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Supporting Information

Proanthocyanidin-crosslinked collagen/konjac glucomannan hydrogel with improved mechanical properties and MRI trackable biodegradation for potential tissue engineering scaffold

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Experimental section

Materials

Highly purified collagen (Type I) used for all experiments in this study was extracted from calf skin as described by Miller[1] with some modifications. Konjac glucomannan powder (purity ≥ 90%, Sheli, Hongkong, China) was further processed to obtain the final purity of no < 98% for all of our experiments. Grape seed-extracted proanthocyanidin (PA) and glutaraldehyde (GA) were obtained from Aladdin (Shanghai, China). Anhydrous alcohol (guaranteed reagent) and neutral balsam were purchased from Sinopharm Chemical Reagent (Shanghai, China). Chloral hydrate and fluorescein diacetate (FDA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phosphate buffered saline (PBS) and all sterile

consumables used in cell experiments were obtained from Corning (USA). CCK-8 kits were provided by Dojindo (Kumamoto, Japan). Bovine serum albumin (BSA), Triton X-100, phalloidin-tetramethylrhodamine B isothiocyanate (phalloidin-TRITC), 4',6-diamidino-2-phenylindole (DAPI), and 4% paraformaldehyde solution were supplied by Solarbio (Beijing, China). Hematoxylin, eosin Y and xylene were bought from Aladdin (Shanghai, China). Fluorochrome-conjugated anti-rat CD68 were purchased from Miltenyi Biotec (Germany). Isoflurane was bought from Lunan Pharmaceutical (Shandong, China). Polyethyleneglycol (PEG, Mw 20 kDa) and other chemicals unless specifically stated were analytical reagent and acquired from Chronchem (Chengdu, China). The ultrapure water from a Milli-Q system (Millipore, Billerica, MA, USA) was used in all procedures of our experiments.

Preparation of Col/KGM/PA hydrogels

Based on our previous work,[2] Col/KGM/PA hydrogel was prepared as described in Scheme1. In brief, an aqueous KGM solution (0.4%, w/v) was added dropwise to an acetic acid collagen solution (pH ~ 2.5) under moderate stirring to obtain a weight ratio of collagen to KGM at 8:2. After thoroughly stirring, the desired hydrogel precursor solution was produced by concentrating the resulted Col/KGM viscous mixture in polyethylene glycol (PEG, 20 k MW) solution until the collagen concentration attained ~ 6.0 mg/mL. Afterwards, so-obtained precursor solution was injected into various moulds, which were placed in a desiccator filled with ammonia ambience and kept for 2 h to regulate the pH of the solution system to 9.0~10.0, and then transferred to incubate for 6 h at 37°C for inducing fibrillogenesis. Such a process resulted in the formation of a composite hydrogel of Col/KGM, which was named as "Col/KGM-hydrogel". Above obtained hydrogels were rinsed several times with ultrapure water and further crosslinked by immersion in PA solution at concentrations of 0.2, 0.6 and 1.0 mg/mL (in PBS solution) for 48 h with continuous shaking to obtain the Col/KGM/PA composite hydrogels, which were labeled as "Col/KGM/PA0.2-hydrogel", "Col/KGM/PA0.6-hydrogel" and "Col/KGM/PA1.0-hydrogel" according to the concentration of PA solution. Meanwhile, the Col/KGM hydrogels crosslinked by GA (0.625%, in PBS solution), labeled as "Col/KGM/GA-hydrogel", were used as controls in biocompatibility (*in vitro* cell study and *in vivo* implantation) and calcification evaluations[3]. In addition, the KGM-free hydrogel samples (i.e., the single component collagen hydrogels crosslinked with the same gradient concentrations of PA, labeled as "Col/PA0.2-hydrogel", "Col/PA0.6-hydrogel" and "Col/PA1.0-hydrogel") also served as the controls to investigate the effects of KGM on the mechanical properties of the hydrogels. All experiment procedures

involving collagen were performed at 4°C unless otherwise specified.

Fourier transform infrared spectroscopy (FTIR)

The principal functional groups of the components of the Col/KGM/PA composites were identified using KBr pellet on a FTIR spectrometer (NEXUS 670 instrument, Thermo Electron, USA) in the range of 4000 to 400 cm^{-1} with a resolution of 1.0 cm^{-1} and 20 scan accumulations.

