Supporting Information

Control of Nanoparticle Release Kinetics from 3D Printed Hydrogel Scaffolds
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Mesoporous silica nanoparticle (MSN) synthesis:

MNS-NH₂ particle synthesis was performed according to a modified synthesis of Rosenholm et al.[S1]

For the particle synthesis the structure directing agent CTAB (Cetyltrimethylammoniumbromide) was dissolved in an alkaline mixture of methanol and water. APTMS (aminopropyltrimethoxysilane) and TMOS (tetramethoxysilane), used as precursors, were mixed under inert atmosphere followed by rapidly addition to the alkaline solution. The final molar composition was TMOS : APTMS : NaOH : CTAB : MeOH : H₂O was 1 : 0.14 : 0.31 : 1.46·10³ : 3.31·10³. The sol was stirred for 12 h at room temperature. For particle flocculation ammonium nitrate was added and the particles were separated by centrifugation (Z 36 HK, Hermle, Wehingen, Germany). To either gain NH₂-functionalyzed or pure silica particles solvent extraction or calcination of the particles was performed to remove the structure directing agent. In the extraction process the particles were dispersed three times in acidic ethanol (0.11 mM, 4 g/L) at room temperature for one hour. Finally, the particles were washed with ethanol and dried over night at 60°C. For calcination the particles were centrifuged, washed with ethanol, and dried at 60°C for 12 h. Subsequently the particles were calcined at 550°C.

MSN functionalization:

COOH-functionalyzed particles were synthesized starting from calcined particles (7.5 mg/mL) which were dispersed in HEPES buffer (25 mM, pH 7.2) and carboxyethylsilanetriol sodium salt (25% in water, 1 mol, 3.33 µmol/mg) was added. After stirring at room temperature particles were centrifuged, washed with ethanol and dried overnight in vacuum.[S2]

Particles were covalently labeled with different ATTO dyes.[S2] Therefore, NH₂-functionalyzed particles (10 mg/mL) were dispersed in borate buffer (200 mM, pH 8.3) and ATTO 647N-NHS solution in DMSO (1 mg/mL; 1 µg/mg) was added and stirred for 1 h. Here COOH-functionalyzed particles (5 mg/mL) were activated by adding a mixture of EDC (77.6 µmol, 1.55 µmol/mg) and NHS (77.6 µmol, 1.55 µmol/mg) in HEPES buffer for 30 min at room temperature. After washing with water, particles (10 mg/mL) were dispersed in borate buffer and mixed with an ATTO 488-amine solution in DMSO (1 mg/mL; 1 µg/mg) and stirred for 1 h. Finally, the functionalized particles were washed with ethanol and dried in vacuum at room temperature for 12 h. All ATTO labeled particles showed a comparable fluorescence intensity.

MSN characterization:

Particles were characterized with nitrogen sorption measurements at 77 K (Quadrasorb-1 SI, Quantachrome Instruments). Particles were under vacuum for 12 h at 140°C.
before measurements. Surface area was calculated based on the BET model. Pore diameters were calculated using the NLDFT (equilibrium kernel) developed for silica. The hydrodynamic particle diameter was determined using dynamic light scattering (Zetasizer Nano-ZS ZEN 3600, Malvern Instruments, Worcestershire, UK). The zetapotential of the particles was determined in PBS (pH 7.4, 25 mM) (Zetasizer Nano-ZS ZEN 3600, Malvern Instruments). To determine the shape of the particles TEM measurements were performed using a Jeol 1400 (Jeol GmbH) at 120 kV HT voltage and 84 µA beam current.

**Figure S1.** Structural characterization of mesoporous silica nanoparticles (MSN). a) Cross section of MSN with a diameter of 400 nm measured with transmission electron microscopy (TEM) for the visualization of the pore system of particles. b) TEM image of 400 nm particles demonstrates a homogenous size distribution. All scale bars correspond to 200 nm. c) Histogram showing the distribution of particle size. All scale bars correspond to 200 nm.

