ADVANCED MATERIALS

Supporting Information

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One Step Creation of Multifunctional 3D Architectured Hydrogels Inducing Bone Regeneration

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With One Step to Multifunctional 3D Architectured Hydrogels Inducing Endogenous Regeneration

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Supporting Information Figures

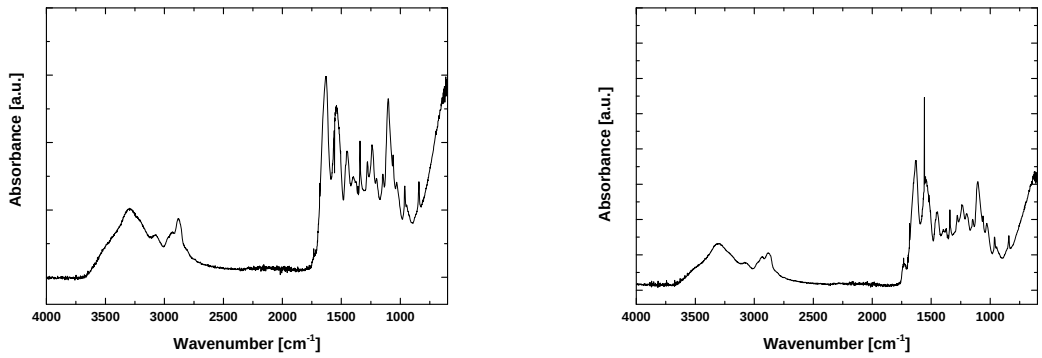

Figure S1. IR spectra of the ArcGels after washing. No peak at 2250 cm^{-1} is present, which would indicate residual isocyanate.

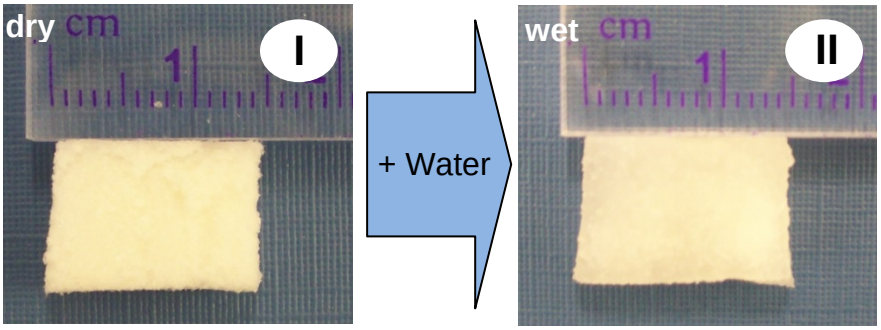

Figure S2. Macroscopic form stability of the gelatin-based ArcGel G10_LNCO8 (corresponding to the direct step from a to f in Figure 2 in the main text). The ArcGel (I, dry) does not change its outer dimensions when taking up water (II, wet).