SEM analyses

The SEM images of the lyophilized hydrogel scaffolds were recorded under field-emission scanning electron microscope (FE-SEM, S-4800, Hitachi, Japan) at an accelerating voltage of 10 kV. The specimens were mounted on sample stages with conductive carbon tape and then sputter-coated with Au to enhance their conductivity.

TEM analyses

A transmission electron microscope (FEI, Tecnai G2 F20 S-TWIN, Hillsboro, USA) was used to explore the influence of the existence of KGM macromolecules on the self-assembly behaviors of collagen molecules. The sponge pieces crushed from the lyophilized hydrogels were ground into fine powder in liquid nitrogen with a pestle and mortar. A few drops of ethanol were applied to the powder to form the suspension, which was then transferred to carbon coated copper grids and stained with 2% phosphotungstic acid (PTA) for 1 h. To evaluate the effects of PA crosslinking on the substructures of collagen fibrils, the suspensions of crosslinked or non-crosslinked collagen hydrogel samples were exposed to an ultrasonic treatment with high intensity for 20 min under Vibra-Cell™ Ultrasonic Liquid Processors (VCX130, SONICS, Newtown, USA).

Mechanical characterization

The viscoelastic properties including storage modulus (G'), loss modulus (G'') and tanδ (G''/G') were measured on a dynamic mechanical analyzer (DMA, TA Instruments Q800, USA) at room temperature. Cylindrical hydrogel samples (modeled in 7.5 mm diameter with 5 mm height) were subjected to compression testing with a preload force of 0.02 N and amplitude of 40 μm at dual-frequency of 1 and 2 Hz. Data scan were repeated three times at every frequency. Accurate sample dimensions were measured

using vernier caliper (digital display) for the diameter (twice perpendicular measurements) and using the axial compression system for the height defined by the preload force.

The micromechanics characterization was performed on a PIUMA Chiaro nanoindenter (Optics11, Amsterdam, Netherlands). The specimens were cylinder-shaped (7.5 mm diameter with 5 mm height). After a calibration according to the operation manual, the tests were conducted with a spherical tip in liquid at room temperature and the parameters are as follows: tip radius = 51 µm , calibration factor = 1.177, maximum displacement of 15 µm. Young's modulus was obtained by fitting the loading curves in Hertz model[4, 5] with the maximum fit range of 85%.

In vitro anti-calcification effect of Col/KGM/PA hydrogel

A simulated body fluid (SBF) assay was applied to investigate the inhibitory effect of PA on biomineralization development since the ion concentrations of SBF are very similar to those of human blood plasma.[3] The hydrogel samples (before and after crosslinking, 7.5 mm diameter with 5 mm height) were separately immersed in 20 mL SBF at 37 °C for 30 days with replacement of fresh SBF every 3 days. Then, the samples were taken out and rinsed several times in ultrapure water, followed by lyophilization for SEM and energy dispersive spectrometer (EDS, Inca Energy 250, Oxford Instruments, UK) analyses.

Radical scavenging activity

For determination of scavenging effect on 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radicals, 600 mm^3 hydrogel or 600 µL ultrapure water (control) was mixed with 1.5 mL DPPH solution (0.1 mM, in anhydrous alcohol) and incubated at room temperature for 60 min in dark. Then the mixture was quantified by absorbance (Abs) measurement at 517 nm on a UV-vis spectrophotometer (U-3900, Hitachi, Japan), and the scavenging effect on DPPH radicals was obtained by the following formula: Scavenging Effect (%) = $(Abs_{control} - Abs_{hydrogel})/Abs_{control} \times 100$.

For hydroxyl radical scavenging assay, 1 mL of $FeSO_4$ solution (9 mM), 1 mL of EDTA solution (9 mM) and 1 mL of salicylic acid–ethanol solution (9 mM) were mixed, then 900 mm^3 hydrogel or 900 µL ultrapure water (control) was added into this mixture solution, to which the reaction was subsequently initiated by the addition of 1 mL H_2O_2 (6 wt%) and carried out at 37 °C for 30 min. 1 mL of ultrapure water instead of H_2O_2 was used as the blank group. Finally, the Abs of the mixture was read at 510 nm, and the scavenging effect on hydroxyl radicals was calculated according to following equation: Scavenging Effect

(%) = [1 - ($Abs_{hydrogel}$ – Abs_{blank})/$Abs_{control}$] ×100.

