

## **Single cell-laden protease-sensitive microniches for long-term culture in 3D**

### **Experimental Section**

#### **Oscillatory shear rheology**

Rheological studies are conducted on a stress-controlled Anton Paar Physica MCR 501 rheometer with a parallel plate–plate geometry (gap size 1 mm, plate diameter 25 mm). For the preparation of the gel samples, 5 or 7.5% (w/v) TG-PEG precursor solution, 30 U mL<sup>-1</sup> FXIII and 30 U mL<sup>-1</sup> thrombin are diluted in CaCl<sub>2</sub>-free DMEM and mixed with either denoted amounts of CaCl<sub>2</sub> (Sigma-Aldrich, cat. no. C5670) or the supernatant from CaCO<sub>3</sub>-nanoparticles (10 mg ml<sup>-1</sup> in CaCl<sub>2</sub>-free DMEM) that prior to centrifugation (5 min at 1000 x g at room temperature) were incubated for 30 min at 37 °C in presence or absence of 5 mM HCl. After mixing, the solution is immediately transferred to the middle of the lower plate at 4 °C. Then, the upper geometry is lowered and the measurement is started. For the first 10 min, each sample is monitored at a constant shearing amplitude and frequency ( $\gamma = 0.01$ ;  $\omega = 0.1$  Hz) to ensure sample equilibration. Then, the temperature is increased to 37 °C to induce gelation and the sample is monitored at a constant shearing amplitude and frequency ( $\gamma = 0.01$ ;  $\omega = 0.1$  Hz) for further 30 min to ensure complete gelation. Finally, a frequency sweep is recorded at constant strain amplitude ( $\gamma = 0.01$ ;  $\omega = 0.01$ –16 Hz) at 37 °C.

#### **Determination of size, zeta potential and polydispersity index of CaCO<sub>3</sub> nanoparticles**

CaCO<sub>3</sub> nanoparticles are prepared as described in the section 'preparation of CaCO<sub>3</sub> nanoparticles'. CaCO<sub>3</sub> nanoparticles are then suspended in distilled water saturated with CaCO<sub>3</sub>, which was prepared alike DMEM-CaCO<sub>3</sub> as described in the section 'preparation of MSCs prior to encapsulation'. The diameter (intensity-average) as well as the zeta potential of these particles are determined by dynamic light scattering experiments using a Malvern zen3600 equipped with a He-Ne laser (633 nm) and a fixed detector oriented at 173°.

#### **Assessment of localization of CaCO<sub>3</sub> nanoparticles on cells**

CaCO<sub>3</sub> nanoparticles are fluorescently labeled by incubation with 0.5 mg mL<sup>-1</sup> tetramethylrhodamine conjugated albumin from bovine serum (Thermo Fisher Scientific, cat. no. A23016) in DMEM complete and then prepared according to the section 'preparation of CaCO<sub>3</sub> nanoparticles'. MSCs are then loaded with labeled CaCO<sub>3</sub> nanoparticles as described in the

section 'Preparation of MSCs prior to encapsulation'. All the following solutions in this paragraph were saturated with  $\text{CaCO}_3$  as described for DMEM- $\text{CaCO}_3$  in the section 'preparation of MSCs prior to encapsulation'. Cells are fixed with 4% (v/v) paraformaldehyde (PFA; Electron Microscopy Science, cat. no. 15710) in phosphate buffered saline (PBS; 2.7 mM KCl, 2 mM  $\text{KH}_2\text{PO}_4$ , 137 mM NaCl, 10 mM  $\text{Na}_2\text{HPO}_4$ , pH 7.4), washed with PBS, permeabilized using 0.2% (v/v) Triton X-100 (Sigma-Aldrich, cat. no. X100) in PBS, washed once with PBS and stained with Hoechst 33342 (1:1000; Life technologies, cat. no. H3570) and Fluorescein conjugated phalloidin (1:100; Biotium, cat. no. 00030) for 30 min at room temperature. Cells were washed twice with PBS and images (pixel size:  $0.13 \times 0.13 \mu\text{m}$ ) of Rhodamine, Hoechst 33342 and FITC are acquired using an upright confocal microscope (Zeiss LSM710).

