly consequence of hemodynamical changes.

Mice were anesthetized with 2% isoflurane using SomnoSuite® (Kentscientific). The right carotid artery was ligated distal to the carotid bifurcation. Carotid ligation was carried out in three groups of animals: 1) Control animals in which no other procedure was carried out (n=5); 2) Animals with systemic treatment, in which two days prior to carotid ligation, an osmotic minipump (model 2004, Alzet), containing either PAP-1 (50 µg/Kg/day) or vehicle (PEG-35 castor oil, 25% in H₂O, Kolliphor® EL, Sigma) was implanted subcutaneously in the dorsal region of the neck (n=6 per group); and 3) Animals with local treatment, with control-ELR or PAP-1-ELR gels formed “in situ” by catalyst free click reactions, applied around the ligation (n=6 per group). In this later group, we mix 15 µl of each ELR solutions (VKV-CO and IK-N3) at 4 °C, and immediately 20 µL of the mixture were placed over the ligation area, at 37°C, so that the hydrogel was formed in less than 2 min.

Ibuprofen (20 mg/100 ml in the drinking water) was administered for 3 days after surgery. After 28 days mice were euthanized in CO₂ chamber to collect right (ligated) and left (control) carotid arteries, that were fixated overnight in 4% formaldehyde. All procedures were approved by the Institutional Care and Use Committee of the University of Valladolid in accordance with the EU Directive 2010/63/EU for animal experiments. Both male and female BPH mice were used as detailed below.

2.11. Morphometric analysis.

Morphometric analysis was performed in 7-µm thick sections of paraffin-embedded hSV and mice carotid arteries stained with Masson trichrome, as described elsewhere [11] and analyzed blinded using computerized morphometry (ImageJ). The analysis of vascular remodeling in vivo was performed at 300 to 500 µm downstream the ligation site. Measures included luminal, intimal, media and vessel area. From these data, the wall area (intima+media area), the intima to media ratio (I/M ratio) and the percentage of stenosis were calculated to define the degree of vessel remodeling [28] (see supporting information for a detailed description).

2.12. Statistical analyses.

Data were plotted and analyzed using Origin and Excel software. Pooled data are expressed as mean ± SEM, and p-values < 0.05 were considered as significantly different. For comparisons between two groups with normal distribution, Student's t test, for paired or unpaired data as required, was used to determined p-values, otherwise Wilcoxon-Mann-Whitney test (for paired or unpaired data) was applied. For comparisons among several groups, one-way ANOVA followed by Tukey's test was employed in the case of normal distributions and equal variances; alternatively Kruskal-Wallis analysis followed by Dunn's test was used. Shapiro-Wilk test and Bartlett's test were used to test normality and homogeneity of variances respectively. R-studio and Excel were used for analyses.

3. RESULTS

3.1. Production, chemical modification and characterization of the ELRs.

Two different ELRs (VKV and IK) were chemically modified through their lysine residues to incorporate different reacting groups (IK bearing azides and VKV bearing cyclooctines), as described in section 2.2. The characterization of both modified ELRs was carried out by MALDI-ToF, NMR, FTIR and DSC (see supporting information).

VKV was chemically modified by amidation reaction of amine groups present in the side chain of lysine amino acids with an N-succinimidyl carbamate derivative carrying a cyclooctine group. Thirteen lysines of the 24 total ones were modified as determined by the increase in molecular weight recorded by MALDI-ToF technic (Figure S1) and corroborated by integral analysis from NMR signals (Figure S2). The number of modified lysines, equal to the number of cyclooctine groups introduced at the VKV-CO molecule, for 0.6 equivalents of reactant is in accord with a 92% conversion yield.

In a similar way, IK was chemically modified also by amidation reaction of lysine amine groups, this time using an N-succinimidyl carbamate derivative carrying an azide group. Twenty lysines of the 24 total ones were modified and an 83% conversion yield was obtained for one equivalent of reactant. The percentage of modification was determined as previously explained for VKV-CO (Figure S3 and S4) and, additionally, the presence of azide groups was corroborated by their characteristic FTIR band at 2100 cm^{-1} (Figure S5).

