SUPPORTING INFORMATION

Cytotoxicity, retention, and anti-inflammatory effects of a CeO_2 nanoparticle-based supramolecular complex in a 3D liver cell culture model

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Experimental Section

Supramolecular complex synthesis

Supramolecular complex was synthesized in three step procedure: 1) synthesis of CeO_2 NPs colloidal solution; 2) synthesis of ZnMFA:CeO_2NPs complex; 3) synthesis of ZnMFA:CeO_2NPs:HPβ-CD complex. The CeO_2NPs colloidal solution was prepared as described elsewhere [1].

Synthesis of ZnMFA:CeO_2NPs complex:

75 ml of the Zn-MK ethanol solution (0.2 mg/ml) was mixed with 75 ml of the CeO_2NPs aqueous solution (0.2 mg/ml) followed by stirring until an opalescent solution was formed. Then, 75 ml of distilled water was added to obtain transparent colorless solution. The solution was poured into a round bottom flask and evaporated to 30 ml using a rotary evaporator at a bath temperature of 50oC. During evaporation, the alcohol was completely removed. The resulting aqueous ZnMFA:CeO_2NPs solution contains 0.5 mg/ml ZnMFA in a complex with 0.5 mg/ml CeO_2 NPs.

Synthesis of ZnMFA:CeO_2NPs:HPβ-CD complex:

30 ml of the ZnMFA: CeO_2NP complex solution was added to 9 ml of an aqueous solution of HPβ-CD (10 mg/ml). The solution was stirred for 10 minutes and left in the dark at room temperature for 24 hours. The solution was evaporated to dryness using a rotary evaporator to obtain a bright-yellow powder which is soluble in water. The powder was dried in a desiccator over $CaCl_2$.

Characterization of the supramolecular complex

Transmission electron microscopy (TEM) was performed using a TEM-125K electron microscope (Selmi, Ukraine) and a 100 kV electron beam. For sample preparation, 200 mesh carbon-coated Cu were used. A 2 μL drop of the test solution was deposited to a grid and the solvent allowed to evaporate. Absorption spectra were measured using a “Specord 200” spectrometer (Analytik Jena, Germany). The temperature of experiments was 25°C. Hydrodynamic diameters were measured using a ZetaPALS/BI-MAS analyzer (Brookhaven Instruments Corp., USA) operated in the phase analysis light scattering mode. Measurements were carried out at the scattering angle of 90° and laser emission at 659 nm. The temperature of experiments was 25°C.

Cell Culture

Hepatocellular carcinoma cells (HepG2; HB-8065, ATCC, USA) were cultivated in Minimal Essential Media (Sigma-Aldrich, Austria) and supplemented with 10% fetal bovine serum (Sigma-Aldrich, Austria) and 1% antibiotic/antimycotic solution (Sigma-Aldrich, Austria) to a confluency of approx. 60-80 % under cell culture conditions at 37°C and 5% CO_2.

Spheroid Generation

Cells for spheroid production were obtained from monolayer cultures. After rinsing with phosphate buffer (1X PBS, Sigma-Aldrich, Austria) and treatment with trypsin/EDTA (Sigma-Aldrich, Austria), cell suspension was centrifuged at 1250 rpm for 5 min, adjusted to a cell density of 15.000 cells/ml and 200 µl were pipetted to each well of the U-bottom ultra-low attachment plate (Corning, Austria). Cells were incubated at 37°C and 5% CO_2 humidified atmosphere and media was changed every 48h.

Intracellular ATP determination

For cell viability determination 3D spheroids were treated with CellTiter-Glo® 3D Reagent and RealTime-Glo™ MT Cell Viability Assay (Promega, Austria) according to manufacturer's protocol.

Live/dead staining

Spheroid viability was qualitatively evaluated using a commercially available fluorescence assay (LIVE/DEAD® Viability/Cytotoxicity Assay, Life Technologies, Austria).