### **Calculation of the number of $\text{CaCO}_3$ nanoparticles per cell**

Cells are loaded with  $\text{CaCO}_3$  nanoparticles as described in the section 'Preparation of MSCs prior to encapsulation'. Bound  $\text{CaCO}_3$  nanoparticles are then completely dissolved by incubation with 20% HCl in distilled water for 5 min at room temperature. Calcium concentration is measured using a calcium detection kit (Abcam, cat. no. 102505) according to the manufacturer's instructions. The moles of calcium originating from one cell are then calculated by dividing the calcium concentration by the used cell concentration. Next, to calculate the corresponding number of  $\text{CaCO}_3$  nanoparticles, moles of calcium from one cell are divided by moles of  $\text{CaCO}_3$  that are contained in a spherical particle with a density of  $2.71 \text{ g cm}^{-3}$  and the measured diameter of 770 nm.

### **Analysis of cell position within microniches**

For the following paragraph, all media changes and washing steps are performed by centrifugation (5 min at  $250 \times g$  at room temperature) of microniches in well plates.

Microniches are fixed with 4% (v/v) PFA in PBS, permeabilized using 0.2% (v/v) Triton X-100 in PBS, washed once with PBS, stained with TRITC-conjugated phalloidin (1:500; Millipore, cat. no. 90228) and Hoechst 33342 (1:1000) nuclear stain for 30 min at room temperature. Samples are washed twice with PBS and embedded in 1.5% (w/v) TG-PEG bulk gels within channels of a channel slide (ibidi, cat. no. 80626) to prevent microniche movement during image acquisition. Z-stack images (voxel size:  $0.13 \times 0.13 \times 0.3 \mu\text{m}$ ) of TRITC, Hoechst 33342 and FITC are acquired using an upright confocal microscope (Zeiss LSM710). To measure the distance from the cell surface to the exterior of the corresponding microniche, a 3D mesh model of the cell and microniche is created based on FITC and TRITC channels of acquired Z-stack images using a semi-automated MATLAB (R2015a, MathWorks Inc, USA) script. Minimal distance is assessed by calculating in 3D space the distance from each of 676 points on the cell surface to 676 points on the microniche exterior using MATLAB.

### **Cell viability assessment**

For the following paragraph, all media changes and washing steps of microniches are performed by centrifugation (5 min at 250 x g at room temperature) in well plates.

To appraise the viability of D1 cells encapsulated in microniches or non-encapsulated cells in well plates, LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells (Life technologies, cat. no. L3224) is used according to the manufacturer's instructions. In brief, samples are incubated at static conditions in 500  $\mu$ L DMEM complete supplemented with 4  $\mu$ M calcein and 8  $\mu$ M ethidium homodimer-1 for 10 min at 37 °C and 5% CO<sub>2</sub> in a humidified atmosphere. Fluorescence images of microniches and their encapsulated cells as well as non-encapsulated cells are acquired using a fluorescence microscope (EVOS FL). For microniches, cell viabilities are evaluated on the basis of at least 100 cell-laden microniches per time point and condition.

### **Osteogenic differentiation**

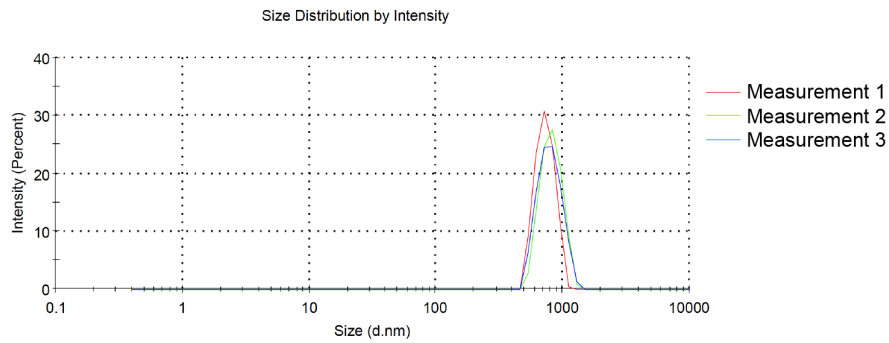
For the following paragraph, all media changes and washing steps of microniches are performed by centrifugation (5 min at 250 x g at room temperature) in well plates.