3.2. PAP-1 embedded hydrogels formulations.

In order to explore the combination of an active compound as PAP-1 with ELRs hydrogels as release devices, two different approaches were used: Nickel grids as DES prototyping model [17], coated using layer-by-layer strategy with PAP-1 embedded click hydrogel, and PAP-1 containing hydrogel beads for *in vitro* analysis and *in situ* injectable hydrogels prepared with the “copper-free click hydrogels” methodology [15].

The layer-by-layer strategy consists of alternatively dip-coating with IK-N3 and then VKV-CO, connected to each other by click reaction between azide and cyclooctine groups (Figure 1A). The resulting Click-ELR film uniformly filled the whole grid (Figure 1B). The presence of PAP-1 in ELRs solutions led to PAP-1 coated grids. PAP-1 amount was estimated by weight difference between uncovered and coated grid.

In situ injectable hydrogels were prepared from embedding of PAP-1 in both IK-N3 and VKV-CO solutions at low temperature (4°C). The injection of the mixture at the injured vessel *in vivo* lead to the formation of a stable well-integrated hydrogel as a store for PAP-1. Hydrogel beads were obtained following the same “copper-free click” hydrogels preparation methodology [17] (Figure 1C) by adaptation to small volumes of ELRs solution mixtures (Figure 1D), resulting in 5 mm diameter semi-spheres. The quantity of ELRs and PAP-1 employed was the same than in the coated grids, so that the results obtained with the two strategies can be compared.

3.3 Click hydrogels characterization.

Physical properties of PAP-1 embedded click hydrogels were characterized to prove their analogy with extracellular matrix since they are going to be injected to form 3D scaffolds for PAP-1 gradual delivery.

ELRs hydrogels at 100 mg/ml porosity value is found to be 88 % with a swelling ratio of 1.6 % at 37°C , similar to those for analogous click hydrogels previously used as injectable *in situ* cross-linkable scaffolds for regenerative medicine [29].

Rheological measurements in the linear viscoelastic range will provide information in conditions close to hydrogel stability [30–32]. Variation of the linear viscoelastic range was measured rheologically in hydrogels at a concentration of 100 mg/mL as those used in *in vivo* experiments. In order to ensure that measurements were carried out within the linear viscoelastic region, rheological tests were performed for 1% strain and 1 Hz of frequency being G' and G'' values represented in figure S6. The gel strength can be estimated from the magnitude of G' and the significant difference between G' and G'' ($G' \gg G''$), which means that the storage modulus is the major contributor. These rheological measurements were performed in triplicate. At 1 Hz, the value of G' and G'' were 220.3 ± 34.1 and 13.2 ± 5.6 Pa, respectively. The quotient between G' and G'' gives us the value of the loss tangent ($\tan \delta$) where δ represents the lag between the stress and the deformation as a measure of viscoelasticity behavior. In this case, a value of δ close to zero (0.06) and kept constant with time indicates that is an elastic material. The hydrogel can be classified as soft material displaying a high degree of elasticity.

3.4. Release and mathematical model.

An *in vitro* kinetic release study was performed to explore the delivery of PAP-1 from grid coatings. Figure 2A depicts the time course of PAP-1 release from the grid up to 90 days. An initial quantity of 1262 nM was released during the first 7 days, which represents 0.5 % of total cargo (determined by parallel PAP-1 release assays using DMSO as solvent, see figure S7). From 7 to 30 days, the release rate decreased, with around 190 nM delivered each seven days. Finally, between 30 and 90 days, a third stage with an even lower release rate (< 50 nM each 7 days) can be observed.