Activated partial thromboplastin time (APTT) and prothrombin time (PT) assessment

Fresh rabbit whole blood was collected and centrifuged at 3000 rpm for 30 min to obtain platelets poor plasma (PPP). 2 mL of PPP was added into one sterile tube containing the hydrogel sample (0.4 g) or without sample (blank group) and incubated at 37 °C for 60 min with shaking (60 rpm). Then the incubated PPP solutions were collected and the values of APTT and PT were acquired on a STA-Compact-Max® automatic coagulation analyzer (Stago, Asnières sur Seine, France) by using the special kits (STA®-C.K. PREST® 5 for APTT test and STA®-Neoplastine CI Plus 5 for PT test, Stago, Asnières sur Seine, France) according to the manufacturer's instructions.

Hemolysis assay

The hemolysis evaluation was performed based on the haemoglobin cyanide (HiCN) method recommended by the International Organization for Standardization (ISO 10993-4:2002) with slight modifications. The fresh whole blood was collected from three healthy New Zealand White Rabbit with anticoagulant treatment and diluted in PBS to a hemoglobin concentration of 10 mg/mL. The hydrogel sample (1.5 g), 7 mL of PBS and 1mL of the diluted rabbit blood were sequentially added to a sterile centrifuge tube and incubated at 37 °C for 3 h with shaking. Then, after centrifugation at 800 g for 15 min, 1 mL of the supernatant was mixed with 1 mL of van Kampen Zijlstra's reagent (Tianjin Institute of Modern High Technology, Shandong, China) and the Abs of the mixture at 540 nm was recorded on a UV–vis spectrophotometer. PBS and ultrapure water were used as the negative and positive control, respectively. The percentages of hemolysis were calculated according to the following relationship: Hemolysis (%) = ($Abs_{hydrogel}$ – $Abs_{negative}$)/($Abs_{positive}$ – $Abs_{negative}$) ×100.

Cell culture and hydrogel preparation

HUVECs were cultured in Ham's F-12K medium supplemented with 0.04 mg/mL endothelial cell growth supplement (ECGS), 0.1 mg/mL heparin, 10 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin in a 5% CO_2 incubator at 37 °C. Cells at passage 4-6 were used for further studies and medium was changed every 2 days. Before cell seeding, the hydrogel samples were immersed in Ham's F-12K medium contained in 24-well plates to equilibrium for 24 hours at 37 °C.

Cell adhesion and proliferation assay

HUVECs were inoculated on the surfaces of "Col/KGM/PA0.2-1.0 hydrogel" or "Col/KGM/GA-hydrogel" (control) at the density of 5×10^4 cells/well in 24-well plates. At predetermined intervals (24 h and 4 days), the cell-inoculated hydrogels were washed twice with PBS and stained with fluorescein diacetate (FDA, 25 μg/mL) in PBS for 5 min. After rinsing with PBS to remove uncombined FDA, the fluorescent images were captured with CLSM. Furthermore, the quantitative analysis was performed via CCK-8 assay. At the end of each culturing period, the medium was replaced with 1 mL fresh medium containing 100 μL of the CCK-8 solution, followed by incubation for another 3 h. The absorbance at 450 nm of the supernatant was recorded on a microplate reader (Varioskan Flash, Thermo Fisher Scientific, USA).

Cell morphology assay

Cells were cultured as described above for 24 h or 4 days, then fixed with 4% paraformaldehyde solution for 30 min, permeabilizated with 0.1% Triton X-100 for 10 min, and subjected to incubation of 5% BSA for 2 h to avoid non-specific binding. Cytoskeleton was stained with 200 nM phalloidin–TRITC solution for 60 min, followed by nuclear staining with 10 μg/mL DAPI for 5 min. Then, fluorescent images were obtained under CLSM.

Subcutaneous implantation for *in vivo* biologic evaluations

A dorsal subcutaneous implantation model was adopted to assess the effects of PA-crosslinking treatments on the long-term interactions of tissue with such collagen-based hydrogels *in vivo*. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Sichuan University and carried out in accordance with the guidelines for animal rights formulated by Sichuan University. The female Sprague-Dawley (SD) rats (170-190 g) were provided by Dossy Experimental Animal Co. (Chengdu, China). After anesthesia by intraperitoneal injection of 10% (v/v) chloral hydrate (0.3 mL/100 g), a subcutaneous skin pocket was made for implantation of the hydrogel samples (10 mm diameter with 3-5 mm height), then the wound was sutured and disinfected carefully. All animals were housed under standard specific pathogen-free (SPF) animal facilities.

