Supplementary Information

Bone regeneration mediated by a bioactive and biodegradable ECM-like hydrogel based on elastin-like recombinamers

Dante J. Coletta, MD,[1#] Arturo Ibáñez-Fonseca, MSc,[2#] Liliana R. Missana, PhD,[3,4#] María V. Jammal, PhD,[3,4] Ezequiel J. Vitelli, MD,[1] Mariangeles Aimone, BSc,[1] Facundo Zabalza, BSc,[1] João P. Mardegan Issa, PhD,[5] Matilde Alonso, PhD,[2] José Carlos Rodríguez-Cabello, PhD,[2] and Sara Feldman, PhD,[1]

[#] These authors contributed equally to this work.

[1] LABOATEM. Osteoarticular Biology, Tissue Engineering and Emerging Therapies Laboratory, School of Medicine, National Rosario University, Rosario, Argentina.

[2] BIOFORGE Lab, University of Valladolid, CIBER-BBN, Valladolid, Spain.

[3] Experimental Pathology & Tissue Engineering Laboratory, Dental School, National Tucumán University, Tucumán, Argentina.

[4] Tissues Laboratory, Proimi-Biotechnology-Conicet, Tucumán, Argentina.

[5] Ribeirão Preto School of Dentistry, University of São Paulo, São Paulo, Brazil.

Supplementary Methods

ELR biosynthesis and characterization

The block co-polymer that constitutes the ELR backbone was designed to comprise a hydrophobic block (containing isoleucine as the guest residue), known as I_{60}, which is able to establish physical interactions above the T_t, i.e. at physiological temperature. Moreover, it includes a hydrophilic block (containing glutamic acid with a carboxylic group), termed E_{50}, which remains hydrated at physiological temperature. Both the ELRs designed and used in this work include an elastase cleavage sequence for biodegradation, as described above. One of them was genetically engineered to include a RGD domain for cell attachment, while the other one included the BMP-2 domain to enhance osteogenesis, thus giving ELR-Elastase-RGD (ELR-E-RGD) and ELR-Elastase-BMP-2, respectively (Fig. S1, Supplementary Information).

The mechanical properties of the hydrogels formed by the ELRs above the T_t were assessed by rheological testing in a controlled stress rheometer (AR2000ex, TA Instruments) equipped with a Peltier plate for control of the temperature and a 12 mm plate diameter for the shear stress. To that end, a mixture of both ELRs (98% (w/w) ELR-E-RGD and 2% (w/w) ELR-E-BMP-2, see below) was dissolved in PBS at 300 mg/mL and 4 ºC for 24 h. Hydrogels were then formed *in situ* by depositing 200 μL of the solution onto the cold Peltier plate and increasing the temperature to 37 ºC. Time sweep experiments were conducted at a constant strain of 0.5% and a frequency of 1 Hz.

Elastase-mediated cleavage of the ELR in solution

SDS-PAGE was performed to evaluate the biodegradation of the samples incubated with elastase, comparing those taken at different time points with the original sample at the

beginning of the experiment. Gels were prepared at a polyacrylamide (Acryl/Bis™, Amresco, USA) concentration of 10%. The protein molecular weight marker used was Pierce Unstained Protein MW Marker (Thermo Fisher, USA). Images were obtained using the Gel Logic 100 Imaging System (Eastman Kodak, USA) and analyzed using Kodak 1D Image Analysis software (Eastman Kodak, USA).

In vivo *experiments*

Pre-surgical preparation

Antibiotic prophylaxis was carried out prior to the surgical procedure by applying cefazolin at a dose of 50 mg/kg/day, administered intramuscularly.

The anesthetic treatment was performed by combining three drugs, which were administered intramuscularly, namely Ketamine Hydrochloride at 35 mg/kg/day, Xylazine Hydrochloride (2.0%) at 18 mg/kg, and Acepromazine Maleate (1.0%) at 1 mg/kg, achieving complete relaxation of the animal. This anesthetic effect lasted for 45-60 minutes.