This experimental profile was fitted with the models described in section 2.4. The release profile was divided in three periods (0-7, 7-30 and 30-90 days). As shown in table I, Peppas-Sahlin equation provided the best fit to the data, which points to a preferred diffusion mechanism for PAP-1 release. The fact that $k_1 \gg k_2$ indicates that the release is little influenced by the relaxation of the polymer chains, while the b coefficient in the Lindner-Lippold equation close to zero suggests that PAP-1 quantity at the surface of hydrogel is insignificant.

The power law release exponent (n) describes the release mechanism. The first period release (0-7 day) would be governed by anomalous or non-Fickian diffusion ($0.5 < n < 1$), because of the controlled relaxation of the polymeric chains. In the second and third periods (7-30 and 30-90 days, respectively), the $n < 0.45$, represents a pseudo-Fickian diffusion mechanism, which indicates that the release rate depends on the diffusion of PAP-1 through the swollen porous polymer matrix. The release of PAP-1 molecules located in the inner layers of the matrix is slow because they have to pass through each of the pores of the matrix. Also, the water absorption mechanism of the hydrogel exerts an opposite force to PAP-1 delivery, contributing to the delay in PAP-1 release (Figure 2B).

3.5. Effect of PAP-1 released from ELR hydrogels in cultured VSMCs.

We next explored the effect of PAP-1 released from ELR hydrogel beads or covered grids on the proliferation rate of cultured VSMCs. Lyophilized hydrogel beads or covered grids (both with control ELRs or with ELR containing PAP-1) were placed in the wells with cultured VSMCs. PDGF-induced proliferation was measured after 24h treatment and compared with proliferation rate in the absence of ELR (Ctrl) or in the presence of 100 nM PAP-1 in the media. The results of these experiments (Figure 3A,B) show that PAP-1 released from either ELR-covered grids or beads was able to inhibit VSMC proliferation to the same extent than 100 nM PAP-1, while in the absence of PAP-1 both devices had no effect on PDGF-induced proliferation. Since 100 nM PAP-1 is a saturating concentration for the inhibition of VSMC

proliferation, these experiments demonstrate that ELR hydrogels release enough PAP-1 to get at least such concentration. We also explore the time course of PAP-1 release from these devices. ELR-PAP covered grids were kept in culture media for different time lengths (from 1 to 12 weeks) before they were used for a 24h treatment of VSMCs. We did not see differences in the blocking effect of the covered grids that had been previously releasing PAP-1 during up to 12 weeks (Figure 3C), indicating that suitable levels of PAP-1 release are maintained for long periods of time.

3.6. Effect of ELR-PAP hydrogel beads on human vessel remodeling.

As PAP-1 released devices are intended for the prevention of IH in human vessels, we next explored their ability to inhibit vessel remodeling in organ culture. 20% FBS incubation for two weeks has been found to induce IH development of human vessels rings in ex-vivo organ culture [11]. This remodeling was characterized by an increased thickness of intimal and media layers together with a decrease of the lumen area and was significantly prevented when 100nM PAP-1 was present in the culture media (see control sections in figure 4). Here we explore in parallel, in sections of the same human saphenous veins (hSV), the effects of incubation for two weeks with control ELR or ELR-PAP hydrogel beads. While control ELR hydrogel beads did not affect IH, ELR-PAP beads had the same effect that direct PAP-1 application to the 20%FBS media (Figure 4): an inhibition of vessel remodeling with a reduced neointima formation, reflected by significant decreases of the % stenosis, the % of intima area and the I/M ratio (figure 4, right plots). Similar results were obtained with ELR covered grids (Figure S9).