Image acquisition

Phase-contrast micrographs of HepG2 spheroids were analyzed and spheroid diameters were measured using Olympus' CellSense Standard® software. Live/dead cells were monitored by a fluorescence microscope (IX83, Olympus, Germany) with fluorescein optical filter (ex 485, em 530) and rhodamine filter (ex 530 nm, em 645 nm).

Inductively coupled plasma mass spectrometry (ICP-MS)

After respective time points, HepG2 spheroids were washed with 1X PBS and fixed with 4% Paraformaldehyde (Sigma-Aldrich, Austria) for 48 hours. Spheroids were embedded in Paraffin and cut into 4 µm sections for histological staining with hematoxylin and eosin (H&E). For ^{140}Ce retention analysis in a 2D monolayer model, microscope glass slides (VWR, Austria)

were coated with 0.25 µg/ml Collagen I (from rat tail, Sigma-Aldrich, Austria), and HepG2 cells were seeded at an initial density of 10^5 cells/ml. HepG2 cells were incubated for 2 days at 37°C and 5% CO_2 until a confluency of approx. 80% was reached prior drug exposure with SMC. After respective time points, HepG2 monolayer were washed with 1X PBS, fixed with 4% Paraformaldehyde (Sigma-Aldrich, Austria) for 15 minutes and stored in 1X TBS buffer (Sigma-Aldrich, Austria) until proceeding with analysis.

For sample ablation a 213 nm frequency quintupled Nd:YAG laser (New Wave 213, ESI, Fremont, CA) was used. Samples are placed into a washout cell with washout times below one second. Ablated material is transported with a Helium gasflow to the coupled iCAP Qc ICP-MS instrument (ThermoFisher Scientific, Germany). The gas flow is mixed with Argon as make-up gas upon introduction to the plasma. For data acquisition the Qtegra software was provided by the manufacturer. Elemental images were acquired through the usage of Epina ImageLab 2.99.

A detailed description of the laser and measurement parameters can be found in Table S-1. Generally, all parameters were held constant with the exception of the laser scan speed and laser spot diameter. For imaging measurements, a spot diameter of 10 µm with a laser scan speed of 30 µm/s was chosen, whereas quantitative measurements were performed with a 40 µm laser spot diameter and 120 µm/s laser scan speed.

Table S-1: Summary of instrumental parameters used for LA-ICP-MS measurements

LA-ICP-MS		*ICP-MS*	
Wavelength	213 nm	Plasma power	1550 W
Pulse duration	4 ns	Cool gas flow	14 L/min
Laser repetition rate	20 Hz	Auxiliary gas flow	0.8 L/min
Laser Fluence	11 J/cm²	Cones	Ni
Laser scan speed	30 - 120 µm/s (imaging/ quantitative)	Monitored isotopes	27Al, 29Si, 31P, 64Zn, 67Zn, 68Zn, 140Ce, 142Ce
Laser spot diameter	10 - 40 µm (imaging/ quantitative)	Dwell time per isotope	10 ms
Carrier gas flow (He)	650 mL/min	Mass resolution (m/Δm)	300

For the quantification a dried droplet calibration approach was applied. Throughout the experiment ultra-pure water with a resistivity of 18 MΩ obtained from a Barnstead EASYPURE II water system (ThermoFisher Scientific, Marietta, OH, USA) was used for dilutions. The standards contained a cerium single standard solution (ThermoFisher, Germany) and di-sodium hydrogen phosphate dodecahydrate (p.a., Carl Roth, Germany) to calibrate cerium and

phosphorus respectively. Six solutions with cerium concentration ranging from 0.3 µg/l to 100 µg/l and di-sodium hydrogen phosphate dodecahydrate concentration ranging from 10 µg/l to 3000 µg/l were produced. 1 µL of each standard was pipetted on a glass substrate which was precoated with octadecyltrichlorosilane to make the surface hydrophobic and subdue the coffee-ring effect.