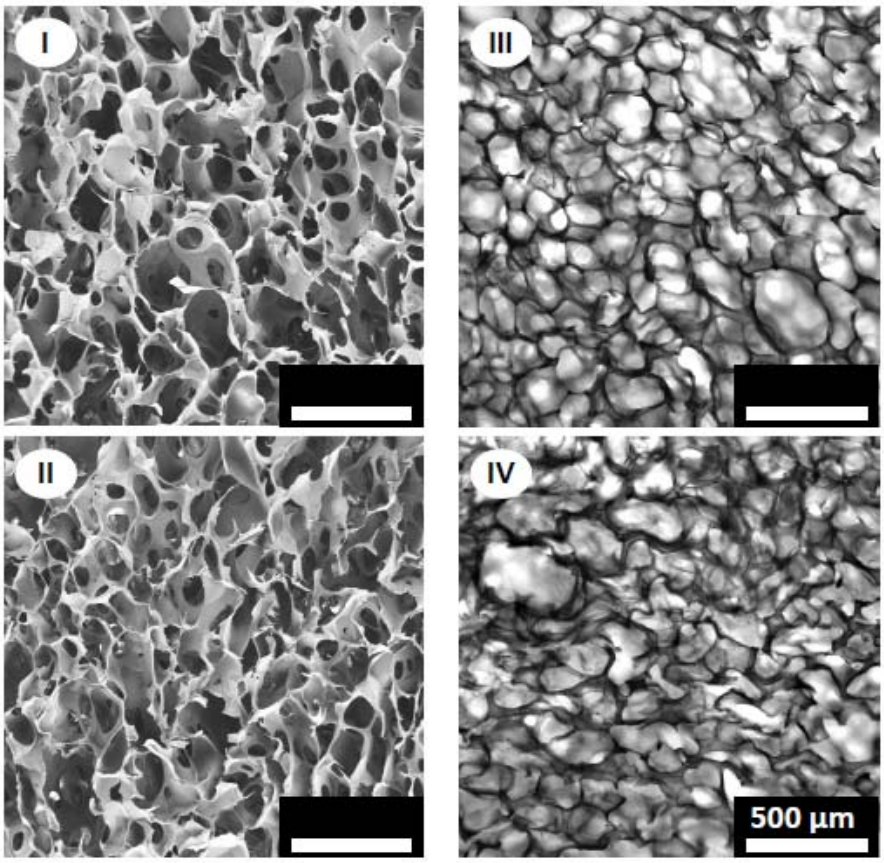

Figure S3. Form stability of pore morphology. The pictures from randomly chosen fields of view of a G10_LNCO5 ArcGel in the dry state (SEM, left, I & II) and wet state (CLSM, right, III & IV) illustrate that neither the pore sizes nor the wall thicknesses change upon water uptake. This can be explained by allocation of water molecules in the large free volume of the material.

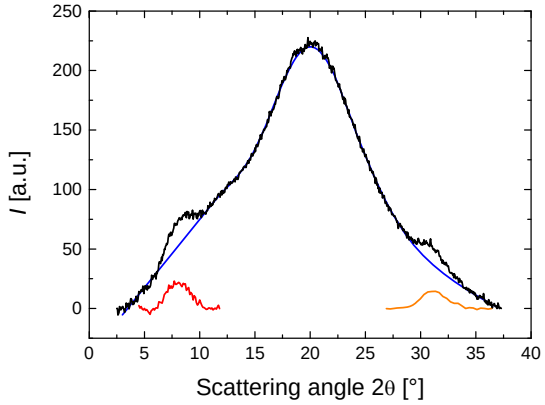

Figure S4. A WAXS spectrum (black) of the ArcGel G10_LNCO3, with peaks at 2θ= 7.5° indicating triple helices, 22° representing the amorphous halo, and 31°, showing single helices. The blue, red, and orange curves represent the theoretical fits, and the relation of the integrated fitted curves give the values presented in Fig. 4b of the manuscript.