Surgical procedure

The area was shaved with an electric shaver, brushed with a 10% povidone-iodine solution, and immediately covered with fenestrated drapes. The intervention began with a longitudinal cutaneous incision of 4 cm in the internal lateral distal metaphysis of the femur, immediately above the medial condyle.

Both the medial and lateral flaps were separated, and a non-muscular aponeurotic plane was opened until the desired bone area had been reached through the divulsion in said tissues. The central point of the perforation was marked with a bradawl, and the first hole

was cut immediately, using a drill with a 3 mm diameter bit attached to a sterile electric motor. Milling was subsequently continued using a 6 mm diameter bit to obtain the desired bone defect of no more than 3 mm in depth. The bone defect was washed with a sterile physiological solution to eliminate detritus, and hemostasis of the lesion was performed using a sterile swab plus gauze. The area was then dried with a sterile gauze and filled with the chilled ELR solution to form a hydrogel *in situ*. To this end, 100 μL of the solution was placed into the hole created in the bone using an automatic pipette and sterile tips, which were stored at 4 ºC to avoid gelification of the ELR before and during implantation. The aponeurotic plane was first sutured using resorbable material type 3/0, then the skin was sutured with 3/0 Nylon, and disinfected with povidone-iodine.

Post-surgical clinical studies

During the study period, the overall status, mobility and food intake of the animals were monitored daily. Body temperature was measured daily during the first week, and then weekly thereafter. Biochemical parameters (complete blood count) were evaluated using standard procedures at days 0, 2, 30 and at the end of the study. Total serum proteins, albumin, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were evaluated using standard commercial kits (Wiener lab Group, Argentina) (data not shown).

Supplementary Figures and Tables

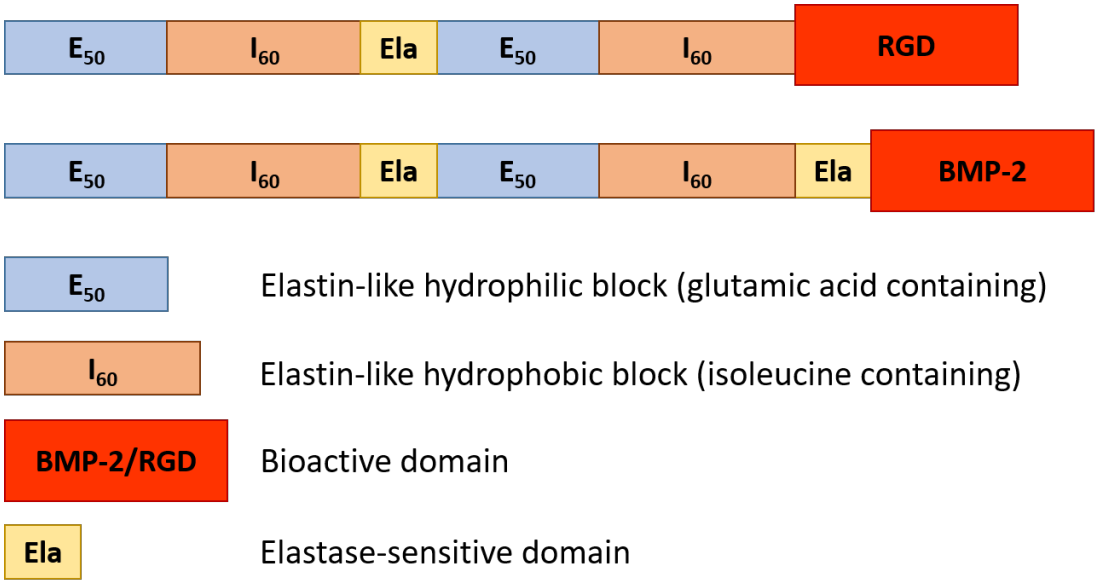

FIG. S1. Original scheme of the design of the ELRs engineered for this work, ELR-E-RGD and ELR-E-BMP-2, respectively.