For sample ablation, a 213 nm frequency quintupled Nd:YAG laser (New Wave 213, ESI, Fremont, CA) was used. Ablated material was transported with Helium gasflow to the coupled iCAP Qc ICP-MS instrument (Thermo Fisher Scientific, Germany). For data acquisition the Qtegra software was provided by the manufacturer. Elemental images were acquired through the usage of Epina ImageLab 2.99. For quantification a dried droplet calibration approach was applied. Raw data was normalized to ^{31}P signal of one spheroid and respective ATP content per cell. For imaging measurements, a spot diameter of 10 µm with a laser scan speed of 30 µm/s was chosen, whereas quantitative measurements were performed with a 40 µm laser spot diameter and 120 µm/s laser scan speed.

Quantification of cytokine secretion

After 5 days of spheroid cultivation, inflammation was induced by 600 µM free fatty acids (FFA; at a molecular ration of 2:1 for oleic: palmitic acid) for 24 hours. Media was changed and HepG2 spheroids were treated with SMC for 48h. After cultivation, spheroid supernatants were collected and frozen at -20°C until ELISA of Human IL-6, Human IL-8 and Human TNF-alpha (Abcam, Germany). ELISA was performed according to the manufacturer's protocols.

Statistical Analysis

Statistical significance among the experimental groups was determined with Student's t-test. A P value $p<0.05$ was considered statistically significant (*). Graphs were plotted and statistical analysis was performed using Prism 8.2.1 (GraphPad Software, USA).

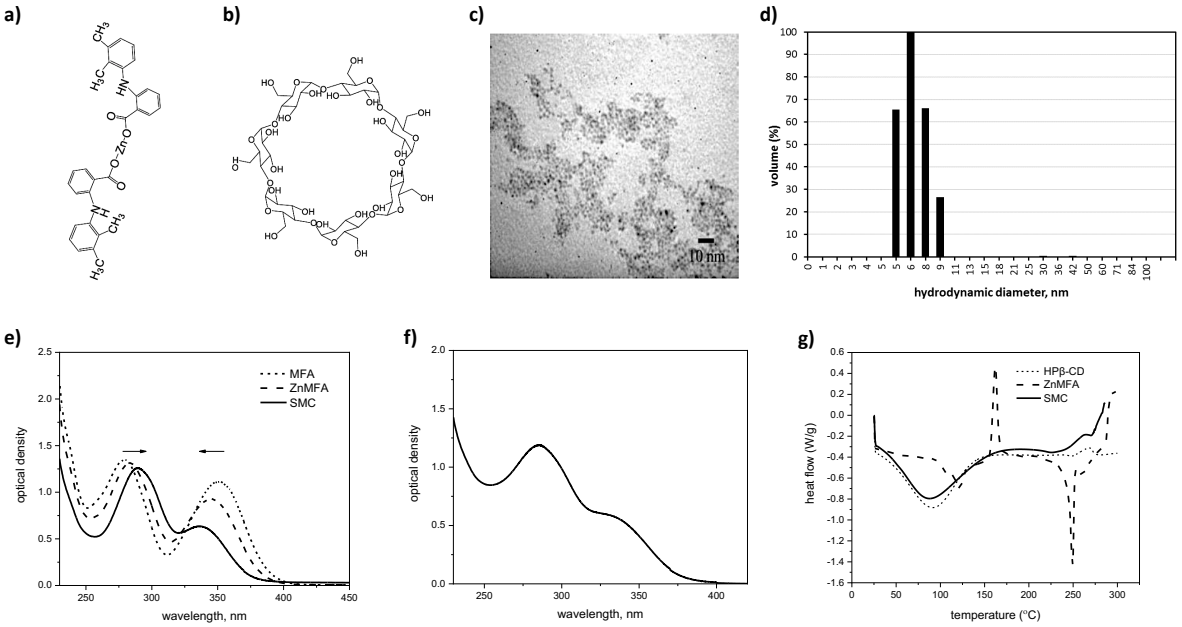


Figure S-1: a) Structural formulas of zinc salt of mefenamic acid, b) hydroxypropyl-β-cyclodextrin and c) TEM image of CeO_2 nanoparticles. d) Size Distribution (DLS) of a colloidal dispersion of the supramolecular complex (SMC) in water. UV/VIS absorption spectra of e) mefenamic acid (MFA), zinc salt of mefenamic acid ZnMFA and SMC in methanol and f) of SMC in water. g) Differential scanning calorimetry DSC curves for SMC and its components.

