Electronic Supplementary Information

When drug nanocarriers miss their target: extracellular diffusion and cell uptake are not enough to be effective

Vincent Pautu,[a] *Heng Zhao,*[b] *Angelika Mielcarek,*[b] *Anna Balasso,*[a] *Patrick Couvreur,*[a] *Christian Serre,*,*[b] *Simona Mura,*,*[a]

[a] Institut Galien Paris-Saclay, UMR 8612, CNRS, Université Paris-Saclay, Faculté de Pharmacie, 5 rue Jean-Baptiste Clément, F-92296 Châtenay-Malabry cedex, France.

E-mail: simona.mura@universite-paris-saclay.fr

[b] Institut des matériaux poreux de Paris, Ecole normale supérieure, ESPCI Paris, CNRS, PSL University, 75005 Paris, France

E-mail: christian.serre@ens.psl.eu

Experimental Section

Materials

Iron (III) nitrate nonahydrate, benzene-1,3,5-tricarboxylic acid (trimesic acid) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl) were purchased from Alfa Aesar (France). 4-nitrobenzenediazonium tetrafluoroborate was obtained by TCI Europe (France). Amine- poly(ethylene glycol) (PEG) (Mw 5000 Da) was purchased from Iris Biotech (Germany). N-hydroxysulfosuccinimide sodium salt (sulfo-NHS), acryl-PEG (Mw 5000 Da) and analytical grade solvents were purchased from Sigma Aldrich (France). Doxorubicin HCl was obtained from Carbosynth (UK). Ultrapure water was obtained with the MilliQ purification system (Merck Millipore, France).

Synthesis of nanoMOFs

MIL-100 (Fe) nanoMOFs were synthesized as previously described by a green synthesis method.[1] Iron (III) nitrate nonahydrate (0.9 mmol, 1.5 eq.) and trimesic acid (0.6 mmol 1 eq.) were dissolved in 45 mL of ultrapure water and stirred for 2 days at room temperature. After distribution in 2 mL Eppendorf tubes, synthesized nanoMOFs were recovered by centrifugation (16,870 *g* for 10 min). Non-reacted residual reagents were removed by washing nanoMOFs twice in water (2 mL) and twice in ethanol (2 mL). At each washing cycle, particles were collected by centrifugation (16,870 *g* for 10 min) and redispersed by sonication for 10 seconds using a VibraCell sonicator Type 75041 (Fisher Scientific, Aalst, Belgium) at 20% power. Stock nanoMOF dispersions in ethanol were stored at 4°C until use.

Doxorubicin encapsulation

Doxorubicin (Doxo) encapsulation was performed by the soaking method as reported previously.[2] Briefly, nanoMOFs (5 mg) were dispersed in 1.5 mL of doxorubicin aqueous solution (10 $mg.mL^{-1}$) and kept under agitation for 24 h in the dark. Particles were then collected

by centrifugation (16,870 *g* for 10 min) and washed five times in ultrapure water (2 mL) in order to remove the free drug. At each washing cycle, particles were collected by centrifugation (16,870 *g* for 10 min) and redispersed by sonication for 10 seconds using a Type 75041 (Fisher Scientific, Aalst, Belgium) at 20% power. Encapsulation efficiency and loading capacity were determined following quantification of doxorubicin in the supernatant by UV-Vis absorbance detection at 480 nm (Lambda 25 spectrometer, PerkinElmer, France).

Synthesis of PEG-functionalized nanoMOFs (Covalent method)

Covalent surface functionalization of nanoMOFs (nanoMOF_Cv) was performed as previously described.[3] Briefly, nanoMOFs (5 mg) were dispersed in ethanol (500 µL). After addition of EDC hydrochloride (4.6 mg) and a catalytic amount sulfo-NHS, a PEG-NH_2 aqueous solution (0.1 µmol, 500 µL) was poured to the nanoMOF dispersion. The mixture was stirred for 30 min at room temperature. Particles were collected by centrifugation (16,870 *g* for 10 min) and washed three times in ultrapure water (2 mL). At each washing cycle, particles were collected by centrifugation (16,870 *g* for 10 min) and redispersed by sonication for 10 seconds using a Type 75041 (Fisher Scientific, Aalst, Belgium) at 20% power.

The same protocol was followed for the surface modification of drug-loaded nanoMOFs (nanoMOF_Cv_Doxo). Loss of doxorubicin during the PEGylation process was determined by drug quantification in the supernatant by UV-Vis absorbance detection at 480 nm (Lambda 25 spectrometer, PerkinElmer, France).

Synthesis of PEG-functionalized nanoMOFs (GraftFast® method)

GraftFast® surface modification (nanoMOF_Gf) was performed according to an already established protocol.[4] Practically, nanoMOFs (5 mg) were resuspended in ultrapure water (500 µL). Acryl-PEG (0.77 µmol) and 4-nitrobenzenediazonium tetrafluoroborate (25.35 µmol) were dissolved in 500 µL of ultrapure water and then poured to the nanoMOF dispersion.

Complete mixture of the reagents occurred after stirring for few minutes. After addition of a catalytic amount of iron powder, the mixture was kept under agitation for 40 min at room temperature. At the end of the reaction, nanoMOFs were recovered with a glass pipette using a 1.1 T magnet to avoid collection of the residual non-reacted iron powder.

Particles were collected by centrifugation (16,870 *g* for 10 min) and washed a first time in ultrapure water (2 mL) and then twice in ethanol (2 mL). A final washing step in water (2 mL) ensured the complete removal of all unreacted reagents. At each washing step, particles were collected by centrifugation (16,870 *g* for 10 min) and redispersed by sonication for 10 seconds using a Type 75041 (Fisher Scientific, Aalst, Belgium) at 20% power. The same protocol was followed for the surface modification of drug loaded nanoMOFs (nanoMOF_Gf_Doxo). Loss of doxorubicin during the PEGylation process was determined by drug quantification in the supernatant by UV-Vis absorbance detection at 480 nm (Lambda 25 spectrometer, PerkinElmer, France).

NanoMOF characterization

Mean diameter and particle size distribution of nanoMOFs were assessed by dynamic light scattering (DLS) at 25 °C (Zetasizer Nano ZS, Malvern Instruments, UK, 173° scattering angle). Particle surface charge was investigated using the same apparatus by zeta potential measurements at 25 °C after dilution with 1 mM NaCl solution applying the Smoluchowski equation. For all measurements nanoMOF concentration was kept constant at 0.1 $mg.mL^{-1}$.