3.7. Effect of ELR-PAP hydrogel in an in vivo model of vascular injury.

Finally, we studied the effects of ELR-PAP hydrogels when applied at the injured vessel in an animal model of vascular lesion. Carotid ligation was used to induce IH in mice. In this model the ligated vessel is still subject to arterial blood pressure, and the hemodynamic changes associated to the loss of laminar flow activate formation of neointima [27]. We first checked that carotid ligation produced a consistent and robust IH lesion in all the animals (left panels in figure 5A). The perivascular application of ELR hydrogel (middle panels) around the ligated artery did not seem to modify vessel remodeling, while ligated arteries treated with ELR-PAP hydrogels (right panels) showed a clear reduction in the development of neointima after 4 weeks. In all cases, morphometric analysis showed that carotid ligation induced inward remodeling, with marked increases of the wall thickness/wall area and a concomitant decrease of the lumen area, so we used these descriptors to define remodeling upon ligation. Figure 5B shows the average data obtained when calculating the lumen area (in % of the vessel area) and the wall thickness in all the conditions depicted in figure 5A. Pairwise comparisons between control and ligated arteries were performed in each group of animals. The decrease of lumen area in the ligated CA is more pronounced in control conditions or in arteries treated with ELR alone than in the presence of ELR-PAP hydrogels. More clear-cut effects of PAP-1 treatment were observed when determining wall thickness, as no significant change was observed between control and ligated CA in this case (right panel in figure 5B).

To investigate possible sex-dependent differences in vessel remodeling, both male and female mice were subjected to carotid ligation. Wall area in ligated CA was $33929 \pm 1538 \mu\text{m}^2$ in males versus $31148 \pm 3096 \mu\text{m}^2$ in females, $p=0.46$, and the lumen decrease was 43.7 ± 5.03 % in males and 39.12 ± 3.04 % in females, $p=0.43$. As no significant sex-related differences were observed data from both male and female were pooled together in subsequent analysis.

We also compared the effects of these hydrogels with systemic application of PAP-1 using minipumps, to release the drug over the 4-week period (Figure 5C). In both cases PAP-1 significantly reduced remodeling, by inhibiting the increase in wall area and preventing lumen reduction, indicating that also *in vivo* PAP-1 release from the ELR hydrogel provides therapeutic levels of PAP-1. No side effects of hydrogel application (with or without PAP) were observed, pointing to a good biocompatibility of these compounds as previously suggested *in vitro* [17,33]. Mice recovered from surgery at the same rate than the control animals (with no ELR application), and their weight gain after surgery was not different in both groups (Figure 6A). In two additional mice, sections of the whole neck were obtained to explore ELR location and look for signs of inflammation, necrosis or any other adverse reaction. Figure 6B illustrates the unreactive nature of ELRs, which did not show any apparent harmful effect.

4. DISCUSSION

Cardiovascular disease is the first cause of death in developed countries. Yet, the burden of these diseases is not confined to mortality, as the increasing levels of hospitalization and surgical intervention represent a high economic and social cost [1]. In the case of patients with symptomatic CAD, implantation of DES releasing anti-proliferative agents had improved the outcomes of these interventions. However, DES had substantial drawbacks, including delayed healing, local hypersensitivity reactions and neoatherosclerosis, which all led to newer-generation DES with different scaffold designs, different polymers and new antiproliferative drugs. This is a very active field of research, and more than 30 different DES are commercially available in Europe and USA [34]. The effective restoration and maintenance of coronary vessel patency is a major problem requiring evaluation. Principal reasons for the disappointingly slow clinical implementation of these therapies are an incomplete understanding of the vascular biology of restenosis, the difficulty of translating findings in animal models into the human setting and the technical difficulties to achieve efficient drug delivery at the lesion place of the vessels.