For assessment of osteogenic differentiation of D1 cells, culture medium is supplemented with 50  $\mu$ g mL<sup>-1</sup> L-ascorbic acid (Sigma-Aldrich, cat. no. A8960), 10 mM  $\beta$ -glycerophosphate (Sigma-Aldrich, cat. no. G9422) and 0.1  $\mu$ M Dexamethasone (Sigma-Aldrich, cat. no. D4902) and is exchanged every other day.

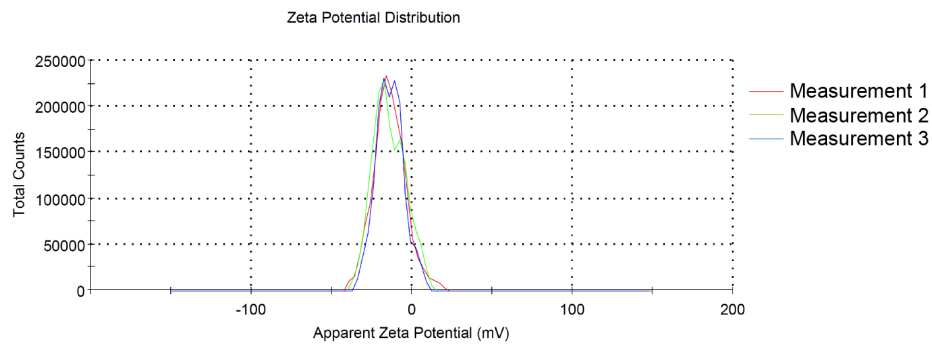
To appraise the osteogenic differentiation capabilities of D1 cells encapsulated in TG-PEG microniches and as adherent cells in well plates, cells are fixed with 4% (v/v) PFA in PBS at indicated time points, washed twice with PBS and stained with Hoechst 33342 (1:1000) nuclear stain for 5 min. Next, samples are washed twice with PBS and equilibrated into alkaline staining buffer (100mM Tris-HCl, 100mM NaCl, 0.1% Tween-20, 50mM MgCl<sub>2</sub>, pH 8.2) for 10 min. For colorimetric staining of alkaline phosphatase (ALP) activity, samples are then incubated in alkaline staining buffer with the addition of 500  $\mu$ g mL<sup>-1</sup> naphthol AS-MX phosphate (Sigma-Aldrich, cat. no. N5000) and 500  $\mu$ g mL<sup>-1</sup> Fast Blue BB (Sigma-Aldrich, cat. no. F3378) for 30 min. Next, microniches and adherent cells are washed twice with PBS and ALP expression is assessed using a fluorescence microscope (Nikon Eclipse E800) equipped with a 40x objective. ALP expression of D1 cells encapsulated in microniches is evaluated on the basis of at least 100 microniches from three independent experiments per time point and condition.