The crystal structure of nanoMOFs was assessed by X-ray powder diffraction (XRPD) analysis on a high- throughput powder diffractometer (D8 Advance, Bruker AXS, Germany) equipped with a CuKα radiation (λ = 1.5418 Å) and a LynxEye detector. Thermogravimetric analyses (TGA) were performed on a PerkinElmer Diamond TGA/DTA STA 6,000. Around 5 mg of sample was heated at a rate of of 5 $°C \cdot min^{-1}$ from 25 °C to 600 °C, under O_2 atmosphere (20

$mL.min^{-1}$). Measurements were repeated at least in triplicate. Method used for the analysis of the raw TGA data is reported on Schema S1.

Fluorescence spectra

Fluorescence spectra of free Doxo and Doxo-loaded nanoMOFs in water (doxorubicin concentration 10 µM) were recorded with a spectrofluorometer (FP 750, Jasco). The emission spectra were collected in the 500-650 nm range after sample excitation at 480 nm.

Doxorubicin release in complete cell culture medium

Doxo-loaded nanoMOFs were incubated (final Doxo concentration 10 µM) in phenol-free Roswell Park Memorial Institute (RPMI 1640) cell culture medium supplemented with 10% fetal bovine serum, penicillin (50 U mL^{-1}) and streptomycin (0.05 mg mL^{-1}). Incubation was carried out at 37°C under gentle agitation. At different time points, each sample (2 mL) was centrifuged (21,060 *g*, 30 min). The supernatant was collected, and the doxorubicin released from the nanoparticles was quantified using an UV-Vis spectrophotometer (ELX 800, BioteK, France).

Doxorubicin release in PBS

Doxo-loaded nanoMOFs (1 $mg.mL^{-1}$) were incubated in phosphate saline buffer (PBS) at pH 5.1 and 7.4. Incubation was carried out at 37 °C under gentle stirring (250 rpm). At different time points, each sample (2 mL) was centrifuged (21,060 *g*, 30 min). The supernatant was collected, and the doxorubicin released from the nanoparticles was quantified using an UV-Vis spectrophotometer (Lambda 45, PerkinElmer, France).

Cell lines

Human epithelial lung carcinoma cells (A549) and human lung fibroblasts (MRC-5) were purchased from ATCC (France) and maintained as recommended. Briefly, A549 cells were maintained in Roswell Park Memorial Institute medium (RPMI 1640, Sigma Aldrich, France) supplemented with 10% fetal bovine serum (FBS, Gibco, France). MRC-5 cells were cultured in Eagle's Minimum Essential Medium (EMEM, Sigma Aldrich, France) supplemented with 10% heat-inactivated FBS and 1% of 200 mM L-glutamine solution (Sigma Aldrich, France). All media were further supplemented with penicillin (50 $U.mL^{-1}$) and streptomycin (50 $\mu g.mL^{-1}$) (Sigma Aldrich, France). Cells were maintained in a humid atmosphere at 37 °C with 5% CO_2. Cells were used below passage 15 after thawing and harvested at a confluence of 70-80%.

Green fluorescent protein (GFP)-expressing fibroblasts were generated by lentiviral transduction according to manufacturer protocols as previously described.[5] Briefly, MRC-5 fibroblasts (9×10^4 $cells.mL^{-1}$) were seeded in 24-well plates (1 mL *per* well) and incubated for 24 h in a humid atmosphere at 37 °C with 5% CO_2. Transduction was performed by cell incubation with pLenti-C-mGFP-P2A-Puro particles (Origene, Germany). A polybrene-containing medium (8 $\mu g.mL^{-1}$) and a multiplicity of infection (MOI) of 10 were used. Transduction particle-containing medium was removed after 24 h and replaced with fresh medium containing puromycin (1 $\mu g.mL^{-1}$) (Thermo Fisher Scientific, France) as selection antibiotic. Puromycin-containing medium was replaced every 3 days until resistant colonies could be identified. Cell fluorescence was measured 48 h after infection and prior to the cryopreservation of selected cells.

NanoMOF cytotoxicity (2D cell culture)

Cytotoxicity studies were performed using the 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. 100 µL of cell dispersion (7×10^4 and 5×10^4 $cells.mL^{-1}$ for

A549 and MRC5, respectively) was seeded in 96-well plates 24 h before the treatment with serial dilutions of nanoMOFs in culture medium. After 72 h incubation, 20 µL of a 5 $mg.mL^{-1}$ MTT (Sigma Aldrich, France) solution in phosphate buffered saline was added to each well. Following 2 h incubation, culture medium was removed and formazan crystals were dissolved in 200 µL of dimethyl sulfoxide (DMSO). Spectrophotometric measurements of the solubilized dye absorbance were performed on a microplate reader (LAB System Original Multiscan MS) at 570 nm. Cell viability was calculated as the absorbance ratio of treated cells *versus* the control ones (*i.e.*, untreated cells). All experiments were repeated at least three times to determine means and standard deviations (SD).

Assessment of Doxo and Doxo-loaded nanoMOF uptake by CLSM (2D cell culture)

A549 cells (4 x 10^5 cells *per* well, 6 well plates) were seeded onto twenty-five mm diameter confocal microscopy glass slides and cultured at 37 °C with 5% CO_2 in complete medium. After 24 h the latter was discarded, and cells were treated with 2 mL of free Doxo (10 µM) or Doxo-loaded nanoMOF (10 µM equivalent in doxorubicin) in complete medium for 24 h. Next, the medium was removed, and cells were washed with PBS (1 mL). Nuclei were stained with Hoechst 33342 solution in PBS (20 min, 37°C). Finally, cells were washed twice with PBS and microscopy glasses were transferred to a live-cell imaging chamber with 1 mL of complete medium. All acquisitions were performed with a Confocal Laser Scanning Microscope (CLSM) TCS SP8 (Leica, Germany) equipped with a CS2 Plan Apochromat 63x/NA 1.4 oil immersion objective lens and a WLL Laser (488 nm) and a 405 nm diode for Doxo and Hoechst 33342 excitation, respectively. Fluorescence was collected with 411-453 nm wide emission slits for the blue signal and 539–601 nm wide emission slits for the red one. Transmission images were realized with a PMT-trans detector. Images were acquired in 1024 x 1024 pixels size, 12-bit depth, 400 Hz scanning speed using the Leica SP8 LAS X software (Version 3.1.5, Leica, Germany).