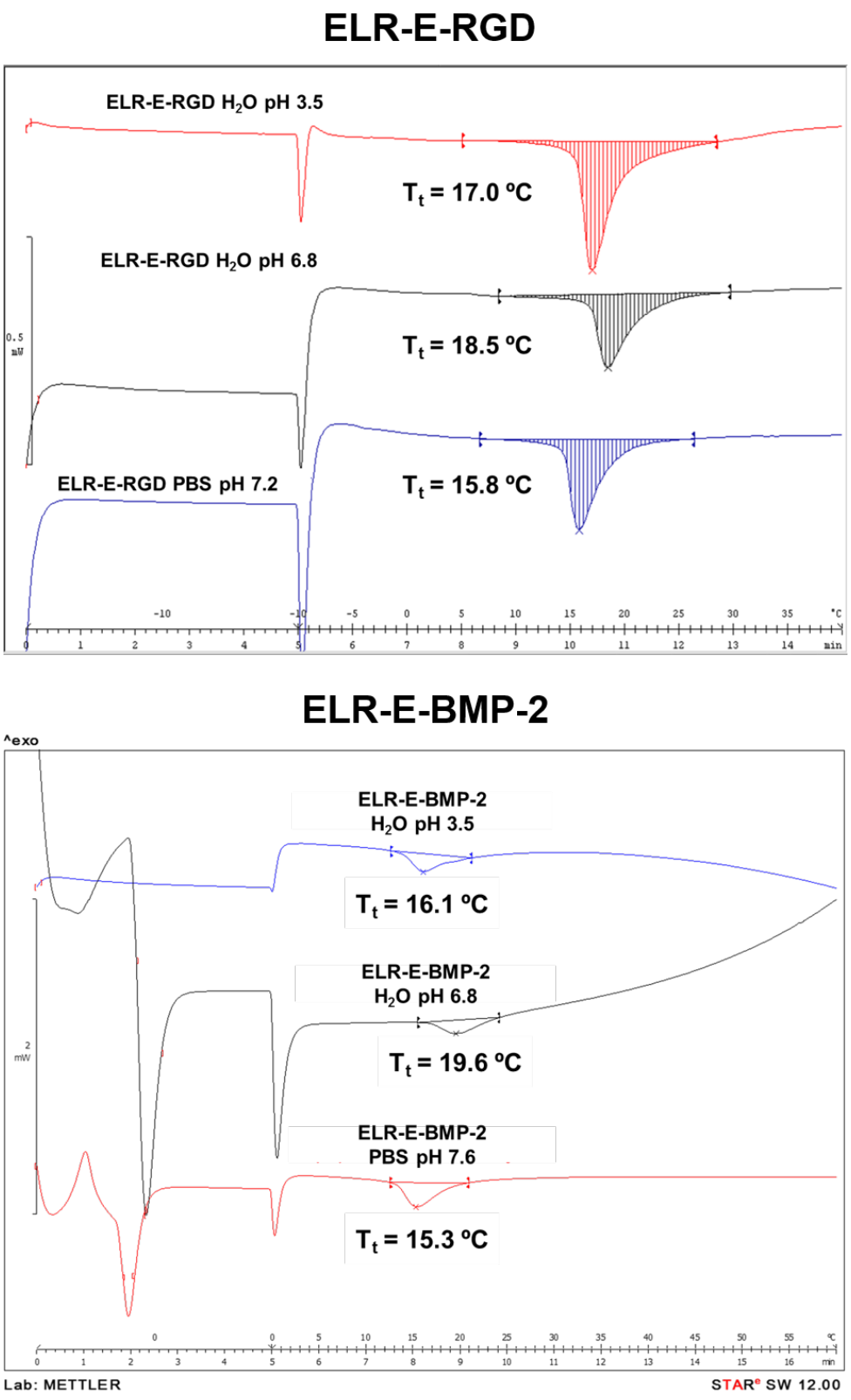

FIG. S2. DSC graphs of ELR-E-RGD (top) and ELR-E-BMP-2 (bottom) showing the T_t of both ELRs in different solvents (water and PBS) and at different pH values.

TABLE S1. Comparison between the predicted number of each amino acid in ELR-E-RGD and the experimental values.