Here we demonstrate that the combined use of a new therapeutical target (Kv1.3 channels) and a new scaffold as the delivery system (ELRs hydrogels) could represent a promising approach for the prevention of restenosis in human vessels. Our data provide evidence that different formats of these ELR-PAP devices allowed a continuous release of PAP-1 at therapeutical levels with an appropriate time course. The small amounts of PAP-1 released make difficult to obtain a reliable determination of the free drug concentration in our experimental settings. However, our functional readout indicates that both the dose and the time course of release are adequate for required tasks (i.e., inhibition of proliferation of human VSMCs in culture, prevention of IH of hSV in organ culture or the inhibition of remodeling in an *in vivo* model of lesion). Determination of free circulating PAP-1 levels along time could be pursued, but they still would not provide a better evidence than morphometric analysis, which reflect that therapeutical levels are obtained locally, at the vessel wall and in the place of injury. In fact, it has been reported that in most durable-polymer DES, the drug might continue to exist beyond the reported release profiles in levels below currently detectable limits within the polymer matrix, and these low levels of drug can have biological consequences related to vascular healing and IH inhibition [34]. Nevertheless, we can make an estimate based on the effects of mini-pump administered PAP-1, which were not different from those with ELR-PAP1. Minipumps released PAP-1 at a rate of 35 ng/h. Considering a blood volume of 0,08 ml/gr, we can get an upper level estimation of PAP-1 concentration of $81 \pm 2,68$ nM, which is in the range of the concentrations used in the *in vitro* experiments.

The choice of these three different biological settings tries to uncover several relevant aspects aimed to provide improved anti-restenosis therapies in the clinics. 1) The *in vitro* systems demonstrates the ability of ELR hydrogels to provide therapeutical levels of PAP-1 on human VSMCs during prolonged times without any apparent side effect; 2) the *in vivo* model shows that PAP-1 released from the ELR hydrogels blocks IH and restenosis in the absence of toxic side effect of the ELR or the PAP-1 released; and 3) the organ culture confirms the results of the animal model in human vessels, which is not a minor point (see below).

4.1. Advantages of PAP-1 as an anti-restenosis drug.

Despite our best efforts, restenosis and in-stent stenosis are still prevalent. Numerous anti-proliferative drugs, many of which are highly specific to their targets and show clear effects in vitro, end up failing to inhibit restenosis in the clinics. One very likely reason for this failure is that restenosis is the consequence of several overlapping biological process with different time courses and different relevance (depending on species and vascular bed). In human arteries, in addition to IH other processes such as inward remodeling of the vessel wall, elastic recoil, inflammatory reactions and thrombus formation and reorganization also contribute to restenosis [35]. Even if neointimal formation were the major cause of restenosis, the role of VSMC proliferation remains controversial. Upon PM, VSMCs undergo proliferation, migration or both, followed by increased ECM synthesis resulting in the IH lesion.

According to this, an ideal anti-restenosis drug candidate should be not only an antiproliferative drug but also a drug that inhibits secretory and migratory properties of dedifferentiated VSMCs. This has been confirmed for Kv1.3 channel blockers such as PAP-1 in different preparations including human vessels ([8,11]. In addition, there is good evidence that Kv1.3 blockers can target other biological processes involved in restenosis, showing anti-inflammatory, anti-thrombotic and immunomodulatory effects [36–38], thus reducing fibrosis, platelet aggregation and inflammation in the lesion. However, these aspects have not been directly addressed in our *in vivo* model. In particular, it will be relevant to explore if PAP-1 treatment does not delay re-endothelization after lesion, thus preventing the in-stent thrombosis associated to the current DES [3], a study will require other models of lesion, such as the mechanically-induced endothelial denudation model [8]. An additional advantage of PAP-1 over the most common drugs used in DES in the clinics (namely mTOR inhibitors and paclitaxel and derivatives, [35]), is its low systemic toxicity, which has been previously characterized [39] and extensively reported in different animal models [40–44] and also proven here with the use of minipumps.

4.2. Advantages of perivascular application and need of sustained release.

The absence of evident toxic side effects of PAP-1 even when administered systemically provides an increased level of safety. However, local drug application combines increased efficacy with low toxicity and is always the first choice for antirestenotic treatments after vascular surgery. In the case of CABG using vein grafts, additional advantages of local (and particularly perivascular) over systemic drug release approaches are clear [7]. Mechanical support and even constriction of the vessel to increase shear stress favors the arterialization of vein grafts, improving their patency [45], as external scaffolds prevented vein dilatation, decreased the development of IH and preserved the architecture of the media layer. This can be the case here, as perivascular application of ELR hydrogel significantly reduced the increased wall thickness observed upon ligation of control untreated arteries (see figures 5B and S10). The change in wall thickness between control and ligated CA was 199 ± 16 % in control mice and 148 ± 4 % in ELR treated vessels ($p < 0.05$). Drug-free devices have proven their efficacy in controlling focal vascular pathologies, although their efficiency is improved

when active compounds are added [46–48]. In our experiments, wall thickness in ELR-PAP treated vessels was $116 \pm 4\%$ of control artery ($p < 0.001$ versus ELR or control, Figure S10).