Intracellular localization of Doxo-loaded nanoMOFs by CLSM (2D cell culture)

A549 cells (4.2 x 10^4 cells *per* well) were seeded onto 8-well polymer µ-Slides (ibidi, GmbH, France) in complete medium and cultured for 24 h. Medium was then discarded, and cells were treated for 24 h with 300 µL of Doxo-loaded nanoMOFs (10 µM equivalent in doxorubicin). Cells were washed with PBS (1 mL) and lysosomes were stained (30 min, 37°C) using the Lysosomal staining Kit blue fluorescence (Abcam, France). Finally, cells were washed twice with PBS (1 mL) and 300 µL of complete medium was added in each well. All acquisitions were made with a CLSM TCS SP8 (Leica, Germany) equipped with a CS2 Plan Apochromat 63x/NA 1.4 oil immersion objective lens and a WLL Laser (488 nm) and a 405 nm diode for Doxo and Hoechst 33342 excitation, respectively. Fluorescence was collected with 411-453 nm wide emission slits for the blue signal and 539–601 nm wide emission slits for the red one. Transmission images were realized with a PMT-trans detector. Images were acquired in 1024 x 1024 pixels size, 12-bit depth, 400 Hz scanning speed using the Leica SP8 LAS X software (Version 3.1.5, Leica, Germany).

Construction of 3D multicellular tumor spheroids (MTCS)

Heterotype A549:MRC-5 MCTS were constructed according to the liquid overlay technique [6] using 96 round-bottomed well plates (CELLSTAR®, Sigma Aldrich, France). Before use, 50 µL of 1.2 % (w/v) poly-2-hydroxyethyl methacrylate (pHEMA, Sigma Aldrich, France) ethanolic solution was added to each well, and solvent was evaporated in sterile conditions. For the spheroid construction, suspensions of each cell type were prepared in RPMI complete medium and then 200 µL of their opportune mixture was transferred into each well. The number of A549 cells was fixed at 5000 cells *per* well. A ratio of 1:2 among A549: MRC-5 has been used. After cell seeding, plates were centrifuged (200 *g*, 5 min, 20 °C) and then incubated in a humidified atmosphere with 5% CO_2 at 37 °C for a minimum of 72 h. For long term culture, half of the

medium was replaced every 3 days. Fluorescently-labelled spheroids were prepared using GFP-expressing MRC-5 fibroblasts.

Spheroid optical imaging

Optical images were acquired using the AxioObserver Z1/Colibri/TIRF (Carl Zeiss, Germany) inverted microscope equipped with a Peltier cooled (-40 °C) CoolSnap HQ2 CCD camera (Photometrics,Tucson, USA) and an XL incubator thermostated at 37 °C providing 5% CO_2. By using a halogen lamp and a motorized stage in automated mode (ZenBlue software / high content acquisitions), transmitted light images of spheroids were collected directly from the pHEMA-coated plates with an EC Plan-Neofluar 2.5x/NA 0.085 dry objective lens. Through an image-processing macro, specifically created with the Image-J® software, the minimum and major diameters of at least 60 spheroids were measured. Spheroid volume (V) was calculated according to the formula $V = \frac{4}{3}\pi r^3$ where $r = \frac{1}{2}\sqrt{d_1 d_2}$ corresponds to the spheroid geometric mean radius.

Light Sheet Fluorescence Microscopy (LSFM) imaging of spheroids

3-day-old fluorescently-labelled spheroids were fixed in 500 µL of 4% paraformaldehyde (Roti®-Histofix 4%) (1 h, room temperature) and then permeabilized with 500 µL of 0.1% Triton X-100 (Sigma Aldrich, France) in PBS (1 h, room temperature). Then, the triton solution was replaced with 200 µL of PBS and cell nuclei were stained overnight with Hoechst 33342 (NucBlue™ Reagent, Thermo Fisher Scientific, France) in the dark at room temperature. MCTS were imaged with the Lightsheet Z.1 Microscope (Carl Zeiss, Germany) equipped with a Plan-Apochromat 20x 1NA water-immersion objective lens with left and right illumination. After sample excitation at 405 nm (Hoechst 33342) and 488 nm (GFP), fluorescence signals were collected using a 420-470 nm band pass (BP) and a 505-545 nm BP emission filter for

Hoechst 33342 and GFP, respectively. Samples were scanned using the Zen 2014 SP1 Black-Edition software (Carl Zeiss, Germany). The 3D rending Imaris® software (Bitplane AG, Zurich - Switzerland) was used to obtain a 3D representation and the optical section of the spheroids.

Spheroid histology and immunohistochemistry

MCTS were fixed for 2 hours in 500 µL of 4% paraformaldehyde (Roti®-Histofix 4%, Roth Sochiel EURL, France) at room temperature. After inclusion in 4% low-melting agarose (UltraPure LMP Agarose, Invitrogen), spheroids were embedded in paraffin and sectioned. Sections (5 µm) were stained with haematoxylin and eosin (H&E) according to standard protocols. For immunohistochemical staining, the heat-mediated antigen retrieval of antibodies was carried out in citrate buffer at different pH (according to manufacturer protocols) and sections were then incubated with monoclonal antibody to cytokeratin AE1/AE3 (mouse, dil 1:50; M351501-2 Dako, France) and fibronectin (rabbit, dil 1:250; ab2413 Abcam, UK). Primary antibodies were detected with peroxidase-conjugated secondary antibodies by using diaminobenzidine as chromogen and haematoxylin as a counterstain.

Doxo and Doxo-loaded nanoMOF cytotoxicity (3D cell culture)

Spheroids were prepared and cultured for 3 days before treatment with several dilution of doxorubicin or Doxo-loaded nanoMOFs in complete culture medium. After 24, 48 and 72 h, viability assessment was performed by ATP quantification using the CellTiter-Glo® 3D reagent (Promega France). Briefly, 150 µL of the medium was discarded and 50 µL of the CellTiter-Glo® 3D reagent was added to each well. Plates were gently shaken under dark for 10 min and samples were kept under dark at room temperature for additional 20 minutes. Samples were then transferred into white opaque 96-well plates and the luminescence signal was measured with a benchtop plate reader (EnSpire Alpha 2390; PerkinElmer, USA) (4 replicates per

condition, n=4). Cell viability was calculated as the ratio of the luminescence of treated well compared to average signal of untreated spheroids.