Amino acid	Predicted	Experimental
ASP+ASN	2+0	2.47
GLU+GLN	21	25.54
SER	7	5.34
HIS	-	-
GLY	530	533.99
THR	2	2.30
ARG	2	1.13
ALA	7	6.01
TYR	-	-
CYS	-	-
VAL	350	344.22
MET	1	0.66
TRP	-	-
PHE	-	-
ILE	160	158.98
LEU	2	2.32
LYS	-	-
PRO	266	267.47

TABLE S2. Comparison between the predicted number of each amino acid in ELR-E-BMP-2 and the experimental values.

Amino acid	Predicted	Experimental
ASP+ASN	6+7	10.30
GLU+GLN	26+2	34.82
SER	9	6.54
HIS	5	3.38
GLY	457	463.15
THR	3	3.01
ARG	2	5.79
ALA	12	14.94
TYR	5	8.43
CYS	7	7.32
VAL	325	313.03
MET	3	2.31
TRP	2	-
PHE	3	2.92
ILE	124	121.54
LEU	10	10.29
LYS	6	3.74
PRO	234	233.16

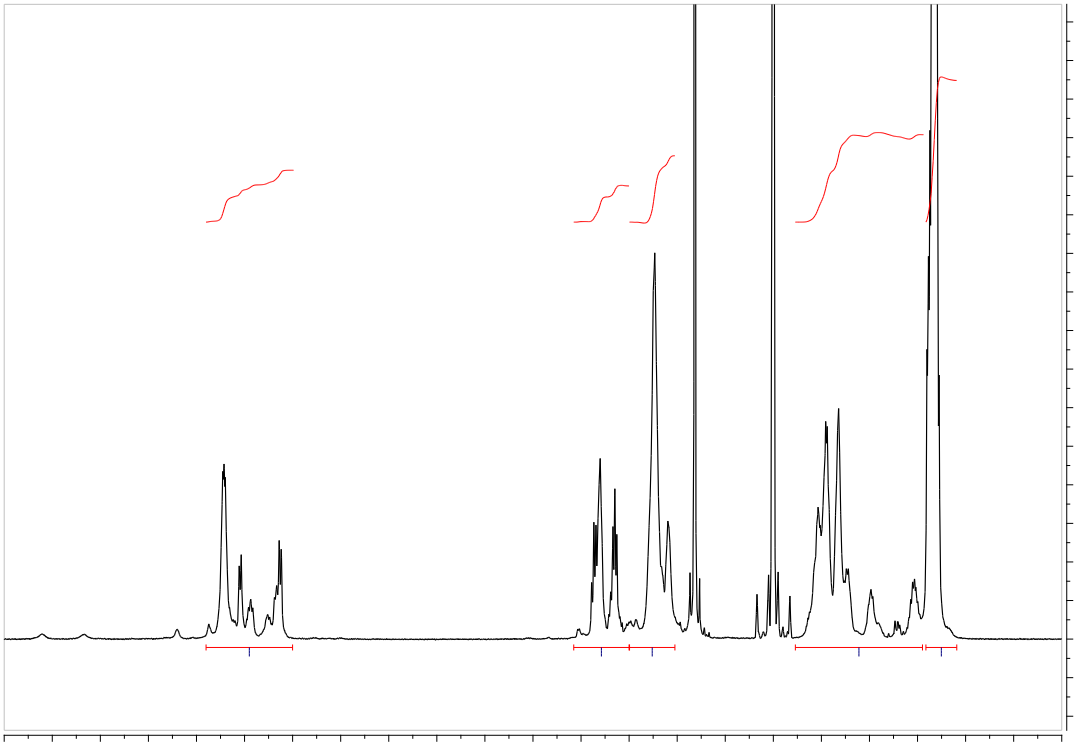

FIG. S3. H-NMR spectrum of ELR-E-RGD showing the integration of the peaks corresponding to the different types of hydrogens.

TABLE S3. Comparison between the predicted value of each type of hydrogen in ELR-E-RGD and the experimental values found by integration of each peak in the corresponding H-NMR spectrum.

