Supplemental Data

Materials

All chemicals were purchased from Sigma Aldrich unless stated otherwise and used as received. Cell culture media, supplements and cell culture consumables were purchased from Sigma Aldrich, Fisher Scientific or VWR.

Silk scaffold production

Silk production

For the silk sponge production cocoons of the silkworm *Bombyx mori L.,* purchased from Sericulture and Agriculture Experimental Station (Vratsa, Bulgaria), were cleaned by removing the remains of the silk moth and the innermost film. The cocoon shells were cooked for 30 min in 0.02 M Na_2CO_3 to remove the sericin coating of the silk fibroin filaments. After the degumming process, the silk was washed several times and dried overnight. The dried silk fibroin was dissolved in a $CaCl_2$:EtOH:distilled water solution (1:2:8 molar ratio) boiling under reflux for 45 min. This silk solution was filled in pre-soaked Spectra/Por™ dialysis tubes, purchased from Spectrum Labs™ (Irving, USA), with a molecular weight cut-off of 6-8 kDa and dialysed against distilled water for 72 h with regular changes. The dialysed solution was collected and centrifuged at 4618 x g at room temperature for 10 min. The supernatant was frozen at -80 °C and then lyophilized. For the preparation of the sponge, the solvent casting and particulate leaching method was used. As porogen, sodium chloride of a particle size of 500-800 μm was filled in a petri dish resulting in 1 cm bed height. The lyophilized silk was dissolved in 1,1,1,3,3,3 hexafluoroisopropanol (HFIP) at room temperature in order to prepare a 16% w/v solution. After the addition of the silk solution to the particle bed, the mold was covered to allow the silk/HFIP solution to infiltrate into the salt bed more homogeneously, by reducing the evaporation rate. This was then put into a 37°C pre-heated oven for 1 h covered and overnight uncovered. To induce β-sheet structure, the dried silk/porogen was exposed to methanol. Afterwards, the porogen was washed out until the sponge got a soft and flexible texture. The sponge was then stored and sterilised in 70% ethanol until its use. Before starting cell culture studies, silk sponges are removed from ethanol and incubated in cell culture media overnight. For the experiments, pieces were cut out using a 6 mm diameter biopsy punch.

Scaffold characterization (pore size, SEM, etc)

The silk scaffold morphology was analysed using scanning electron microscopy (SEM). Samples of a size of 5x5x2 mm were dried in an oven over night and fixed using conductive tape on aluminium stubs. A Q150R ES from Quorum Technologies Ltd. (East Grinstead, UK) was used for the gold sutter coating of the samples for 120 s at 50 mA. For the SEM imaging a JEOL JSM-6510 scanning electron microscope of JEOL GmbH (Eching/Munich, Germany) was applied. For FT-IR measurements, a platinum ATR alpha from Bruker (Billerica/USA) was used.

Scaffold toxicity measurement

After sterilizing the prepared functionalized-silk sponges an indirect MTT assay was performed by using a so-called leach-out medium. Therefore, three punches of each cell sponge type were placed into a falcon, covered with 5 mL of DMEM complete, and incubated for 24 h at 37°C. Besides that, 24-well cell culture plates (Cyto-One) were prepared by adding 30,000 MG-63 cells per well. For that reason, cells were

counted and added to an end volume of 500 µL. The plates were then incubated for 24 h at 37°C. DMEM was removed and 500 µL of leach-out medium was added on top of the cells (8 wells per leach-out medium/biological replicates). This was done for every sponge type and in addition to a negative control was added by removing the old DMEM complete and adding a fresh medium instead of a leach-out medium. The 24-well plates were incubated again for 24 h in the cell culture incubator. Afterwards, the medium was removed and 500 µL of a prepared MTT working solution was added (650 µg/mL). The MTT stock solution (5 mg/mL) was diluted 1:7.7 in DMEM without supplements. This was incubated for approximately 1 h, afterwards the plate was checked for violet crystal formation. MTT working solution was discarded and 2 mL DMSO was added to each well of the 24-well plate, followed by a 15-min incubation step on a shaker at room temperature, covered with aluminum foil to protect from light. Then 50 µL of each well was transferred into a 96-well plate (technical triplicates) and further diluted with 50 µL of DMSO. The absorbance at 540 nm vs 650 nm was measured at a TECAN plate reader. The measured values were then statistically analyzed by using One-way ANOVA with Tukey's and Dunnett's multiple comparison test.