Spheroid optical clearing

Clearing solution. A clearing solution was prepared by mixing under stirring fructose (45.03 g) (Sigma Aldrich, France), glycerol (50 mL) (Sigma Aldrich, France) and ultrapure water (10.6 mL) until complete fructose dissolution. Then, water was added to obtain 100 mL final volume.

Light sheet microscopy embedding solution. In order to image cleared spheroids by LSFM, an embedding solution was prepared by dissolving 200 mg of low melting agarose (UltraPure LMP Agarose, Invitrogen) in 5 mL of ultrapure water at 60°C under stirring. Obtained solution was cooled to 40°C, and then 5 mL of fructose/glycerol clearing solution was added. The mixture was stirred until perfectly clear.

Assessment of Doxo and Doxo-loaded nanoMOF penetration into spheroids by CLSM

Spheroids were prepared and cultured for 3 days before incubation with the free drug or drug-loaded nanoMOFs in complete medium at a Doxo concentration of 10 µM. After 24 h, spheroids were harvested and transferred in a microtube. Residual medium was carefully removed and MCTS were washed twice with PBS, fixed with 500 µL of 4% paraformaldehyde (Roti®-Histofix 4%) (1 h, room temperature) and then permeabilized with 500 µL of 0.1% Triton X-100 (Sigma Aldrich, France) in PBS (1 h, room temperature). Then, spheroids were washed once with PBS and cell nuclei were stained overnight with Hoechst 33342 (NucBlue™ Reagent, Thermo Fisher Scientific, France) in the dark at room temperature. Spheroids were then cleared according to a previously published protocol.[7] Briefly, spheroids were harvested and placed on a glass slide (SuperFrost™ plus, VWR). As much as possible of the buffer was removed and 100 µL of fructose/glycerol clearing solution was then added. After 20 min incubation,

spheroids were transferred in fresh clearing solution on a new glass slide and covered with a cover glass. Flattening of spheroids was prevented placing a double side tape between the two slides.

All acquisitions were made with a Confocal Laser Scanning Microscope TCS SP8 (Leica, Germany) equipped with CS2 Plan Apochromat 40x/NA 1.1 water immersion objective lens with 0.75 numerical zoom, a 405 nm diode for Hoechst 33342 and a WLL Laser (488 nm) for Doxo excitation, respectively. Fluorescence was collected with a 411-453 nm wide emission slits for the Hoechst signal and a 539–646 nm wide emission slits for the Doxo one. Transmission images were realized with a PMT-trans detector. Images were acquired in 1024 x 1024 pixels size, 12-bit depth, 400 Hz scanning speed using the Leica SP8 LAS X software (Version 3.1.5; Leica, Germany). Z-series optical sections were collected with a step of 4 µm using the LAS X software.

Assessment of Doxo and Doxo-loaded nanoMOF penetration into spheroids by LSFM

Spheroids were prepared and cultured for 3 days before incubation with the free drug or drug-loaded nanoMOFs in complete medium at a Doxo concentration of 10 µM. After 24 h, spheroids were harvested and transferred in a microtube. Residual medium was carefully removed and MCTS were washed twice with PBS, fixed with 500 µL of 4% paraformaldehyde (Roti®-Histofix 4%) (1 h, room temperature) and then permeabilized with 500 µL of 0.1% Triton X-100 (Sigma Aldrich, France) in PBS (1 h, room temperature). Then, spheroids were washed once with PBS and cell nuclei were stained overnight with Hoechst 33342 (NucBlue™ Reagent, Thermo Fisher Scientific, France) in the dark, at room temperature. Spheroids were then embedded in a light-sheet embedding solution as previously described.[7]

Firstly, spheroids were harvested and placed on a glass slide (SuperFrost™ plus, VWR).Then, as much as possible of the buffer was removed and 50-100 µL of light sheet embedding solution was added to the spheroids. Before solidification of the embedding solution, each spheroid was

aspirated using a light sheet cylindrical glass capillary. After solidification, capillaries were placed in a tube containing the fructose/glycerol clearing solution to allow the sample to settle before analysis. Acquisitions were realized with a Lightsheet Z.1 Microscope (Carl Zeiss, Germany) equipped with a Plan-Apochromat 20x/NA1 Clearing - dipping objective lens. Samples were scanned using the Zen 2014 SP1 Black-Edition software (Carl Zeiss, Germany). 405 nm and 488 nm lasers were used for Hoechst 33342 and Doxo excitation, respectively. Fluorescence signals were recorded using a 420-470 nm band pass (BP) emission filter for Hoechst and a 575-615 nm BP emission filter for Doxo. 3D representation and optical section of the spheroids were obtained using the 3D rending Imaris® software (Bitplane AG, Zurich - Switzerland).

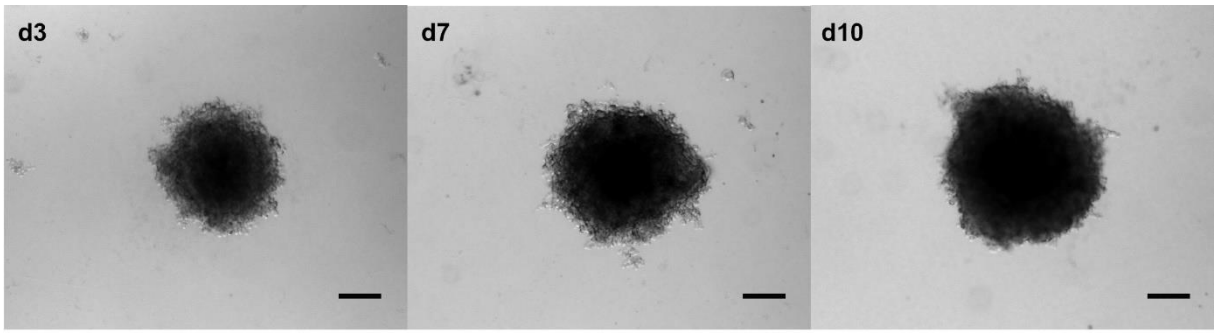


Figure S1. Representative optical imaging of A549:MRC-5 spheroids at day 3, 7 and 10 post seeding. Scale bars: 200 μm.