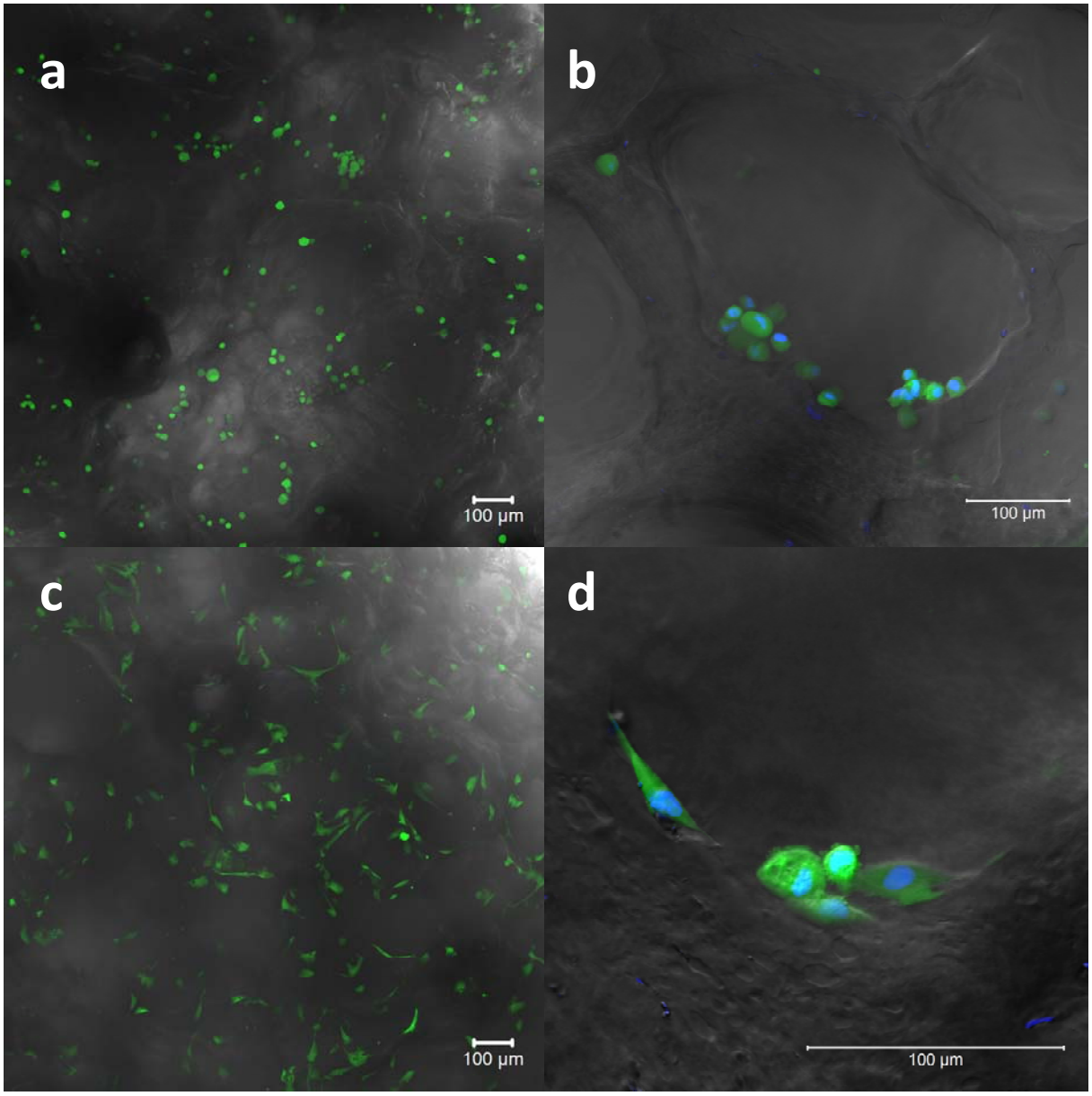

Figure S5. Initial stage of hMSC adhesion on G10_LNCO3. MSCs were stained with CFSE (cytoplasm = green, viable cells), Hoechst 33342 (cell nuclei = blue), and propidium iodide (red, dead cells) at 3 hours (a, b) and 24 (c, d) hours.

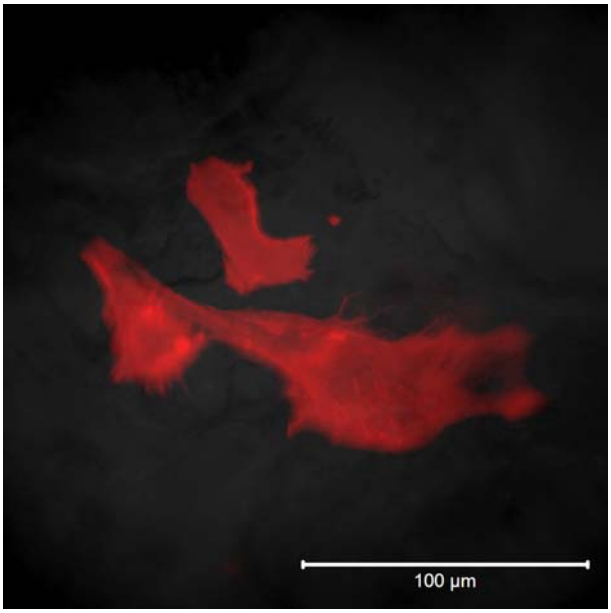

Figure S6. Cytoskeleton organization of hMSCs on the ArcGel G10_LNCO3 after 24 h. Representative confocal image of F-actin staining (Red).