**Figure S1**

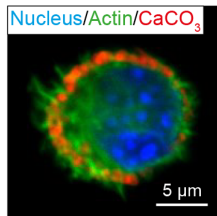
**A**



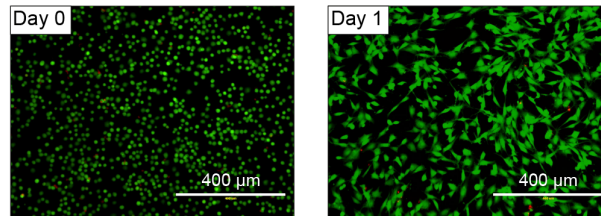
**B**



**C**

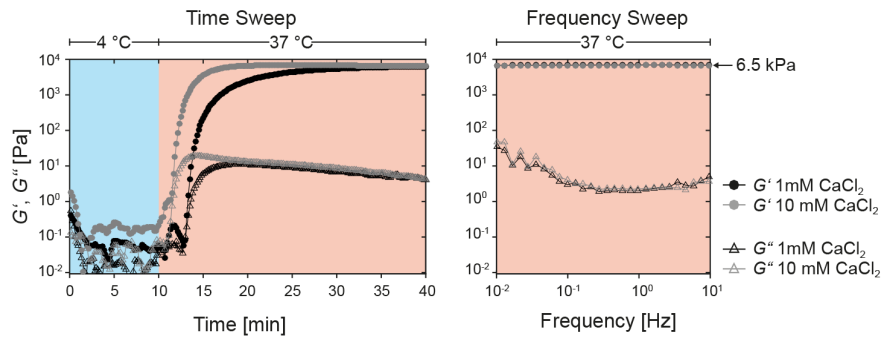


**D**



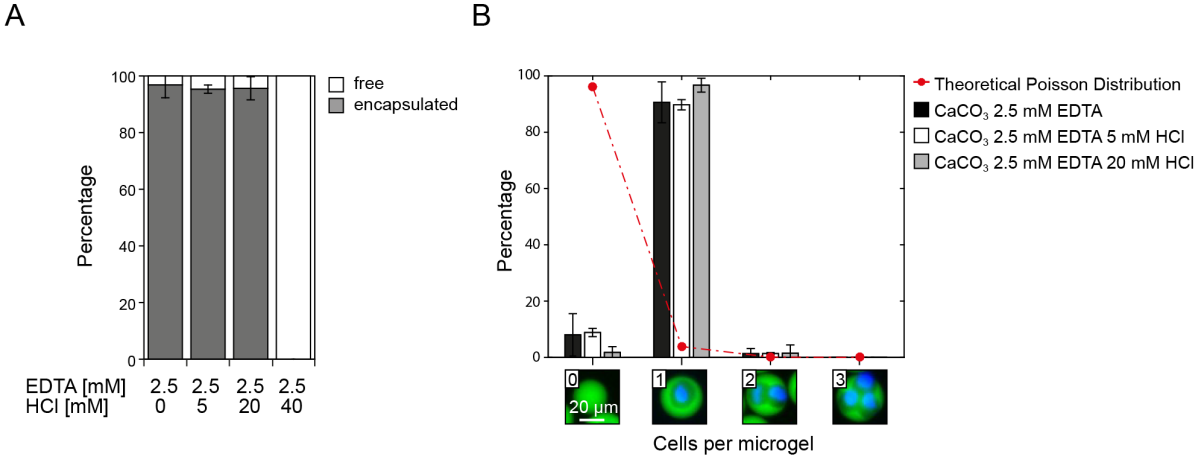
**Characterization of physical and biological properties of CaCO<sub>3</sub> nanoparticles.** Size distribution profiles (A) as obtained by dynamic light scattering as well as zeta potential distributions (B) of CaCO<sub>3</sub> nanoparticles from three independent measurements are shown. C) Confocal image of a Fluorescein-phalloidin (Actin) and Hoechst 33342 (Nuclei) stained MSC after being loaded with rhodamine-labeled CaCO<sub>3</sub> nanoparticles. D) Fluorescent images of live (green) and dead (red) cells 2 hours and 1 day after loading with CaCO<sub>3</sub> nanoparticles.