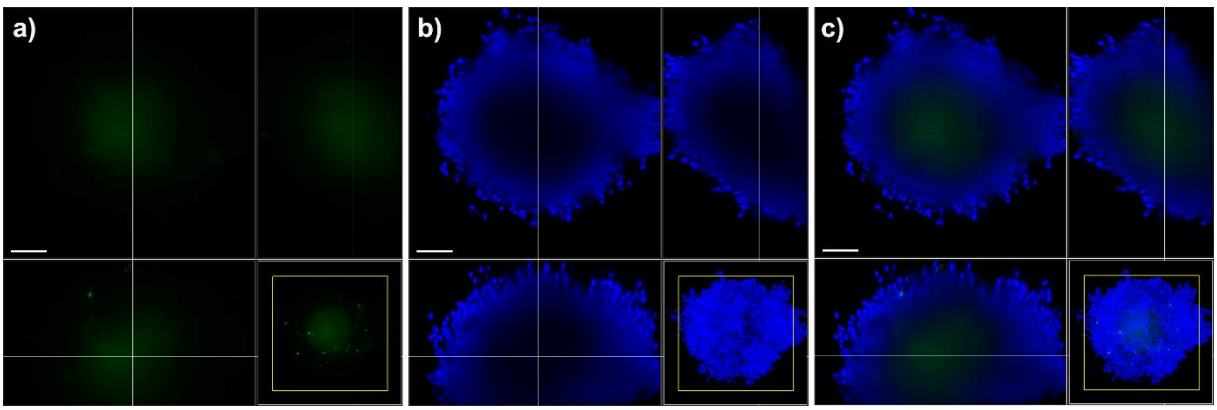


Figure S2. LSFM imaging of A549:MRC-5 spheroids at day 7 post seeding: (a) single green channel (GFP-labeled fibroblasts); (b) single blue channel (Hoechst 33342, nuclei) and (c) overlay of blue and green fluorescence. Scale bars: 100 μm.

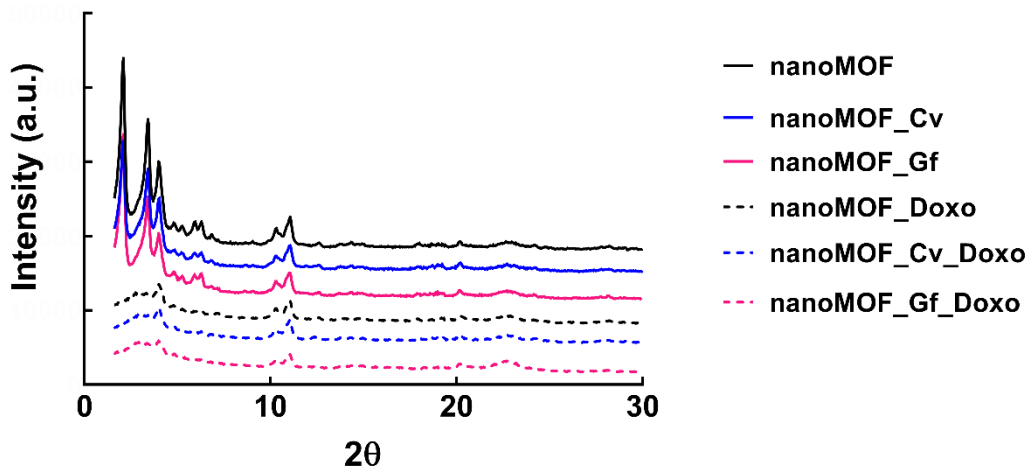


Figure S3. XRPD patterns of empty (solid lines) and Doxo-loaded (dashed lines) nanoMOFs.

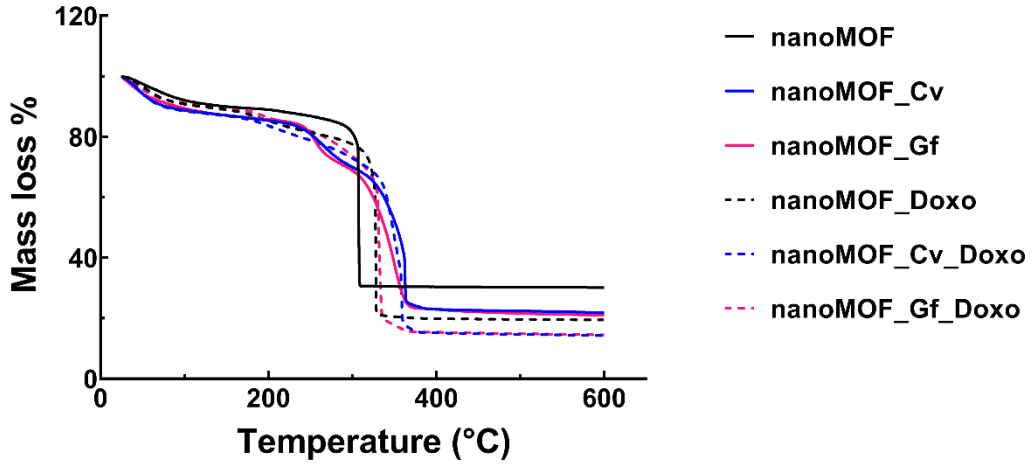


Figure S4. Thermogravimetric analysis of empty (solid lines) and Doxo-loaded (dashed lines) nanoMOFs under oxygen flow at a measuring rate of 5 °C.min^{-1}.

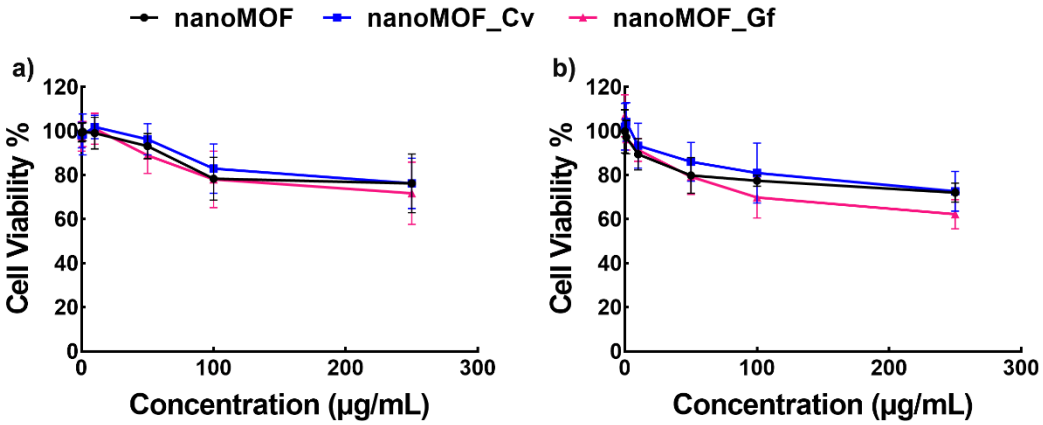


Figure S5. Cell viability of (a) A-549 and (b) MRC-5 cells treated with increasing concentrations of nanoMOFs for 72 h at 37 °C. Values represent mean ± standard deviation. (n=3)