**Table S1:** Physiochemical characterization of MSN-COOH and MSN-NH₂

|                | zeta-potential in PBS (25mM) [mV] | BET surface area [m²/g] | mesopore diameter [nm] | mesopore volume [cm³/g] | particle diameter DLS [nm] | particle diameter TEM [nm] |
|----------------|----------------------------------|-------------------------|------------------------|-------------------------|---------------------------|---------------------------|
| MSN-COOH       | -21                              | 870                     | 3.0                    | 0.47                    | 395                       | 357                       |
| MSN-NH₂        | +20                              | 845                     | 3.2                    | 0.54                    | 410                       | 381                       |

**Polymer Synthesis**

Poly(allyl glycidyl ether-co-glycidyl) P(AGE-co-G)
P(AGE-co-G) was synthesized via anionic polymerization of ethoxy ethyl glycidyl ether (EEGE) (10 mL, 68.0 mmol) and allyl glycidyl ether (AGE) (1.3 mL, 11.2 mmol) with potassium tert-butoxide (KOBu) (1.0 mL, 1 M) as initiator according to literature.\textsuperscript{[S3]} The reactants were mixed under inert atmosphere and the reaction mixture was stirred for 24 h at 60 °C. 1 mL Ethanol was added to stop the polymerization. Subsequently, the EEGE units of P(AGE-co-EEGE) were deprotected. The polymer was dissolved in THF (80 mL/g polymer) and hydrochloric acid (32%, 2.2 mL/g polymer) was added to the mixture and stirred for 3 h at room temperature. After deprotection, THF was decanted and the polymer was dissolved in deionized water (10 mL/g polymer). For purification the polymer solution was dialyzed (Biotech Cellulose Ester dialysis membrane, MW cut-off 3500 Da, spectrumlabs.com) for 3 d in deionized water and freeze-dried (Alpha 1-2 LD, Christ, Osterode am Harz, Germany). P(AGE-co-G) was received as a colorless oil with 14%-allyl functionality (\textsuperscript{1}H-NMR; 14% of all monomer units in the backbone bear an allyl functional group, Figure S2).

\textsuperscript{1}H NMR (300 MHz, D\textsubscript{2}O): δ = 6.02-5.93 (m, 15 H, -O-CH\textsubscript{2}-CH\textsubscript{=CH\textsubscript{2}}), 5.41-5.30 (m, 30 H, -O-CH\textsubscript{2}-CH\textsubscript{=CH\textsubscript{2}}), 4.12-4.10 (d, 30 H, -O-CH\textsubscript{2}-CH\textsubscript{=CH\textsubscript{2}}), 3.80-3.67 (m, 516 H, backbone-H), 1.26 (s, 9 H, tBu-H) ppm. GPC (set 2): M\textsubscript{n} = 2322 Da, D = 1.80.

Figure S2. \textsuperscript{1}H NMR spectra and structural formula of P(AGE-co-G).
Thiol-functionalized poly(glycidol) (PG-SH)

PG-SH was received through thiol-functionalization of P(AGE-co-G) with 8%-allyl functionality according to literature.\textsuperscript{[S3]}

The functionalization occurred in a two-step reaction. The allyl groups of P(AGE-co-G) reacted first in a thiol-ene photo reaction with thioacetic acid. Therefore, P(AGE-co-G) (4.30 g, 0.64 mmol) was dissolved in 92 mL degassed ethanol and 2,2-dimethoxy-2-phenylacetophenon (705 mg, 2.75 mmol) and thioacetic acid (1.57 mL, 22.0 mmol) were added to the polymer solution. The reaction mixture was stirred at room temperature under UV irradiation (365 nm, UV LEDs, 4 x 11 W, Polymerschmiede, Aachen, Germany) for 2 h. Ethanol was removed under reduced pressure and the polymer was dissolved in ethanol and precipitated in cold diethylether (1/10 polymer/diethylether). The thioester was deprotected in a second step. Diethylether was decanted and the rest was removed in vacuum. The remaining polymer was dissolved in 1 M sodium hydroxide solution (30 mL) and heated to 100°C for 2.5 h. The clear orange solution was cooled down to room temperature and neutralized with hydrochloric acid. Tris(2-carboxyethyl)phosphine hydrochloride (2.88 g, 10.0 mmol) were added to the polymer solution to reduce disulfide bonds to thiol bonds. After stirring for 12 h the mixture was dialyzed (Biotech Cellulose Ester dialysis membrane, MW cut-off 3500 Da, spectrumlabs.com) in degassed deionized water for 3d and subsequently freeze-dried. A light yellow oil with a thiol-functionality of 8% was received (characterized with \textsuperscript{1}H NMR; Figure S3).