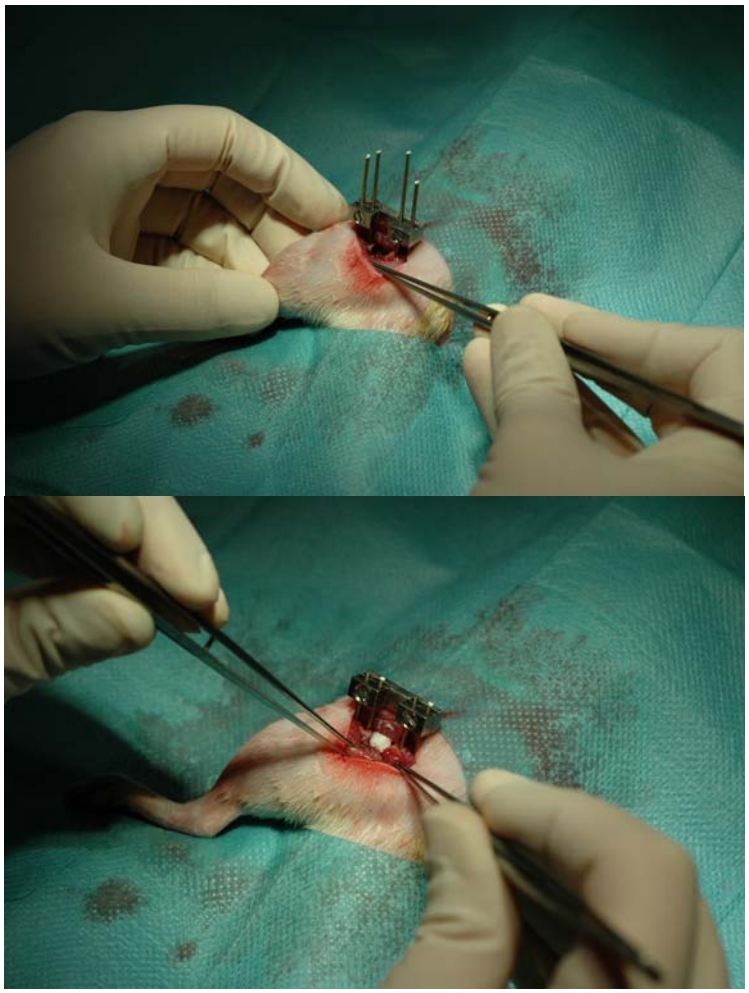

Figure S7. Defect site (top) and implantation of cancellous bone graft (bottom).

Experimental Section

Materials

The materials were purchased from the following companies: Gelatin (Typ A, 200 bloom) with low endotoxin content from GELITA AG (Eberbach, Germany) PEO-PPO-PEO tri-block copolymer (Pluronic® F-108; short: PEPE) from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). LDI from Shanghai Infine Chemical Co., Ltd. (Shanghai, China), and PBS buffer chemicals from Merck KGaA (Darmstadt, Germany).

Methods

One-step ArcGel Synthesis

LDI was added to an aqueous solution of gelatin and PEPE under mechanical stirring in a flat flange cylindrical jacketed vessel with bottom outlet valve (HWS Labortechnik, Mainz, Germany) at 45 °C, and after 3 to 6 minutes the obtained ArcGel was freeze-dried.

Hydrolytic Degradation

Investigation of degradation behavior of the ArcGels in aqueous environment was performed in freshly prepared 1x PBS buffer solution (pH 7.4) at 37 °C in a moving water bath. Samples were cut in approximately 1.5 cm x 1.5 cm x 1 cm blocks (6 replicas per time point and composition for 10 weeks) and stored in 50 mL centrifuge tubes loaded with 14 mL of buffer solution undergoing slight shaking. PBS buffer was exchanged once every week. At each time point, six samples were removed from the water bath and freeze dried. Mass loss, water uptake, pore sizes (SEM & μCT) and if possible compressive moduli were determined by methods detailed below and are reported as average±standard deviation.

Scanning Electron Microscopy (SEM)

Lyophilized ArcGels were sputtered with Gold-Palladium (Au 80% - Pd 20%) with a Polaron SC7640 sputter coater (Quorum Technologies Ltd, Sample preparation division, Ashford, Kent, UK) and investigated using a SUPRA 40 VP electron microscope with a Schottky emitter at an acceleration voltage of 3 kV (Carl Zeiss NTS GmbH, Oberkochen, Germany).

Water uptake (*H*)

Lyophilized ArcGels (1 cm x 1.5 cm x 0.5 cm) were immersed in water for 24 h, and water from the pores was removed by carefully blotting from each side with filter paper. The mass change was used to determine *H* using equation 1,

$$H = (W_s - W_d)/W_s \times 100 \quad \textbf{(1)}$$

with W_s and W_d being the weights of the swollen and dry samples, respectively. For each composition, three replicas were used for the determination of *H* reported as average±standard deviation.