In the case of venous grafts, the increased pressure of the arterial territory is what drives graft arterialization, so it is unlikely that the prevention of PM (reducing VSMCs migration to the intima) would lead to inferior mechanical properties of the graft. In agreement with this, our data in hSV (figure 4) show that the lumen reduction with PAP-1 is mainly due to the decreased of the intima area, with small changes in the media layer. Nevertheless, further *in vivo* studies using venous grafts will be needed to confirm these results.

Both the active compound and its delivery at the right place and with the right kinetics are critical. The time course of the drug released needs to match the development of the pathology. In the case of hSVs, a sustained release over 2-4 weeks is necessary. The initial inflammatory reaction within the first 24h leads to PM of VSMCs, and VSMCs proliferation and migrations leads to IH lesion after 2-4 weeks, which then progresses at a slower rate during the following weeks or months [6].

While the *in-vivo* approach used here could be applicable to perivascular coating of vein grafts, an alternative formulation based on our model of ELR-covered grids could be developed for their use as a new DES. The comparison between the present approach and current DES characteristics is difficult due to their large diversity. The most commonly utilized stent coating techniques, using spray coating to optimize the release profile, show a logarithmic release curve, with an initial burst release (due to the release of the drug present at the boundary between the stent and the vessel) followed by a slower release of drug to enable long-term therapeutic effects [49]. This behavior is not very different from the release profile observed from the ELR hydrogels (see figure 2A). In addition, the click technology allows ELRs chains crosslinking at physiological conditions with no byproducts, creating biocompatible depots for perivascular administration. The use of PAP-1 among other selective Kv1.3 blockers in the ELR hydrogel have some additional advantages, as PAP-1 is a lipophilic molecule with good tissue retention. Despite the low release rate, the presence of PAP-1 occurs at effective doses for more than 12 weeks (Figure 3C).

4.3. Conclusions

We conclude that PAP-1 is not just another agent that inhibits VSMC proliferation *in vitro* but it is also effective inhibiting IH and restenosis in the ligated carotid artery. This *in vivo* model can help to define the coordinated biological processes involved in restenosis and in the vessel response to drug therapy. Still, there are many reasons why success in animal studies may not convert to success in humans. Both the injury and the response to injury may be very different in diseased versus normal vessels and in small muscular coronary arteries versus large elastic peripheral arteries [50]. Also, while in rodent vessels there is a high contribution of VSMC proliferation and IH to restenosis, in human restenotic lesions constrictive inward remodeling is a major determinant of vessel lumen size, reflecting important qualitatively and quantitatively differences in the restenosis mechanisms [50,51]. For all these reasons, we studied the remodeling hSV rings from patients undergoing CABG. Although the organ culture lacks important mechanical and chemical regulatory mechanisms of the whole vessel *in situ*, it offers the advantage to study the IH development and its response to treatment in the same vessels of the same patients undergoing CABG surgery for CAD. Altogether, our results indicate that their use in combination with PAP-1 as the active released anti-restenotic agent offers important advantages for the treatment of unwanted remodeling after vascular interventions. We studied here the effects of perivascular application, but as ELRs have been already proposed as well-suited scaffolds for coating stents, future studies will be needed to define

whether this combination of polymer and drug for a new DES provides superior results compared with the current therapies, using mainly everolimus as active principle. In particular, it will be interesting to see if endovascular PAP-1 application will favor endothelial healing, thus minimizing the late in-stent thrombosis associated with current treatments.