Non-intrusive tracking of hydrogel biodegradation in vivo by MRI

A system of superparamagnetic iron oxide (SPIO)-labeled Col/KGM/PA hydrogel was developed to monitor the biodegradation process of the hydrogels *in vivo*. The dextran-coated SPIO nanoparticles used in this study were prepared with the widely used strategies.[6-8] The hydrogel precursor solution was fully mixed with SPIO stock solution (final Fe_3O_4 concentration ranging from 0.05 to 0.2 mM), and then the same procedures of gelatification and PA-crosslinking were conducted according to Section 2.1 to construct the labeled composite hydrogels, which were implanted subcutaneously on the backs of the rats. On the postoperative time points of 1 day, and 2, 4, 8, and 12 weeks, MRI monitoring for implanted hydrogels was respectively performed using a clinical 3 T MR scanner (Skyra, Siemens, USA) equipped with a quadrature rat volume coil. The rats were anesthetized by 1-1.5% isoflurane mixed with oxygen atmosphere. Transverse T_2-wighted images were acquired using a turbo spin echo (TSE) sequence with fat suppression: repetition time (TR) = 2500 ms, echo time (TE) = 69 ms, field of view (FOV) = 60 × 60 mm, number of signal averages (NSA) = 3, matrix = 256 × 256, gap = 0.0 mm, and slice thickness = 2.0 mm. Coronal T_2-wighted images were acquired using a multi-echo TSE sequence for T_2-mapping with the following parameters: TR = 2500 ms, TE = 12, 23, 35, 46, 58, and 69 ms, FOV = 60 × 60 mm, NSA = 7, matrix = 256 × 256, gap = 0.0 mm, and slice thickness = 1.0 mm. Images were analyzed by MicroDicom software. To estimate the volume, the area of the implants was measured upon coronal images and then multiplied by the thickness obtained on the transverse images. Data from multiple TE scans within regions-of-interest (ROI) were fit to calculate the T_2 values. Then the graphical visualization of T_2 value of the implants, including coronal T_2-maps and the corresponding T_2 value distribution, were achieved by an internal program developed by our lab upon MATLAB (MathWorks, USA) as our previous publication.[9]

Histological assessment

At every post-implantation time point of week 2, 4, 8, 12 and 20, the implanted materials along with the surrounding dermal tissues were retrieved from three animals per group, fixed with 4% paraformaldehyde, then dehydrated in gradient ethanol followed by clearing in dimethylbenzene, finally embedded in paraffin and sliced with a microtome (Leica RM2235, Germany). The tissue sections (5 um) were stained with Hematoxylin-Eosin (HE) for pathological analyses and with Von Kossa for *in vivo* anti-calcification detections. CD68 immunofluorescent staining was adopted to visualize the macrophages around the implants. All staining procedures were performed with standard protocols. The fluorescent images were captured by CLSM. The histological images were acquired under a digital slide scanner

(Pannoramic 250 Flash III scanner, 3DHISTECH, Hungary) with its special CaseViewer software (v2.1, 3DHISTECH). After implantation, the local biological effects were further evaluated upon a histological scoring system based on the international safety standard ISO 10993-6:2016 (Table S1). All histologic analyses were conducted by three independent board-certified pathologists.

Statistical analysis

All quantitative values are presented as mean ± SD. The statistical analysis was performed by a one-way ANOVA using Microsoft Excel 2016.