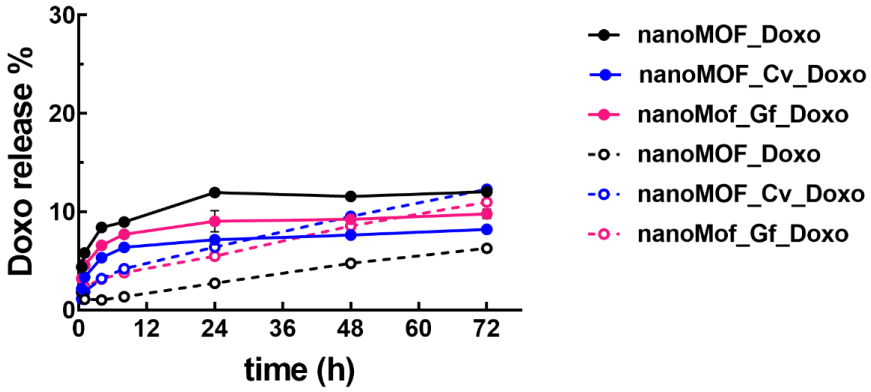


Figure S6. Release profile of doxorubicin from nanoMOFs in PBS buffer at pH 5.1 (solid lines) and 7.4 (dashed lines). Values represent mean ± standard deviation. (n=3)

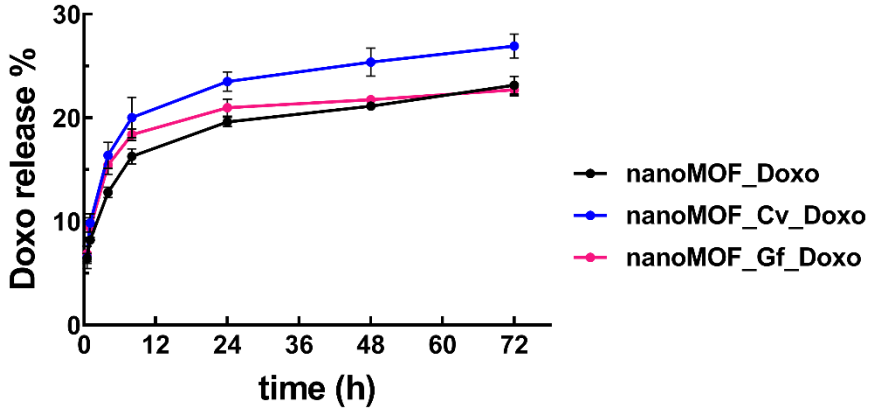


Figure S7. Release profile of doxorubicin from nanoMOFs in complete cell culture medium at 37°C. Values represent mean ± standard deviation. (n=3)

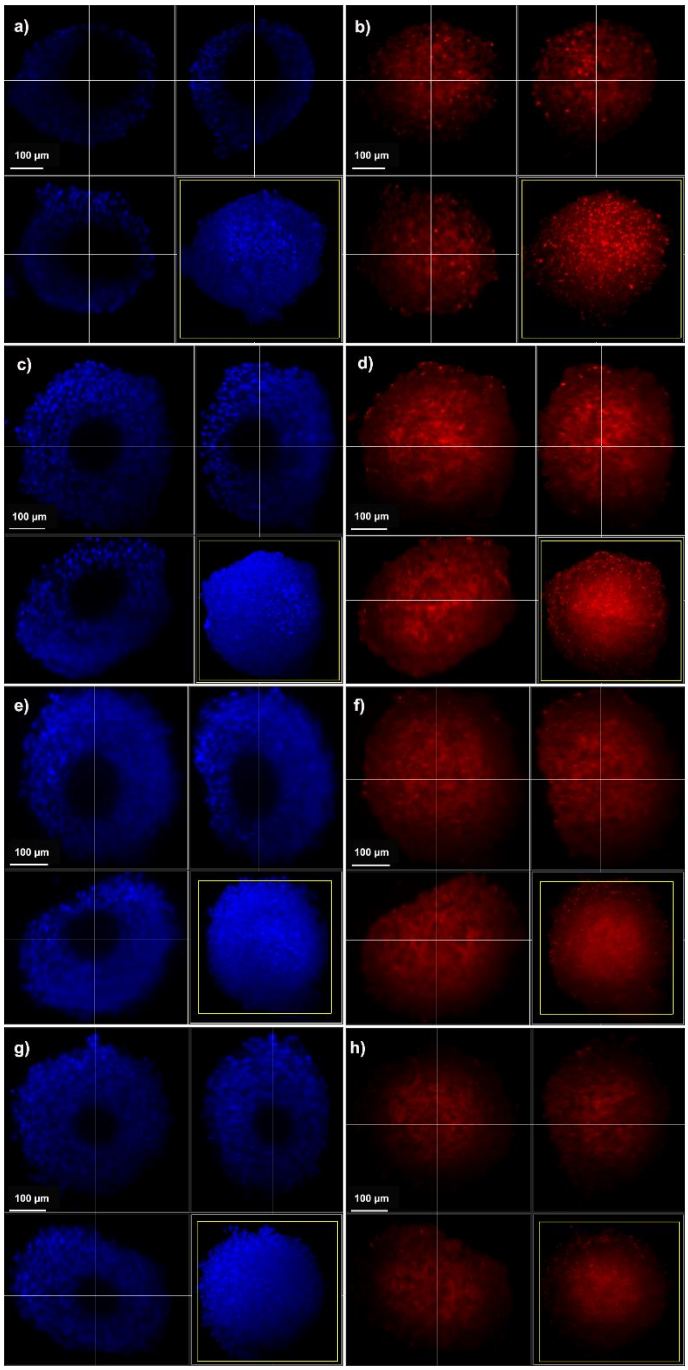


Figure S8. LSFM imaging of MCTS treated for 24 h with (a, b) free Doxo; (c, d) nanoMOF_Doxo; (e, f) nanoMOF_Cv_Doxo and (g, h) nanoMOF_Gf_Doxo at a Doxo concentration of 10 µM. (a, c, e, g) single blue signal (Hoechst 33342, nuclei); (b, d, f, h) single red signal (doxorubicin). Images represent orthogonal section at the middle of the spheroids. For each condition, the 3D tomography of the spheroid is included in the yellow square. Scale bars: 100 µm.