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AUTHOR CONTRIBUTIONS (CRediT roles)

Conceptualization, MS, JRLL, MTPG and FJA; Methodology: PC, MTPG and FJA, Formal Analysis: SME, SS, PC, MS and MTPG; Investigation: SME, SS, MAM, PC, JRLL. MS, MTPG and FJA; Writing – Review & Editing: SME, SS, JRLL, MS, MTPG and FJA; Visualization: SME, SS, MS, MTPG; Supervision: PC, JRLL, MS, MTPG and FJA; Funding Acquisition: JRLL, MTPG and FJA

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TABLES

	Peppas-Sahlin				Lindner-Lippold			
Periods	k_1	k_2	n	R^2	k_1	b	n	R^2
I (0-7 day)	31,594	-5,138	0,643	0,999	28,473	-0,077	0,272	0,992
II (7-30 day)	21,990	5,997	0,220	0,995	27,553	0,151	0,286	0,994
III (30-90 day)	29,445	-2,060	0,335	0,996	30,063	-0,597	0,253	0,993

Table I.- Fitting of PAP-1 release profile from covered grids to the Peppas-Sahlin and Lindner-Lippold equations (see text for details).

FIGURE LEGENDS

Figure 1. ELR-hydrogels formulation and characterization. **A.** Procedure for the fabrication of the click-ELR covered grid, showing the scheme of the Layer-by-Layer methodology. **B.** Microphotograph of an uncovered and covered grid. Scale bars: 0.4 mm. **C.** The scheme depicts the injectable click-ELR hydrogel beads, representing the “copper-free click” hydrogels preparation methodology. **D.** Microphotograph of a hydrogel bead obtained with this method. Scale bar: 1 mm.

Figure 2. In vitro release of PAP-1 from ELR-hydrogel **A.** Time course of the PAP-1 released from covered grids. Grids were placed for up to 90 days at 37°C and 60 rpm in 100 µL distilled H₂O, and cumulative PAP-1 release (in nM) was determined by UPLC at the indicated points. Each data point is the mean ± SEM of 3 independent determinations. The line shows the fit by eye of the data. To analyze the kinetics of the release, the profile was divided in three periods (P-I to P-III) as indicated by the colored areas. **B.** Diagram of the PAP-1 delivery kinetics showing the first period of anomalous or non-Fickian diffusion, followed by a second period of

lower pseudo-Fickian diffusion mechanism which depends on the diffusion of PAP-1 through the swollen porous polymer matrix. The third period shows an even lower pseudo-Fickian diffusion as PAP-1 remains in the innermost layers and it takes longer to cross the hydrogel layer that separates it from the outside (see text for more details).

Figure 3. Effect of PAP-1 released from ELR hydrogels in cultured VSMCs **A.** Proliferation of human saphenous vein (hSV) vascular smooth muscle cells (VSMCs) in primary culture was determined by EdU (5-ethynyl-2'-deoxyuridine) incorporation, after a 24-h treatment in serum-free conditions (0% FBS) or in the presence of 20 ng/mL PDGF in the indicated experimental conditions: PDGF alone (Ctrl), plus 100 nM PAP-1 (PAP), or in the presence of grids coated with ELR alone or ELR-PAP-1 (Grid-Ctr and Grid-PAP respectively). Each bar represents the mean $\pm$ SEM, and the number of samples (n) is also shown in the bar. This n was obtained from at least 6 independent experiments. Positive and negative controls (0% FBS and PDGF 20ng/mL) were included in all experiments, and data was normalized to the proliferation rate observed in control (20 ng/mL PDGF) conditions. ***P<0.001 with respect to PDGF alone; ### P<0.001 with respect to Control grid. The right panels show representative images of Hoechst- labelled nuclei (blue) and EdU+ cells (green) from the same fields in the indicated experimental conditions. Scale bar 60 μ m. **B.** The same experimental design as in A was carried out to explore the effect of lyophilized hydrogel beads made of ELR alone (Bead-Ctr) or containing PAP-1 (ELR-PAP). Mean $\pm$ SEM of the indicated data points obtained from at least 4 different cell cultures. ***, ### p<0.001 with respect to Ctr (PDGF alone) or Bead-Ctr respectively. **C.** The inhibitory effect on PDGF-induced proliferation of PAP-1 released from ELR-covered grids was explored in another set of similar experiments (EdU incorporation experiments in VSMC cultures treated for 24h with these grids) but in this case the covered grids were kept for the indicated time in culture media before their application to the VSMCs. No statistically significant differences were observed among the different points.