Unspecific binding to plastic parts

Non-specific binding of carbon-11, fluorine-18 and gallium-68 tracers to different types of plastic, such as polypropylene (PP), polyethylene (PE) and polyethylene terephthalate (PETE) was determined following the described setup. A filtration tube was outfitted with a filter at the bottom, 20 mg of plastic granules (> 1 mm) and a combi-stopper. The plastic tubes were prepared in triplicates. A plastic granules free column was used as a reference. The respective radiotracer was added to PBS (total volume: 5 ml) using activity concentrations adjusted to their radioactive half-life (fluorine-18: 100 kBq/ml, gallium-68: 100 kBq/ml; carbon-11: 1000 kBq/ml) and vortexed thoroughly. 500 µl of the radioactive PBS was added to each column, after equilibration for 30 min, the activity was removed with a syringe using a female-2-female luer-lock. After three washing steps with PBS, the entire column was measured in a gamma counter. The obtained counts per minute [CPM] were corrected for radioactive decay and corrected for background.

Unspecific binding to column parts

Determination of non-specific binding of ^{11}C-labeled PET radiotracers to a silk sponge was performed using the following protocol: A filtration tube was outfitted with a filter at the bottom and a piece of silk-sponge (6 mm diameter) on top. The respective radiotracer (100 kBq/ml, 50 kBq/ml, 25 kBq/ml and 10 kBq/ml) was added to 1 ml of cell culture media and thoroughly mixed using a vortex mixer. After saturation of the filter and sponge with pure medium, 500 µl of the radioactive solution was pipetted on top of the sponge and left to slowly diffuse through the column. Subsequently, the column was washed three times with cell culture media by letting it slowly diffuse through. Then, the remaining solution was removed with a syringe using a female-2-female luer-lock, before the column was disassembled and the frit, sponge and filtration tube were measured separately in the gamma counter. Measured CPM values were corrected for radioactive decay.

Cell culture

A431 (human epidermoid carcinoma) and HT29 (human colorectal adenocarcinoma) were obtained from American Type Culture Collection (ATCC). HCT116 (human colorectal carcinoma) and HT1080 (human

fibrosarcoma) were kindly provided by Michael Jakupec of the Institute for Inorganic Chemistry, University of Vienna and identified *via* STR profiling by Multipexion (Heidelberg, Germany).

MDCKII Pgp and MDCKII WT cell lines were purchased from the Netherlands Cancer Institute (NKI, Amsterdam, Netherlands), both lines were cultivated in DMEM GlutaMAX™ (Gibco® 61965-026), 10% FCS (Gibco® 10270-106) PC3 PSMApos and PC3 PSMAneg were obtained from ATCC and cultivated in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum, 2 mM Glutamine and 1% penicillin (10,000 U/mL)/streptomycin (10,000 µg/mL).

A431 cells were grown in DMEM supplemented with 10% heat inactivated fetale bovine serum (FBS) and 1% L-glutamine (200 mM). HTC116 and HT1080 cells were grown in RPMI-1640 and HT29 cells in MEM all supplemented with 10% FBS and 1% L-glutamine. All cells were cultured without antibiotics and kept at 37°C under a humidified atmosphere at 5% CO_2. Cell culture plates, dishes and flasks were purchased from Greiner Bio-One (Cellstar®, Frickenhausen, Germany), if not stated otherwise.

Cells were kept under sterile conditions and were incubated at 37°C, 5% CO_2 in a humidified atmosphere.