\textsuperscript{1}H NMR (300 MHz, D\textsubscript{2}O): \(\delta = 3.80-3.67 \text{ (m, 311 H, backbone-H)}, 2.70-2.63 \text{ (m, 10 H, } -O-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{SH}), 1.94-1.90 \text{ (m, 10 H, } -O-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{SH}), 1.26 \text{ (s, 9 H, } \text{tBu-H)} \text{ ppm. GPC (set 1): } M_n = 1897 \text{ Da, } D = 1.71.\)
Figure S3. $^1$H NMR spectra and structural formula of thiol-functionalized polyglycidol.

Polymer characterization
Polymers were characterized via $^1$H NMR and GPC measurements. A Bruker Biospin 300 MHz spectrometer (Bruker, Billerica, MA) was used for $^1$H NMR measurements with D$_2$O ($\delta = 4.79$ internal reference) as a solvent.

GPC measurements were performed on an aqueous GPC system from Malvern (Herrenberg, Germany) which consists of a Viscotek GPCmax (in-line degasser, 2-piston-pump and autosampler), column oven at 35 °C, refractive index detector (Viscotek), viscosity detector (Viscotek 270 detector) and Viscotek A-Columns (set 1: A3000 + A2000 and set 2: 2 x A6000M) for aqueous GPC/SEC (Length = 300 mm, width = 8 mm, porous poly hydroxymethacrylate polymer, particle size A3000 = 6 µm and A2000 = 8 µm or A6000M = 13 µm). The eluation rate was set at 0.7 mL/min and deionized water was used as solvent (8.5 g/L NaNO$_3$ and 0.2 g/L NaN$_3$). Calibration was performed with poly(ethylene glycol) standards (Malvern, Herrenberg, Germany).

Gold Nanoparticle (AuNP) functionalization:
In order to obtain COOH- and NH$_2$-functionalized gold nanoparticles (AuNP) 950 µL of 30 nm citrate-AuNP (136020 ppb Au$^{197}$) in double-distilled water were incubated with
200 µL 3 mM PG-COOH resp. PG-NH₂ solution in PBS (pH 7.4, 25 mM) at room temperature for 12 h. The AuNP were purified by two successive centrifugation- (30 min, 14000 rpm, 4 °C; Mega Star 1.6R, Thermo Scientific, Waltham, MA) and re-suspension cycles in 500 µL PBS (pH 7.4, 25 mM).

**AuNP characterization:**

The particle concentration was determined via inductively coupled plasma mass spectrometry (ICP-MS; Varian, Darmstadt, Germany). AuNP were dissolved with aqua regia (HCl:HNO₃/3:1) afterwards gold ions were measured against standard solutions (100 ppb, 1000 ppb and 10000 ppb). The dispersability of the surface functionalized AuNPs was characterized by dynamic light scattering (Zetasizer Nano-ZS ZEN 3600, Malvern Instruments) and UV-Vis spectroscopy (Thermo Fisher, Waltham, MA, USA). The zetapotentials of the particles were determined in PBS (pH 7.4, 25 mM) (Zetasizer Nano-ZS ZEN 3600, Malvern Instruments, Worcestershire, UK) showing a zetapotential of +26 mV for AuNP-NH₂ and -27 mV for AuNP-COOH.

**AuNP release:**

The preparation of the ink was performed as described in the experimental section. The concentration of COOH- and NH₂-functionalized AuNP in the supernatant was measured by ICP-MS at day 1, day 5 and day 9. Table S2 shows the percentage of released AuNP in the supernatant with respect to the initial particle concentration. Further, as depicted in Fig. S4 UV-Vis absorbance of the supernatant at 530 nm on day 1, day 5 and day 9 was determined.

**Table S2.** The percentage of released AuNP in the supernatant with respect to the initial particle concentration analyzed by ICP-MS at the different time points and cumulative release.

|            | AuNP-COOH [%] | AuNP-NH₂ [%] |
|------------|---------------|--------------|
| Day 1      | 7.6 ± 0.2     | 2.3 ± 0.9    |
| Day 5      | 6.6 ± 0.8     | 3.2 ± 0.8    |
| Day 9      | 4.2 ± 1.0     | 1.9 ±0.7     |
| cumulative | 18.4          | 7.4          |
Figure S4. UV-Vis spectroscopy analysis of AuNP release. The absorbance of the supernatant was analyzed via UV-Vis on day 1, 5 and 9.