Wide angle X-ray scattering (WAXS)

WAXS measurements were performed with a two-dimensional detecting Bruker D8 Discover X-ray diffraction system from Bruker AXS (Karlsruhe, Germany). X-rays were generated at operating voltage of 40 kV and working current of 40 mA, producing Cu Kα-radiation with a wavelength of 0.154 nm.

ArcGels were investigated in transmission geometry with a collimator opening of 0.8 mm at a sample-to-detector distance of 15 cm. Integration of the two-dimensional scattering pattern gave the intensity as a function of the scattering angle.

Compression tests

Compression tests of the ArcGels were performed on a Zwick Z2.5 (Zwick GmbH, Ulm, Germany). Specimens were cut in block shapes (1.5 cm x 1.5 cm x 1 cm). Samples were equilibrated in distilled water for 24 h and compressed with a cross-head speed of 2 $mm \cdot min^{-1}$ to 50% of the original sample height (performed in triplicate, values reported as average±standard deviation).

The macroscopic compression modulus E_c was determined in the elastic regime of the stress-strain curve (at strains of 0.05-0.25%) using the equation $E_c = \sigma/\varepsilon$, where σ corresponds to the force applied per sample area and ε corresponds to the compression distance divided by the original sample length.

The shape fixity ratio R_f (from compression in the dry state) and shape recovery ratio R_r (determined from compression in the wet state) have been calculated according to the equations below.

$$R_f = \frac{h_{fix} - h_0}{h_{load} - h_0} \qquad R_r = \frac{(h_0 - h_{fix}) - (h_0 - h_{rec})}{(h_0 - h_{fix})}$$

with h_0= initial sample height, h_{load} = height after compression (with load), h_{fix} = height after compression and removal of load, and h_{rec}= height after elastic recovery.

Micromechanical analysis of pore walls of ArcGels

AFM experiments were performed using a system combining an optical light microscope (Zeiss Observer Z1, Jena, Germany) to locate the site of indentation and a MFP-3D-Bio AFM (Atomic Force F&E GmbH, Mannheim, Germany) for performing the indentation tests using a pyramidal I-Drive / BL-TR 400 PB AFM tip (Atomic Force F&E GmbH). Sample pieces for the micromechanical analyses were cut to 3 mm x 10 mm x 1 mm with a freshly cleaned razor, degassed, and equilibrated in water at room temperature for 24 h.

Samples were fixed on the bottom of the AFM cell and completely covered with water. Suitable investigation areas on the sample surface were determined by optical light microscopy followed by topographic AFM scanning to confirm a flat surface area. Measurements (500 mV trigger point, 300 – 500 nm max. indentation) were performed within a 6 x 6 points indentation grid (30 x 30 μm) and performed again for up to 4 additional locations giving a maximum of 180 data points. The reduced elastic moduli E_r were calculated with Igor Pro AFM evaluation software, using a modified Hertz model (eq. 2) for conical indenter geometry:

$$P = \frac{2}{\pi} E_r \tan(\phi) \cdot \delta^2 \quad \textbf{(2)}$$

where ϕ is the half-opening angle of the cone, δ is the indentation depth, and P is the loading force. Equation 1 was fitted to the force-distance curves (Figure 3 of the main manuscript) and local Young's moduli were then calculated from E_r using the equation 3:

$$E_r = \left(\frac{1-\nu_1^2}{E_1} + \frac{1-\nu_2^2}{E_2} \right)^{-1} \quad \textbf{(3)}$$

where E_1 is the Young's Modulus of the material and ν_1 the Poisson ratio of the material, while E_2 and ν_2 describe properties of the indenter. For the evaluation of the results obtained for the ArcGel micromechanics, the histograms of relative frequency of the measured local E moduli showed a bimodal distribution. The reported values for E are averaged from the more populated (generally lower E modulus) value range. The set of data points with higher moduli are likely to be systematic errors of the measurements, e.g. the cantilever and not the tip interacting with the material. For G10_LNCO5, G10_LNCO8, and G13_LNCO8, values with >40% frequency were found, with the remaining maxima each accounting for <5% of the total counts. The values measured for G10_LNCO3 varied over a broader range, and the reported value for G10_LNCO3 accounted for 26% frequency of the output.