Spheroid cell culture

Spheroid formation

For the formation of tumor spheroids, non-tissue culture treated u-bottom 96-well plates (Corning, USA) were coated with 1.5% w/w agarose gel. Therefore, 675 mg of agarose were suspended in 44.3 mL of sterile water. The suspension was heated in a water bath to 70-80°C for 1 h until a clear solution was obtained. The wells were quickly coated avoiding air bubbles and plates were stored at 4°C. 100 µL of a cell suspension of 3000, 3000, and 500 cells/well for HTC116, HT1080, and HT29, respectively, were pipetted into the coated 96-well plates. The plates were incubated at 37°C without any disturbance for at least 48 h. Afterwards, the formed spheroids were grown until they reached the size of choice and cell culture medium was changed every 2-3 days. In general, HTC116, HT1080, and HT29 spheroids reached approximately 500 µm of diameter after 4, 4, and 10 days, respectively. The size of the spheroids was measured with an Olympus IMT 2 microscope by determining the diameter of the spheroids. For the evaluation of spheroid symmetry, both the length and width were measured and correlated.

Immunohistochemical analysis of multicellular tumor spheroids

The multicellular tumor spheroids (MTS) were embedded in Tissue-Tek® O.C.T., cut with a cryo-microtome (Thermo Scientific Micron HM560) and thaw-mounted onto adhesion slides (Menzel-Gläser SUPERFROST® PLUS, Thermo Scientific). The cryoslices were stained with hematoxylin and eosin following published procedures.[28]

Live-dead-stain: Fluorescence staining and fluorescence microscopy

The MTS were washed two times with PBS prior to incubation with a 1:1000 dilution of propidium iodide (1 mg/mL) in the respective cell culture media and incubated for six hours at 37°C in a humidified atmosphere. 15 min prior to the end of incubation, calcein acetoxymethylester (calcein-AM, 4 mM in DMSO, 100 UL) was added in a dilution of 1:400. Afterwards, the solution was removed and the MTS were washed twice with PBS. The MTS were measured at 517 nm (G filter, calcein-AM) and 617 nm (B filter, propidium iodide) with an Olympus IMT 2 microscope.

Cell viability: Alamar blue assay and MTT

MTS with an approximate size of 500-600 µm were transferred into non cell-culture treated u-bottom 96-well plates in the respective cell culture media of 100 µL. A stock solution of the test compounds with a maximum concentration of DMSO of 1% was prepared and diluted stepwise. 100 µL of the double-concentrated stock solutions were added in triplicates to each well. Subsequently, the 96-well plates were incubated at 37°C and 5% CO_2 for 24 h. 20 µL of a freshly prepared solution of resazurin sodium salt (110 µg/mL) in PBS were added for 6 hours. Subsequently, the 96-well plates were measured by means of a BioTek Synergy HTX device. Fluorescence was measured at 530 ± 25 nm and 590 ± 20 nm for excitation and emission wavelength, respectively.

Scaffold cell culture

Silk-embedded spheroid culture

Skin cutter with a diameter of 5 mm were applied to trim the silk scaffold to the required dimensions. Multicellular spheroids with a size of 500-600 µm were embedded into the silk scaffold and carefully introduced in the 6 mm diameter columns. In between each embedded spheroid, a PE-based filter frit was placed for unambiguous detection of the spheroids. For short-time storage, the column was flushed with around 10 ml of fully supplemented cell culture media, and spheroids were allowed to settle and adhere for at least 2 hours.

Silk-embedded single cells culture

The silk scaffold was trimmed as previously described by applying a 6 mm diameter skin cutter. The single cell suspension was prepared in the required cell concentration and carefully applied onto the silk scaffold for HT29, HCT116, HT1080, MDCKII Pgp and MDCKII WT as well as PC3 PSMA pos and PC3 PSMA neg cells. The procedure included to push the sponge with a spatula to remove the old media. Then the silk sponge was slowly released by simultaneously applying 1 ml of the cell suspension. The cells were allowed to settle and grow on the scaffold for 24 hours in a cell incubator. Afterward, the column was carefully assembled for further processing.