Additional figures

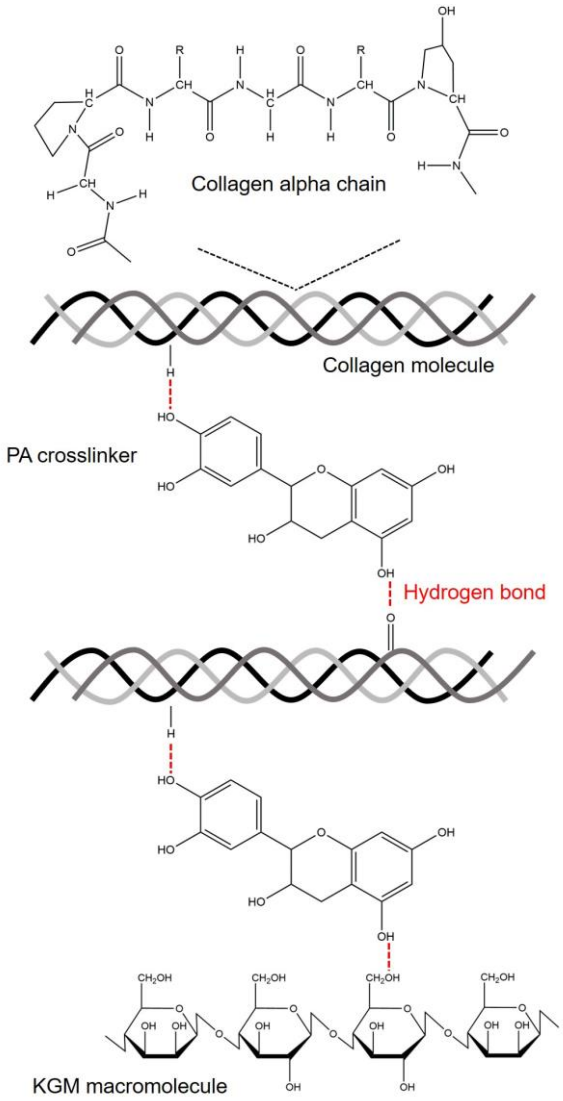

Fig. S1 The reaction scheme for the crosslinking of Col/KGM with PA.

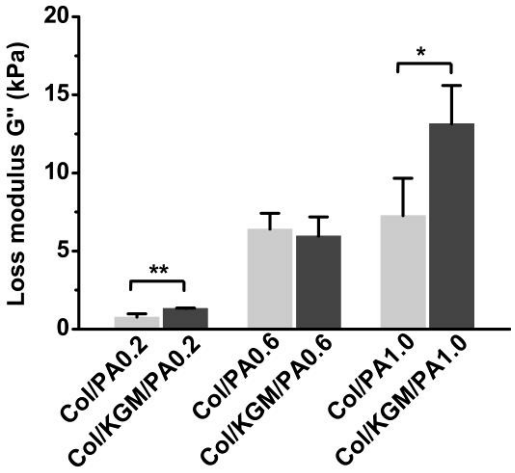

Fig. S2 The loss modulus (G'') of "Col/PA-hydrogel" and "Col/KGM/PA-hydrogel" at the test frequency of 1 Hz. *P < 0.05, **P < 0.01, ***P < 0.001, N.S: not significant; n = 5 per group.

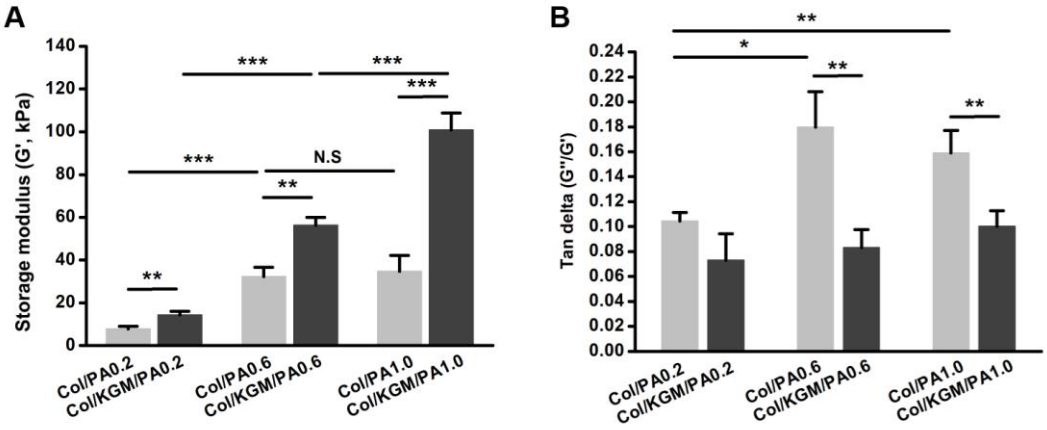

Fig. S3 The **(A)** storage modulus (G') and **(B)** tan delta (G''/G') of "Col/PA-hydrogel" and "Col/KGM/PA-hydrogel" at the test frequency of 2 Hz. *P < 0.05, **P < 0.01, ***P < 0.001, N.S: not significant; n = 5 per group.