Supporting Table S1. Averaged Young's moduli determined from individual indentation experiments. Primary and secondary Young's moduli, as well as Young's moduli of G10_LNCO3 and G10_LNCO8 differ significantly ($p < 0.0001$).

Composition	Primary E [kPa]	Contribution	Secondary E [kPa]
G10_LNCO3	1252 ± 140	26 %	231 ± 34
G10_LNCO8	56 ± 28	40 %	-

hMSC experiments

Bone marrow samples were taken after written consent according to the guidelines approved by the Ethic Committee on the Use of Human Subjects at the University of Rostock (approval no. A21/2007). hMSCs were isolated and characterized following the protocol described in R. Gaebel, D. Furlani, H. Sorg, B. Polchow, J. Frank, K. Bieback, W. Wang, C. Klopsch, L.L. Ong, W. Li, N. Ma, G. Steinhoff, Cell Origin of Human Mesenchymal Stem Cells Determines a Different Healing Performance in Cardiac Regeneration. PLoS ONE 6, e15652 (2011). For proliferation experiments, ArcGels were cut into cubes (edge length of ~5 mm) and seeded with $2x10^4$ hMSCs. hMSCs were stained for the initial cell attachment with carboxyfluorescein succinimidyl ester (CFSE) and Hoechst 33342, and counter stained with propidium iodide (PI) for their viability. F-actin organization was examined by ActinRed 555 ReadyProbes (Life technologies). Samples were imaged with a confocal microscope (Carl Zeiss, LSM 780, Jena, Germany). Images were sampled at a resolution of 1024 by 1024 pixels, using a 10x objective, a 0.6-4 times software zoom and a z-step size of 2.57 µm with a pinhole at 1 Airy unit. Maximum intensity projections of Z-stacks were generated by ZEN 2012 software (Carl Zeiss, Jena, Germany). After 9 days, hMSCs were stained with Hoechst 33258, quantified in randomly chosen three dimensional fields (630× magnification) and analyzed with a confocal laser scanning microscope (Carl Zeiss, ELYRA PS.1. LSM 780,

Jena, Germany). For the osteogenic differentiation experiments, osteogenic supplement was added to basal medium and applied to hMSCs within the ArcGels (R&D Systems, Minneapolis, MN, USA). After three weeks, cells were stained for osteocalcin (polyclonal rabbit anti-osteocalcin, R&D Systems) followed by anti-rabbit Alexa-Fluor 488 (Invitrogen, Carlsbad, CA, USA) conjugated secondary antibody and counterstained with Hoechst 33258. For quantification, the percentage of osteocalcin$^+$ cells in three-dimensional fields (630× magnification) was determined.

Bone grafts harvesting and treatment

The cancellous bone graft was from a 65 year old male donor, who died from cardiac arrest (kindly provided by the University Tissue Bank, Institute of Transfusion Medicine, Charité - Universitätsmedizin Berlin). Fat and connective tissue were removed with scalpels and surgery forceps. Cancellous bone cylinders (2.5 mm diameter, 4 mm height) were obtained using a bone marrow trephine, and were rinsed under pressure immediately with prewarmed (37 °C) water to remove adhering material or blood. Bone cylinders were stored at ≤ -70 °C for at least 6 days. After thawing, cancellous bone cylinders were rinsed for 5 min with sterile distilled water and transferred to an exsiccator containing a mixture of 2 volumes of chloroform and 1 volume of methanol. The cancellous bone cylinders were covered with the chloroform/ methanol mixture. After shaking for 2 h at RT, the cancellous bone cylinders were rinsed eight times with methanol in an ultrasonic bath to remove chloroform, each time for 15 min, followed by two washing steps with distilled water. Chloroform/methanol extracted cylinders were dried under a laminar air flow hood and stored at ≤ -18 °C. After defatting, cancellous bone cylinders were rinsed in sterile distilled water and then treated with PES (2 volumes of peracetic acid 20 g/l H_2O, 1 volume of 96% ethanol and 1 volume of aqua ad injectionem) for 4 h at RT on a rocking platform. In the production process, the cylinders were rinsed several times with Soerensen buffer (physiological sodium chloride solution) to

remove PES. Finally, they were lyophilized and conserved in sterile double bags under sterile conditions (clean room class 100).