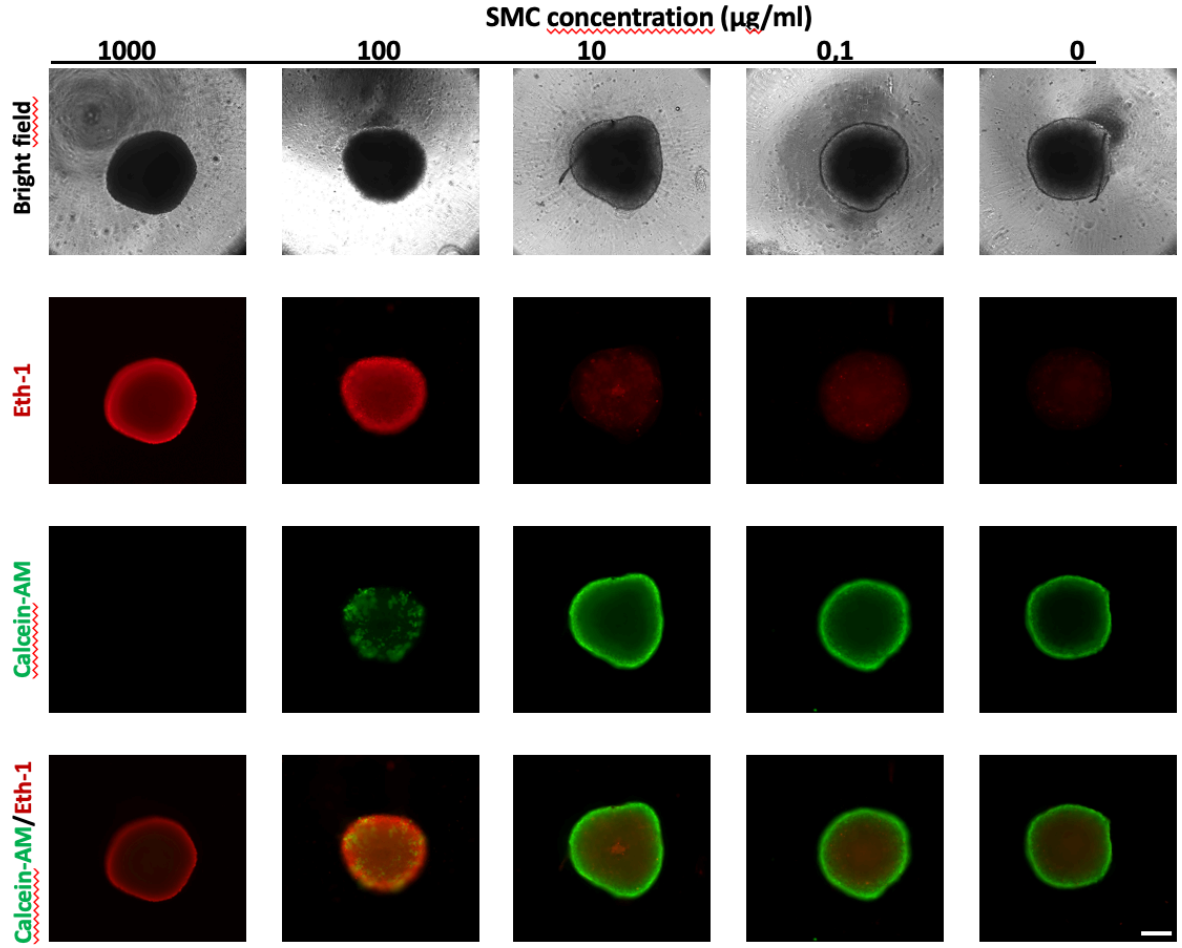
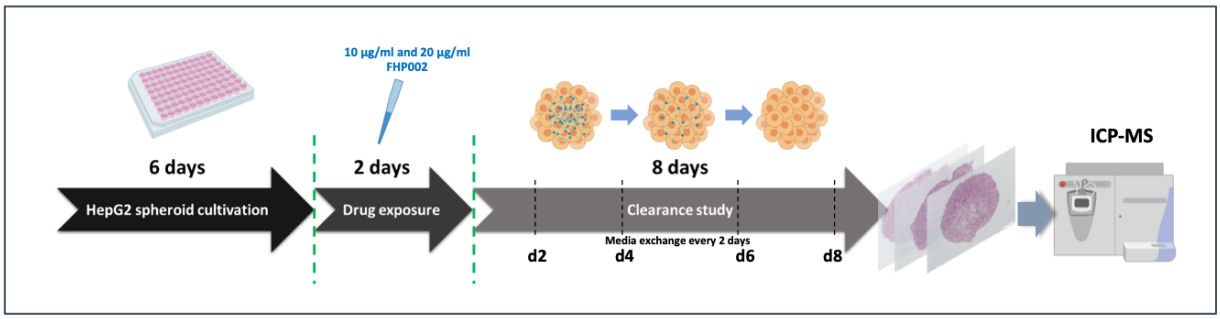