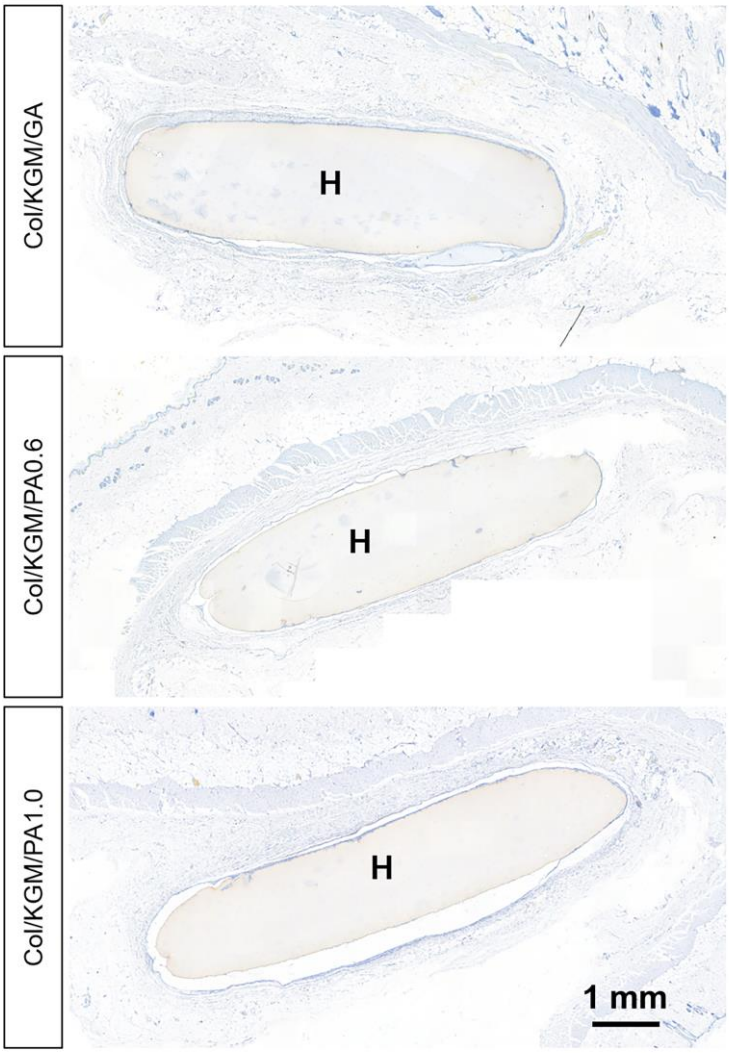

Fig. S4 Von Kossa staining showing *in vivo* calcification state of GA-crosslinked implants of "Col/KGM/GA-hydrogel" and PA-crosslinked implants of "Col/KGM/PA0.6-hydrogel" and "Col/KGM/PA1.0-hydrogel" after subcutaneous implantation for 20 weeks. H: hydrogel; n = 4 animals per group.