**Figure S2**



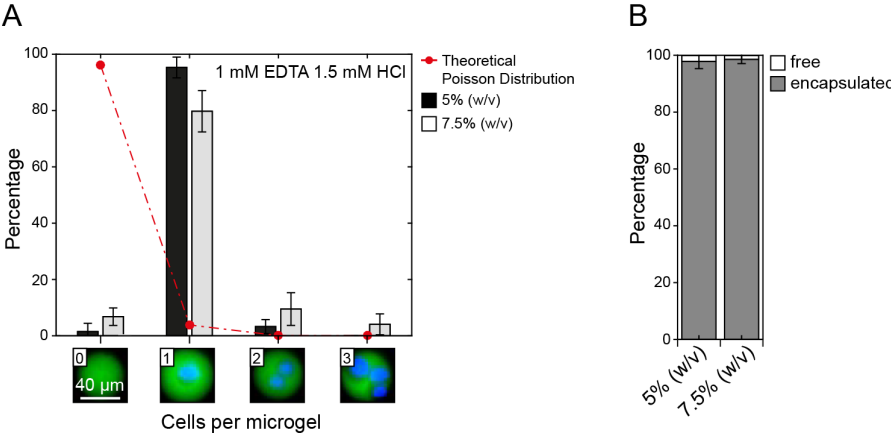
**Investigation of parameters regulating gelation kinetics and final mechanics in TG-PEG microniches.** Rheology data are shown for TG-PEG precursor solutions (7.5% (w/v)) that are covalently crosslinked by FXIII enzymatic activity with  $\text{CaCl}_2$  as calcium source. Gelation is induced by an increase in temperature from 4 °C (blue background) to 37 °C (red background). Plots show the elastic (circle,  $G'$ ) and viscous (triangle,  $G''$ ) parts of the complex shear modulus as a function of time (left panel) and test frequency (right panel).

**Figure S3**



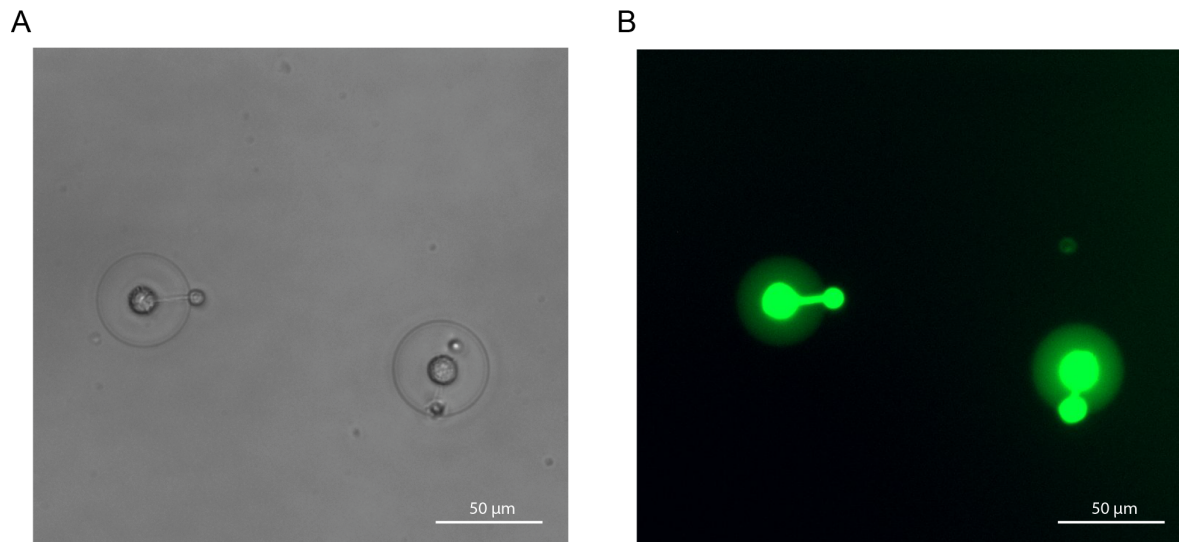
**Optimization of HCl concentration in microdroplets.** A) Percentage of MSCs encapsulated in microniches in dependence of initial HCl and EDTA concentration is shown. Channel dimensions at X-junction are 20  $\mu\text{m}$  x 20  $\mu\text{m}$ . B) Theoretical Poisson distribution (red line) and experimental distribution (bars) of MSCs in TG-PEG microniches (5 % (w/v)) after breaking the emulsion and transfer to cell culture conditions. Data in all panels is depicted as mean  $\pm$  SD (number of analyzed microniches > 200).