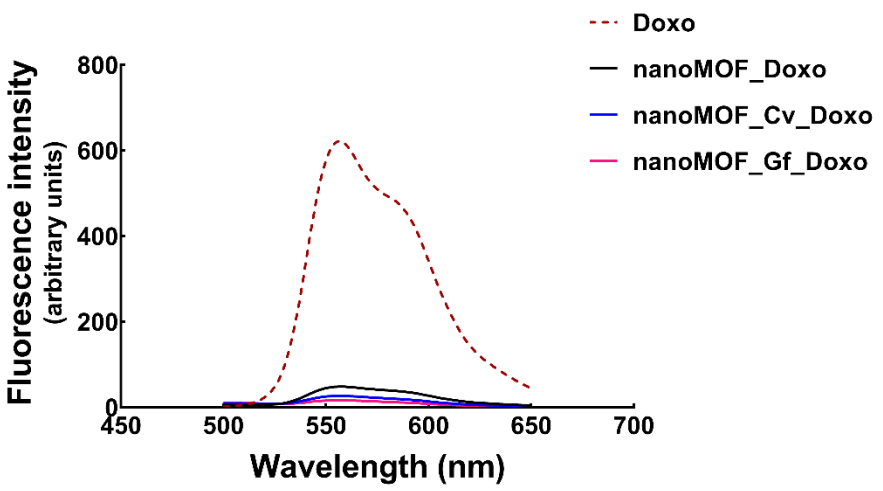


Figure S9. Fluorescence spectra of free doxorubicin (Doxo) and Doxo-loaded nanoMOFs at a concentration of 10 µM in water.

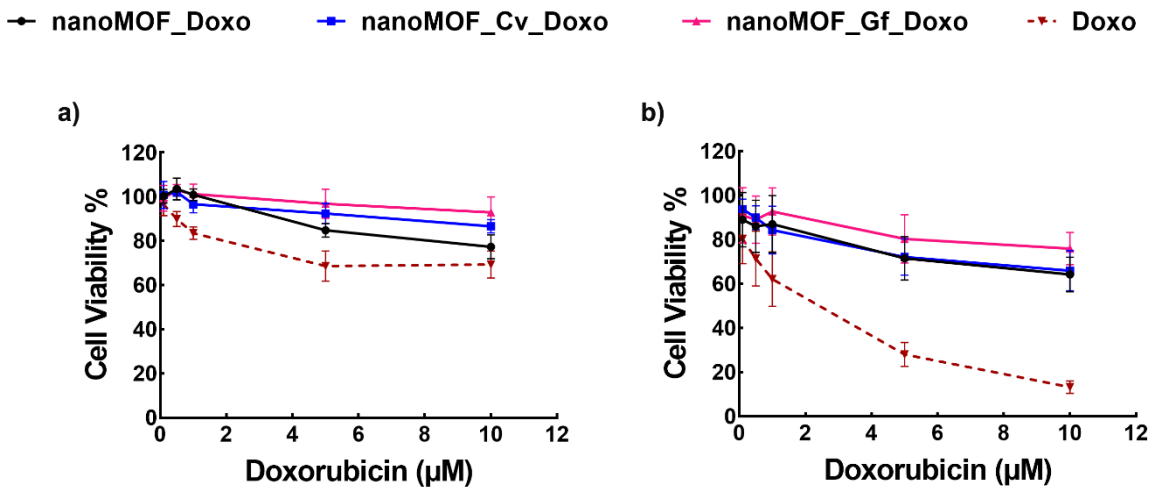


Figure S10. Spheroid viability following exposure to increasing concentrations of free doxorubicin (Doxo) or Doxo-loaded nanoMOFs for (a) 24 h and (b) 48 h at 37 °C. Values represent mean ± standard deviation. (n=3)

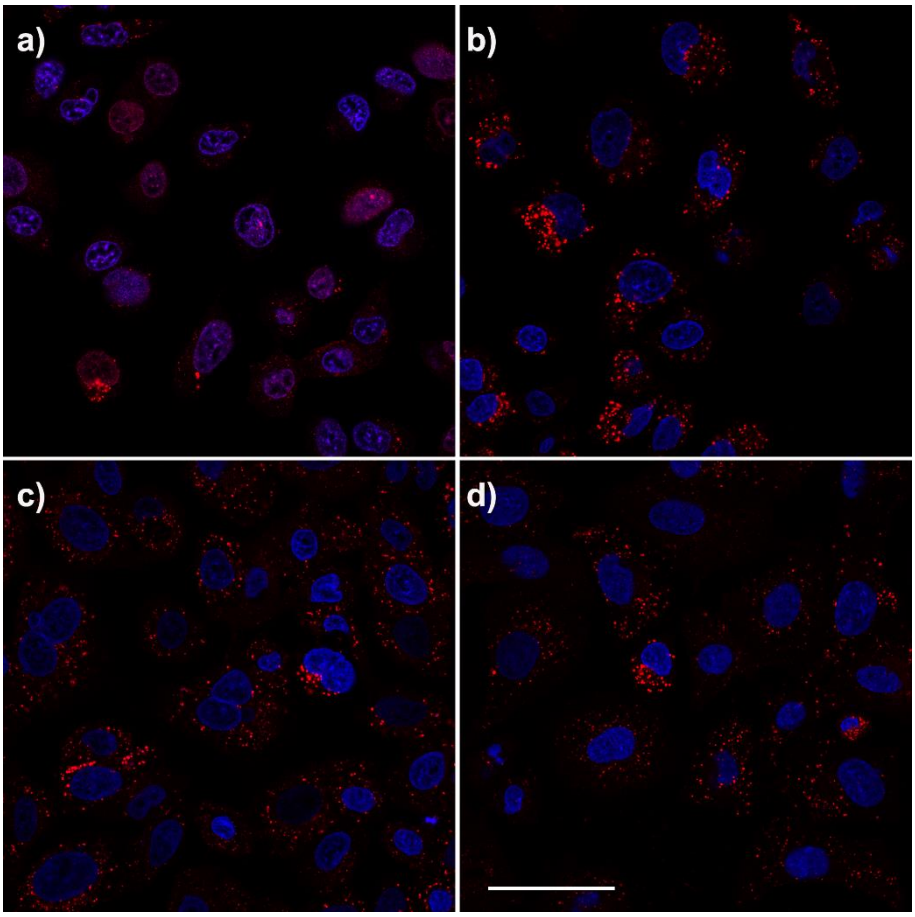


Figure S11. 2D cellular uptake of free Doxo and Doxo-loaded nanoMOFs assessed by CLSM imaging. A549 cells were incubated with (a) free Doxo; (b) nanoMOF_Doxo; (c) nanoMOF_Cv_Doxo and (d) nanoMOF_Gf_Doxo at a Doxo concentration of 10 µM, for 24 h at 37 °C. Cell nuclei were stained with Hoechst (in blue). Red signal corresponds to doxorubicin. Scale bar: 50 µm.