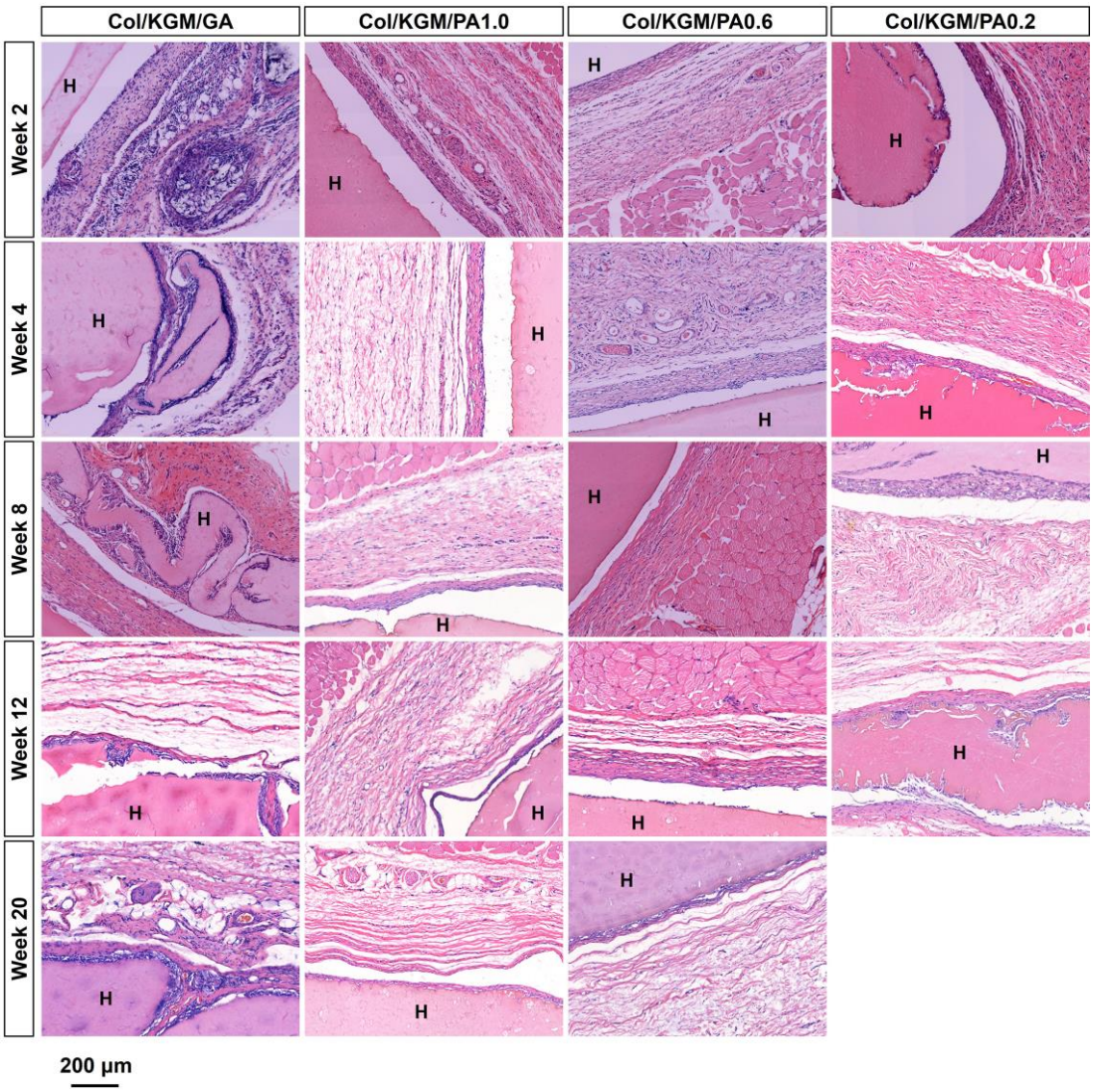

Fig. S5 Representative H&E-stained sections of PA-crosslinked implants ("Col/KGM/PA0.2-1.0-hydrogel") and GA-crosslinked implants ("Col/KGM/GA-hydrogel") along with surrounding tissues at high magnification. The "Col/KGM/PA0.2-hydrogel" was not retrieved at week 20 due to its complete resorption *in vivo*. H: hydrogel; n = 4 animals per group per time point.

Table S1 Histological scoring system

Cell type/response	**Score**				
	0	**1**	**2**	**3**	**4**
Polymorphonuclear cells	0	Rare, 1 to 5/phf [a]	5 to 10/phf	Heavy infiltrate	Packed
Lymphocytes	0				
Macrophages	0				
Fibrosis	0	Narrow band	Moderately thick band	Thick band	Extensive band
Neovascularization	0	Minimal capillary proliferation, Focal, 1 to 3 buds	Groups of 4 to 7 capillaries with supporting fibroblastic structures	Broad band of capillaries with supporting fibroblastic structures	Extensive band of capillaries with supporting fibroblastic structures

[a] phf = per high-powered (400×) field

Table S2 Histological score for the different implants

Implant	Time (week)	Polymorphonuclear cells	Lymphocytes	Macrophages	Fibrosis	Neovascularization
Col/KGM/GA	2	3	3	2	4	3
	4	1	2	2	2	2
	8	1	2	2	4	4
	12	0	1	1	3	2
	20	0	1	1	1	1
Col/KGM/PA0.2	2	1	3	1	3	3
	4	0	1	1	3	3
	8	0	2	2	2	4
	12	0	1	2	2	3
	20	\	\	\	\	\
Col/KGM/PA0.6	2	1	2	1	3	3
	4	0	1	0	4	3
	8	1	1	1	3	4
	12	0	1	0	3	2
	20	0	0	0	2	1
Col/KGM/PA1.0	2	1	2	2	3	3
	4	0	1	1	3	3
	8	0	1	1	3	4
	12	0	1	0	3	3
	20	0	0	0	2	2

The “Col/KGM/PA0.2-hydrogel” was not harvested at week 20 due to complete resorption *in vivo*. n = 4 per group per time point.

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