Operative procedure and *in vivo* experiments.

The operative procedure has been performed according to M. Mehta, H. Schell, C. Schwarz, A. Peters, K. Schmidt-Bleek, A. Ellinghaus, et al. Arch. Orthop. Trauma Surg. 2011, 131, 121-9. In brief, female Sprague Dawley rats were administered an intraperitoneal injection of a solution containing ketamine hydrochloride (60 $mg \cdot kg^{-1}$, ketamine 50 mg, Actavis®, Island) and medetomidine (0.3 $mg \cdot kg^{-1}$, Domitor®, Pfizer, Karlsruhe). The antibiotic clindamycin-2-dihydrogenphosphate (45 $mg \cdot kg^{-1}$, Ratiopharm, Ulm) was also administered subcutaneously. An incision was made across the lateral aspect of the thigh, through the fascia, exposing the femur by separating the gluteus superficialis and biceps femoralis muscles. Four threaded titanium pins were manually screwed into the femur after pre-drilling with a 0.9 mm drill bit resulting in a firm fixation. The external fixator was attached, allowing a 7.5 mm offset. A 5 mm defect was created in the middle of the femur using an oscillating saw by performing a double transverse osteotomy, after which time the fascia and skin were sutured. The analgesic tramadol hydrochloride was administered subcutaneously during the surgery (20 $mg \cdot kg^{-1}$, Grünenthal, Aachen) and over 3 days diluted in the animals' drinking water (25 $mg \cdot L^{-1}$, Grünenthal, Aachen). Immediately after surgery, the rats were allowed to resume normal activity and given unrestricted access to food and water.

ArcGels (n = 6 per composition) or allogenic bone grafts (n = 5) were implanted into 5 mm (critical-size) mid-diaphyseal femoral defects of 23 female Sprague Dawley rats (weight at operation, 250 to 300 g). All animal experiments were carried out according to the policies and procedures approved by the local legal representative (LAGeSo Berlin, G0210/08). At 2,

4, and 6 weeks post-operation, radiographs (LX-60, Faxitron Bioptics, Tucson, USA) of the defect site were taken.

External fixator design

The femoral defects were stabilized by an external unilateral fixator (M. Mehta, H. Schell, C. Schwarz, A. Peters, K. Schmidt-Bleek, A. Ellinghaus, et al. Arch. Orthop. Trauma Surg. 2011, 131, 121-9). The fixator included a crossbar of stainless steel (22 mm x 5 mm x 2 mm), set at a 7.5 mm offset, the free length of the pins between the lateral surface of the rat's femur and the inner side of the fixator bar. Four titanium threaded (0.65 mm core diameter/1.2 mm outer diameter) pins, spaced 8 mm apart, were held between the two sides of the fixator bar under compression. Previous *in vitro* experiments using rat femurs with these external fixators have shown that with loading in axial direction of the bones, the load-deflection curves showed a stiffness of 50 $N \cdot mm^{-1}$.

***In vitro* Micro-Computed Tomography for mineralized tissue formation and density**

At 6 weeks post-operation, bone healing was assessed by microcomputed tomography (vivaCT 40, Scanco Medical, 70 kV, 114 µA) at an isotropic resolution of 10.5 µm. For each femora, the volume of interest was located in the periosteal and endosteal callus, excluding the cortical bone. The volume of interest (VOI) included the 5 mm defect region and 0.5 mm in the proximal and distal directions from the borders of the original osteotomy. A global threshold of 50% of the tissue mineral density from the contralateral femur, equivalent to 353 mg hydroxyapatite per cm^3 was used to distinguish mineralized tissue (bone and calcified cartilage) from poorly mineralized and unmineralized tissue. Bone healing parameters included mineralized callus volume (BV), total callus volume (TV), mineralized callus volume fraction (BV/TV), tissue mineral density (TMD) and tissue mineral content (TMC), defined as BV·TMD, with TMD measured using only voxels whose intensity exceeded the

threshold. Also, two voxels were removed from all bone surfaces prior to computing TMD in order to reduce partial volume effects. Calculation of TMD and TMC was made possible by density calibration data obtained from scans of a hydroxyapatite phantom provided by the system manufacturer.