Figure 4. Effect of ELR-PAP hydrogel beads on human vessel remodeling. **A.,** Representative microphotographs of human saphenous vein (hSV) kept in organ culture for 2 weeks in serum-free conditions (0% FBS), or in the presence of 20% FBS alone or combined with PAP-1 (100 nmol/L) in control conditions or in the presence of lyophilized control ELR or ELR-PAP beads as indicated. The yellow line marks the internal elastic lamina which defines the intima/media border. Images were taken with a 4 $\times$ objective (NA=0.13), scale bars 500 μ m. **B.** Remodeling was characterized using several measures as indicated in the plots. The upper plot shows the percentage of the vessel area occupied by the intima layer (%intima); the middle graph shows the degree of lumen stenosis calculated as % stenosis = 100 $\cdot$ (Intima area–Lumen area)/Intima area, and the lower plot shows the intima to media ratio (I/M ratio, Intima area/Media area). Each data bar is mean $\pm$ SEM of 6-10 different vessels. Positive and negative controls (0% and 20% FBS) were included in all experiments, and vessel rings used for the different conditions were obtained from consecutive vessel segments of similar diameter. * p<0.05; ** p<0.01; *** p<0.001 with respect to 20% FBS. # p<0.05; ## p<0.01; ### p<0.001 with respect to 20%FBS+bead control.

Figure 5. Effect of ELR-PAP hydrogel in an in vivo model of vascular injury. **A.** Representative microphotographs of carotid arteries stained with Masson's trichrome. Paired arteries of the same animal, left unligated carotid arteries (CA) and right ligated CA are shown. The ligated CA was left untreated or treated with ELR or ELR-PAP hydrogel as indicated (20 $\times$ objective. scale bars 50 μ m). Red blood cells can be observed in the lumen of the some arteries (more clearly in the control ligated CA). **B.** The bar graphs plot the differences in lumen area and wall thickness between control, unligated (grey bars) and ligated arteries (red bars). Mean $\pm$ SEM of

5-6 animals in each group; * $p<0.05$; ** $p<0.01$; *** $p<0.001$ for paired data. **C.** Bar plots representing % lumen decrease (upper graph) and wall area (lower one) of ligated carotid arteries. Both values were used to compare remodeling of ligated arteries between control, untreated animals (white bars) and PAP-1 treated animals (green bars) in two independent set of experiments. In the minipump experiments, the control animals were infused with vehicle, and in the ELR experiments, control animals have perivascular application of ELR-hydrogel. Analysis was performed averaging the data from 3-6 different cross section per vessel. Mean $\pm$ SEM; n=5-7 animals per group. * $p<0.05$; ** $p<0.01$ compared to their own controls.

Figure 6. Absence of harmful side effects of ELR-hydrogels in vivo. **A.** Plot of the weight gain observed during 4 weeks (from surgery to end-point procedure) in untreated animals or animals treated with perivascular ELR hydrogels. Data are mean $\pm$ SEM of 7-10 animals per group, no significant differences were observed. **B.** Cross section of the neck of a mice obtained 4 weeks after perivascular application of ELR hydrogel around the right CA. Section was stained with Hematoxylin-eosin. The dashed line shows the area occupied by the ELR-hydrogel. Scale bar 1 mm).

Figure 1
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Figure 2
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Figure 3

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