Type of hydrogen	Predicted value	Measured value
-C<u>H</u>$_3$	1030	Reference
-C<u>H</u>- and -C<u>H</u>$_2$-	1475	1381.2
-N<u>H</u>$_2$	363	377.1

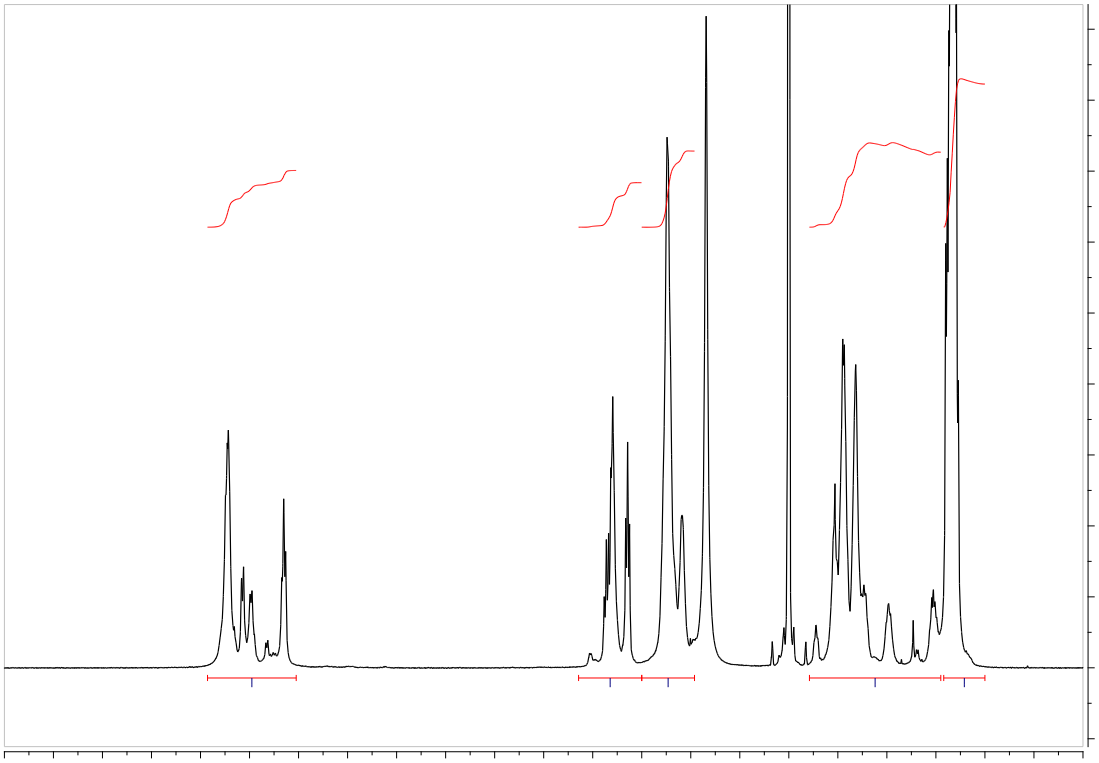

FIG. S4. H-NMR spectrum of ELR-E-BMP-2 showing the integration of the peaks corresponding to the different types of hydrogens.

TABLE S4. Comparison between the predicted value of each type of hydrogen in ELR-E-BMP-2 and the experimental values found by integration of each peak in the corresponding H-NMR spectrum.

Type of hydrogen	Predicted value	Measured value
-C<u>H</u>$_3$	936	Reference
-C<u>H</u>- and -C<u>H</u>$_2$-	1370	1279.1
-N<u>H</u>$_2$	355	370.9