Table S1. Characterization of empty nanoMOFs.

NPs	**Mean diameter** (nm ± SD)	**PdI**	**Zeta potential** (mV ± SD)
nanoMOF	173 ± 18	0.17 ± 0.04	- 28 ± 3
nanoMOF_Cv	162 ± 7	0.15 ± 0.01	6 ± 5
nanoMOF_Gf	165 ± 10	0.14 ± 0.01	- 29 ± 1

Table S2. Characterization of doxorubicin-loaded nanoMOFs.

NPs	**Mean diameter** (nm)	**PdI**	**Zeta potential** (mV)	**Loading capacity** (%)[a]	**Encapsulation efficiency** (%)[b]
nanoMOF_Doxo	1029 ± 406	0.29 ± 0.07	4 ± 1	36 ± 5	19 ± 4
nanoMOF_Cv_Doxo	175 ± 21	0.13 ± 0.03	8 ± 3	34 ± 2	17 ± 1
nanoMOF_Gf_Doxo	164 ± 27	0.14 ± 0.03	-20 ± 1	31 ± 1	15 ± 1

[a]The loading capacity LC (*wt%*) was determined according to the following relationship:

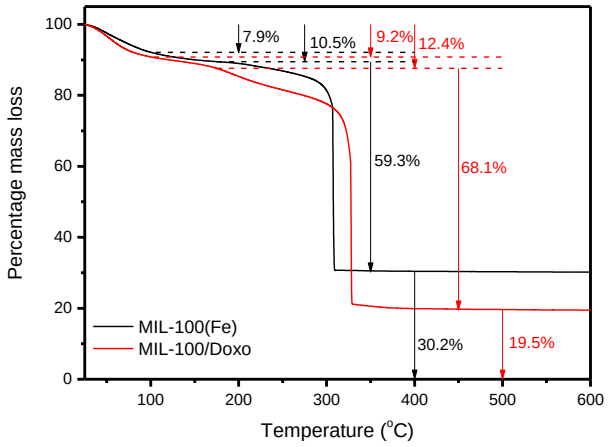
$$LC\ (\%) = \frac{encapsulated\ Doxo\ (mg)}{nanoMOFs\ (mg) + encapsulated\ Doxo(mg)}\ x\ 100$$

Note that, for the surface-decorated nanoMOFs the added PEG is not taken into account.

[b]The encapsulation efficiency EE (*wt%*) was determined according to the following relationship:

$$\text{EE}\ (\%) = \frac{encapsulated\ Doxo\ (mg)}{added\ Doxo\ (mg)}\ x\ 100$$

Schema S1. Method used for the analysis of the raw TGA data. The presented results were obtained in a representative experiment with empty nanoMOFs (black line) and Doxo-loaded nanoMOFs (red line).



Experimental results

Sample	Free water T=100 °C	Free water + bound water T=175 °C	BTC (%)	BTC + Doxo (%)	Fe_2O_3 (%)
MIL-100(Fe)	7.9	10.5	59.3	-	30.2
MIL-100/Doxo	9.2	12.4	-	68.1	19.5

During Doxo loading, the amount of iron was kept constant, and the loss of BTC has been considered as negligible thus results were normalized with respect to the final content of Fe_2O_3.

Normalized results

BTC + Doxo % = $\frac{68.1\% * 30.2\%}{19.5\%} = 105.5\%$

Doxo % = $105.5\% - 59.3\% = 46.2\%$

Experimental Doxo % = $\frac{\text{Experimental (BTC+Doxo)} * \text{Normalized Doxo}}{\text{Normalized (BTC+Doxo)}} = \frac{68.1\% * 46.2\%}{105.5\%} = 29.8\,\%$

The powder having been dried at 100°C for the analysis, the loss of water has been included in our calculation. Accordingly:

Doxo DL % = $\frac{29.8}{(100-9.2)} = 32.8$

References

1. M. Panchal, F. Nouar, C. Serre, M. Benzaqui, S. Sene, N. Steunou, M. G. Marquez, **2017**. United States Patent Application 2020/0102337, "Low Temperature Process for the synthesis of MOF carboxylate nanoparticles"
2. P. Horcajada, T. Chalati, C. Serre, B. Gillet, C. Sebrie, T. Baati, J. F. Eubank, D. Heurtaux, P. Clayette, C. Kreuz, J.-S. Chang, Y. K. Hwang, V. Marsaud, P.-N. Bories, L. Cynober, S. Gil, G. Férey, P. Couvreur and R. Gref, *Nat. Mater.*, 2010, **9**, 172-178.
3. A. Zimpel, T. Preiß, R. Röder, H. Engelke, M. Ingrisch, M. Peller, J. O. Rädler, E. Wagner, T. Bein, U. Lächelt and S. Wuttke, *Chem. Mater.*, 2016, **28**, 3318-3326.
4. M. Giménez-Marqués, E. Bellido, T. Berthelot, T. Simón-Yarza, T. Hidalgo, R. Simón-Vázquez, Á. González-Fernández, J. Avila, M. C. Asensio, R. Gref, P. Couvreur, C. Serre and P. Horcajada, *Small*, 2018, **14**, 1801900.
5. G. Lazzari, V. Nicolas, M. Matsusaki, M. Akashi, P. Couvreur and S. Mura, *Acta Biomater.*, 2018, **78**, 296-307.
6. G. Lazzari, P. Couvreur and S. Mura, *Polym. Chem.*, 2017, **8**, 4947-4969.
7. J. F. Dekkers, M. Alieva, L. M. Wellens, H. C. R. Ariese, P. R. Jamieson, A. M. Vonk, G. D. Amatngalim, H. Hu, K. C. Oost, H. J. G. Snippert, J. M. Beekman, E. J. Wehrens, J. E. Visvader, H. Clevers and A. C. Rios, *Nat. Protoc.*, 2019, **14**, 1756-1771.