**Figure S4**



**Investigation of parameters for fabrication of microniches with a diameter of 45  $\mu$ m.** MSC are encapsulated in 5 and 7.5% (w/v) microniches with 1 mM EDTA and 1.5 mM HCl as starting conditions. X-junction channel dimensions are 30  $\mu$ m x 30  $\mu$ m. A) Theoretical Poisson distribution (red line) and experimental distribution (bars) of MSCs in microniches after breaking the emulsion and transfer to cell culture conditions are shown. B) Percentage of MSCs encapsulated in microniches is shown. Data in all panels is depicted as mean  $\pm$  SD (number of analyzed microniches > 200).

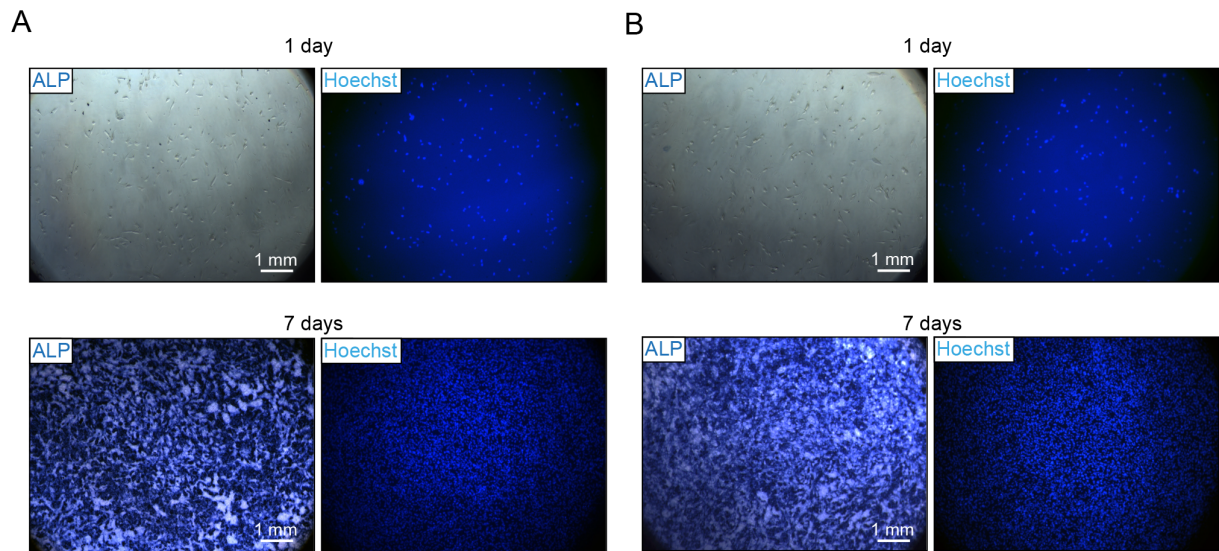
**Figure S5**



**Cell escape occurs through micrometer-sized channels.** MSC are encapsulated in 5% (w/v) microwells with 1 mM EDTA and 1.5 mM HCl as starting conditions. X-junction channel dimensions are 30  $\mu\text{m}$  x 30  $\mu\text{m}$ . Phase contrast (A) and fluorescent microscopy image (B) of live (green) and dead (red) stained cells after 3 days in culture.



**Figure S6**



**Loading of MSCs with CaCO<sub>3</sub> nanoparticles does not affect osteogenic cell differentiation.**

Untreated MSCs (A) and MSCs that were loaded with CaCO<sub>3</sub> nanoparticles (B) are cultured in differentiation medium in tissue culture plates. After 1 and 7 days in culture ALP expression is assessed.