Rheology

The rheological properties of the different ink formulations were tested using an Anton Paar Physica MCR301 rheometer in cone-plate geometry (60 mm diameter, 0.5°, gap 0.058 mm). Solvent evaporation was limited using a solvent trap and all experiments were performed at room temperature. Both oscillating and rotational experiments were applied. Amplitude sweeps (Figure S5) were performed at a frequency of 10 Hz to determine the linear viscoelastic region for the frequency sweeps (Figure S6) obtained at an amplitude of 1%. Recovery analysis (Figure S7) was measured in rotation (high shear rate 5 rad/s, low shear rate 0.01 rad/s for 100 s each).
Figure S5. Amplitude sweep at 10 Hz to determine the linear viscoelastic region.

Figure S6. Frequency sweep at 1 % amplitude.
Figure S7. Test of recovery behavior of inks using rotational rheological experiments with alternating shear rates.

Robotic Dispensing

The inks were printed with a 3D bioprinter (3DDiscovery, regenHU) equipped with an electromagnetic valve dispenser and a cell friendly printhead. To ensure a good printing fidelity the constructs were crosslinked during printing using UV light from a source (bluepoint 4, hönle) at 60 % intensity. Each construct was irradiated with seven pulses each having a duration of 2 s. An optical analysis of the printing quality was performed with a Carl Zeiss SteREO Discovery.V20 post–fabrication.
Figure S8. Schematic drawing of the double printing process. A) Printable hydrogel inks containing differently charged MSN were loaded into two separate dispensing print heads, allowing for printing of MSN-hydrogel strains with different geometries. B) Printing patterns generated to examine nanoparticle mobility (see also Figure 3).
Sample preparation for cell culture experiments

Samples were casted into silicone molds and illuminated with the same lamp used for crosslinking the printed samples. They were irradiated seven times at 2 s pulses and afterwards they were removed from the molds and stored in PBS. The discs (diameter 6 mm, height 1 mm) where thoroughly rinsed with PBS before cell experiments.

Cell culture studies

C2C12 cells (Sigma-Aldrich, Chemie GmbH, Munich, Germany) were maintained in DMEM (Gibco, life technologies, Carlsbad, California) supplemented with 10 % fetal bovine serum, 2 mM L-glutamine and antibiotics in an atmosphere of 5 % CO₂ at 37°C. Crafted hydrogel scaffolds were placed in one corner of an µ-Slide 8 well (ibidi GmbH,
Martinsried, Germany) and incubated with 300 µL of a cell suspension (3300 cells/mL). The cell cultivation medium was exchanged every two days. After 1, 3 and 5 days the samples were fixed with 3.5 % paraformaldehyde solution and stained with DAPI and Phalloidin-TRITC for CLM measurements (Leica Dmi8 microscope, HC APO C52 63x/1.40 OIL objective). TEM measurements were performed using a Jeol 1400 microscope operated at 120 kV and 65 µA beam current.

Figure S10. Time-dependent cellular uptake of differently charged MSN (0.05 mg/ml) by C2C12 cells grown on a glass surface. Different to the previous experiments both particles (MSN-NH$_2$ and MSN-COOH) were labeled with ATTO 647N. Via software the particles were colored artificially in red and green to comply with the color code used in the rest of the experiments. Amino-functionalized, positively charged MSN-NH$_2$ were taken up much stronger by C2C12 cells illustrated by a stronger fluorescence signal (shown in red) around the nucleus illustrated especially in the zoom pictures. The uptake amount of COOH-functionalized, negatively charged MSN-COOH was much lower illustrated by a lower fluorescence signal (shown in green) inside the cells. Particle uptake occurred already after two hours for MSN-NH$_2$ whereas for MSN-
COOH after 4 and 24 hours only single particles inside cells could be observed. All scale bars correspond to 20 µm.

**Figure S11.** Stability measurements of particle dispersions (0.1 mg/ml) in cell culture media including 10% FCS. Both particles (positively and negatively charged) show no increased agglomeration in cell culture media during 24 h.

**References supplementary information**

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