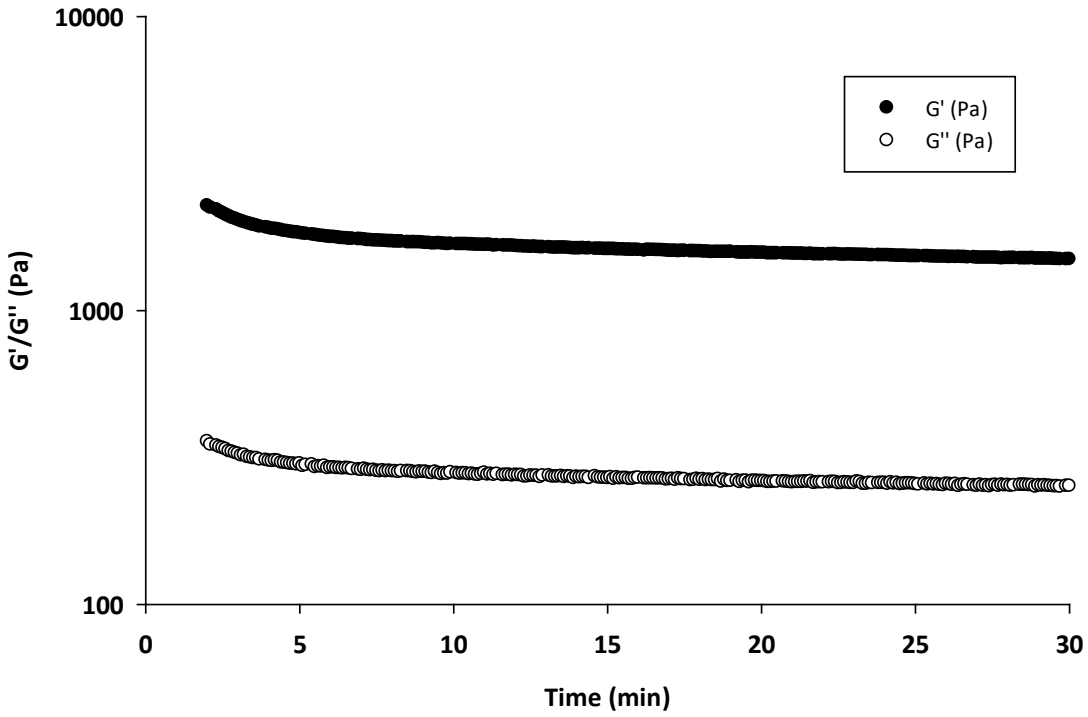

FIG. S5. Graph showing the mechanical properties of a hydrogel made of a mixture of ELR-E-RGD (98% (w/w)) and ELR-E-BMP-2 (2% (w/w)) at 300 mg/mL in PBS.