Figure S-2: Bright field and fluorescence micrographs of a live (green)–dead (red) assay on HepG2 spheroids after exposure time 96 hours with 1000 µg/ml, 100 µg/ml, 10 µg/ml and 0.1 µg/ml and 0 µg/ml SMC at day 6 post-seeding. Scale bars, 200 µm.



Scheme S-1: Schematic workflow of nanodrug retention study including HepG2 spheroid cultivation, exposure of 10 µg/ml and 20 µg/ml of supramolecular complex, periodic washing steps of spheroids with cell culture media for 8 days post-treatment, histology followed by quantification of ^{140}Ce by ICP-MS. The figures were exported under a paid subscription. Created with BioRender (www.biorender.com).

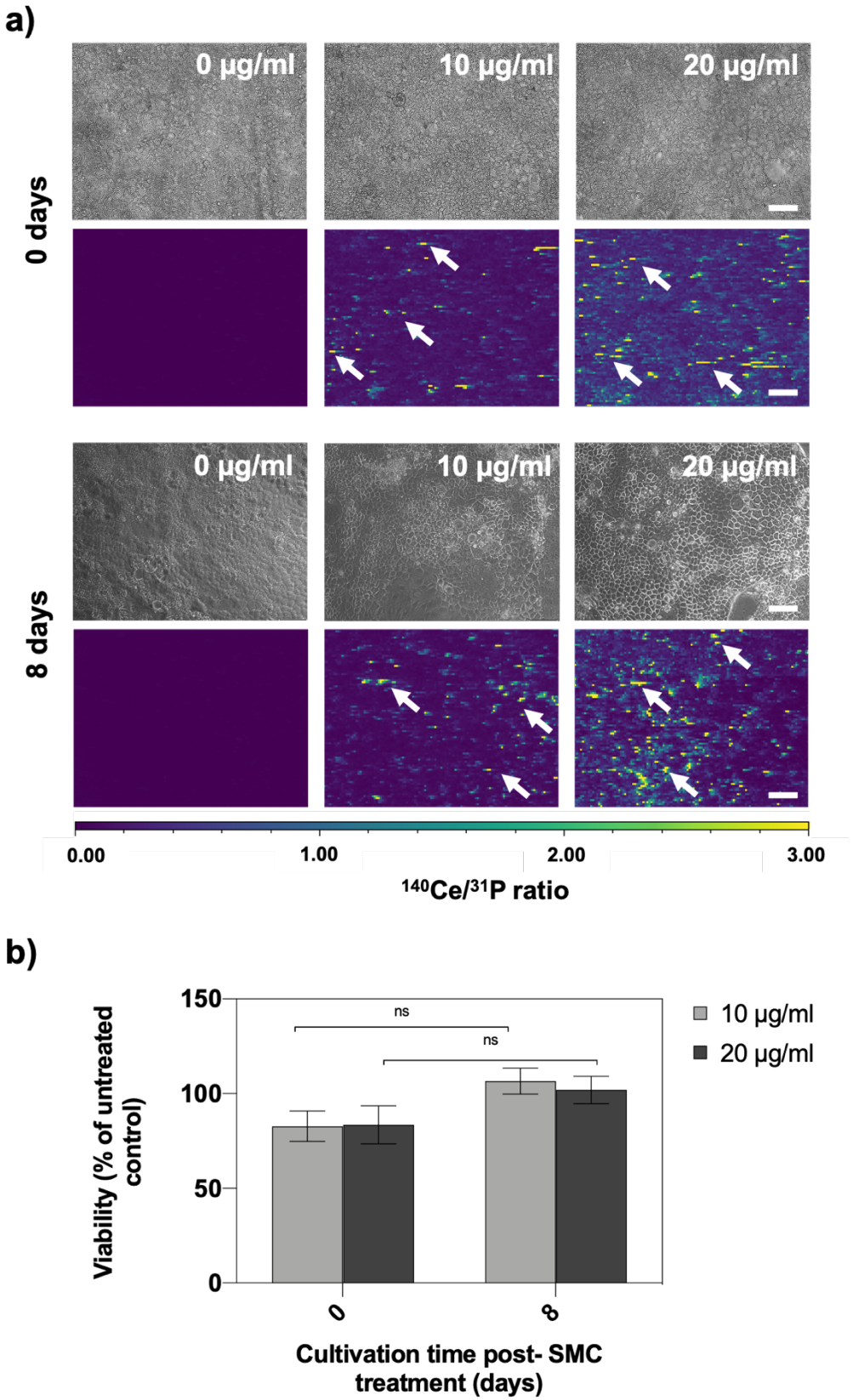


Figure S-3: a) Bright-field and LA-ICP-MS micrographs of $^{140}Ce/^{31}P$ signal ratio distribution in HepG2 monolayer culture after 0- and 8-days post-treatment with 0, 