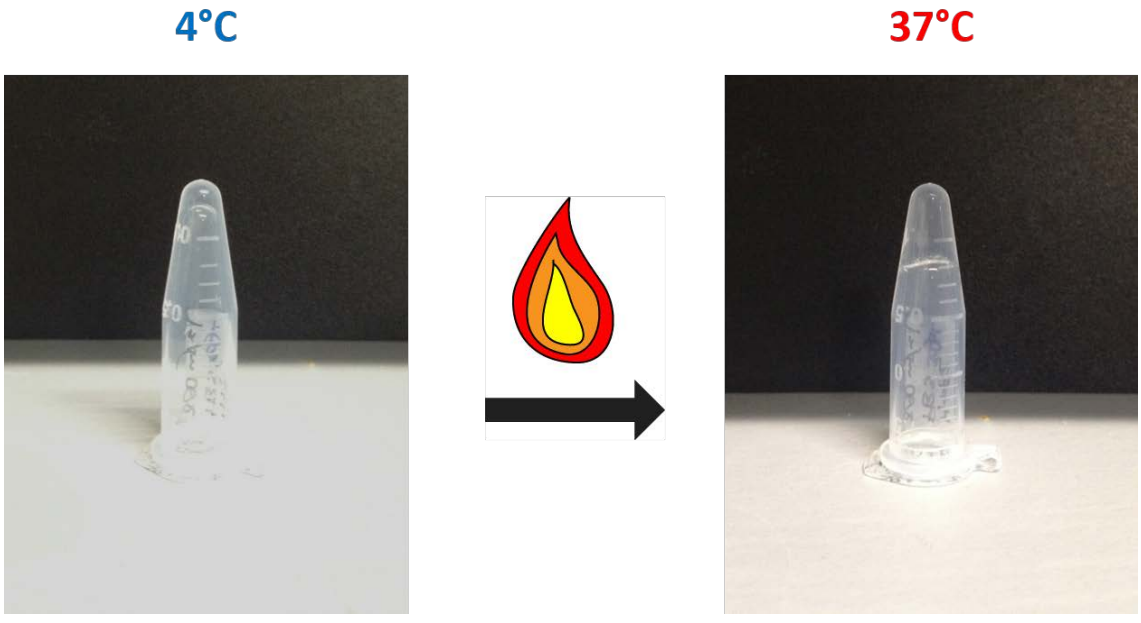

FIG. S6. Hydrogel formation due to a temperature increase above the T_t of a mixture of ELR-E-RGD (98% (w/w)) and ELR-E-BMP-2 (2% (w/w)) at 300 mg/mL in PBS. The

solution was first kept in an ice bath until complete dissolution. Then, it was incubated at 37ºC for 5 minutes to allow the formation of the hydrogel.

TABLE S5. Table showing the expected molecular weight, the molecular weight plus 20%, and the calculated (experimental) *Mw* of the bands appearing upon elastase-mediated degradation.

Nascent bands	**Expected *Mw* (kDa)**	**Expected *Mw* + 20% (kDa)**	**Calculated *Mw* (kDa)**
Nascent band 1	66.5-65.5	79.8-78.6	80.8
Nascent band 2	48.2-46.7	57.8-56.0	54.2

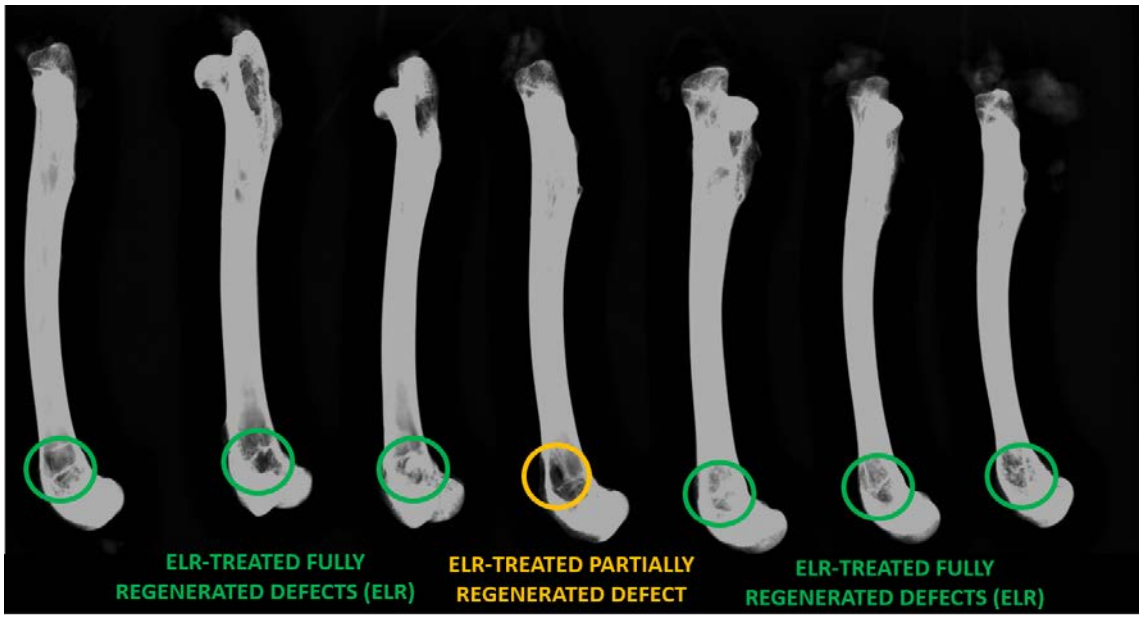

FIG. S7. Radiography showing the seven femora extracted at the end of the experiment. Green circles correspond to fully regenerated defects (six out of seven samples) and the yellow circle to a partially regenerated defect.