10, and 20 µg/ml SMC. Scale bar, 100 µm. b) Cellular viability of HepG2 monolayer culture after 0- and 8-days of exposure at SMC concentrations of 10 µg/ml and 20 µg/ml, n=3 ± SD.

Table S-2: Table of the anti-inflammatory capacity of the supramolecular complex in an untreated and free fatty acid (FFA) treated HepG2 spheroid model.

Inflammatory factor	*control (pg/ml)*	*+ FFA (pg/ml)*	*Effective nanodrug doses (µg/ml)*	*+ FAA +SMC (pg/ml)*	*p-value (treated vs. inflamed)*	*Reduction of cytokine secretion (%)*
TNF-α	676.5 ± 44.0	895.1 ± 100.5	0.1	653.6 ± 67.3	$p < 0.05$	30.9 ± 1.7
IL-6				No effect		
IL-8	133.1 ± 5.2	182.4 ± 3.8	0.1	170.6 ± 4.6	$p < 0.05$	6.4 ± 3.6
			1.0	157.0 ± 4.0	$p < 0.01$	13.9 ± 0.7

References

(1) Vladimir K., K.; Anna V., G.; Olga O., S.; Yuri V., M. Colloids and Surfaces A: Physicochemical and Engineering Aspects 2012, 409, 176-182.