Radiotracer production

2-[^{18}F]FDG (2-[^{18}F]Fluorodeoxyglucose), [^{18}F]FMISO ([^{18}F]fluoromisonidazole), [^{18}F]FET ([^{18}F]fluoroethyl)-l-tyrosine), [^{18}F]FEC ([^{18}F]fluoroethylcholine], [^{18}F]NaF and [^{11}C]MET ([^{11}C]methionine), [^{11}C]MCP ([^{11}C]methionine), [^{11}C]PHNO ([^{11}C](+)-4-propyl-9-hydroxynaphthoxazine), [^{11}C]PIB (Pittsburgh compound B), [^{11}C]DPMARE, [^{68}Ga]DOTANOC (DOTA-nal3-octreotide), [^{68}Ga]PSMA (prostate specific membrane antigen) and [^{68}Ga]$GaCl_3$ were produced at the PET centre of the General Hospital of Vienna. All PET radiotracers meet the quality criteria stated in the European Pharmacopoeia.[27]

Accumulation studies in 2D and 3D culture

2D cell culture accumulation experiments at 37°C and 4°C

HCT116, HT1080, and HT29 cells were seeded in 100 mm^2 cell culture dishes at a concentration of 2.5x10^5 cells/mL. After 24 hours, the cells were incubated with 3 mL of a 6 MBq/ml concentrated solution of the respective radiotracer for 50 min at 37°C and 4°C. After the incubation time, 200 µL of the incubation media were transferred to Eppendorf tubes (applied dose). Cells were washed two times with 3 mL of cell

culture media and subsequently 500 µL media was added. The cells were detached from the plastic dish using a cell scraper. 200 µL of the cell suspension was transferred to Eppendorf tubes. The blank dishes were treated the same way.

Spheroid accumulation experiments at 37°C and 4°C

Cellular accumulation experiments of spheroids were performed according to Monazzam *et al.* at 37°C and 4°C, respectively. As soon as the HCT116 spheroids reached a size of 500-600 µm, they were transferred to a 12-well plate in 500 µL. The plates required for the 4°C experiments were cooled on ice in advance for around 30 min. Then, 500 µL of the radiotracer solution (6 MBq/mL) were added and plates were incubated for 50 min at 37°C or 4°C. 20 µL of the incubation medium was measured as a reference solution, 20 µL of the last washing medium was measured as background control and 20 µL of the respective sample including the MTS were transferred to Eppendorf vials and measured with a calibrated γ-counter (2480 automatic gamma counter, Perkin Elmer, Wizard^2 3").

Scaffold accumulation experiments at 37°C and 4°C

1×10^6 HCT116 cells per mL were seeded on silk scaffold and the column was assembled. For the 4°C experiments, the columns were cooled on ice in advance for around 30 min. 10 mL (approx. 1 MBq/mL) of the respective radiotracer solution was applied. The radioactivity was measured that remained in the column. The cells were incubated for 50 min either at 37°C or 4°C, afterwards, the column was washed with 50 mL of medium. The trapped radioactivity in the total column was measured and the distribution between the different compartments was assessed by µPET/CT.

Column assembly

The columns were carefully assembled by transferring the pre-cut silk sponges into the column and slowly pushing them down to the bottom. It has to be assured, that the sponge is not compressed at all times in order to avoid physical stress on cells. Between each compartment, a PE-based filter frit was set to keep the silk sponge in place. The filter plates additionally reduce the turnover from cells in different departments. The columns were closed with a male connector and 10 ml of pre-warmed cell culture media were slowly applied (approx. 1 mL/min) in order to completely fill the column with the aqueous solution. The columns were stored for max. 2-3 h prior to µPET measurements.

µPET/SPECT/CT imaging and image reconstruction and data post-processing

µPET activity limitation tests and calibration

Increasing radioactivity of 2-[^{18}F]FDG in the range of 1-1000 kBq per 100 µL was pipetted into Eppendorf tubes. The total radioactivity was measured in a calibrated dose calibrator followed by µPET and µCT for compartment visualization and attenuation correction (Siemens Inveon Multimodal µSPECT/CT, dedicated µPET; Siemens Medical Solutions, Knoxville, USA).

Activity administration stop-flow mechanism

Approximately 1 MBq of the respective radiotracer in 10 mL fully supplemented cell culture media was applied into the column *via* a syringe. Once the column was filled the system was allowed to equilibrate for 20 min (carbon-11), 30 min (gallium-68) or 30 min (fluorine-18) at room temperature. Subsequently,

10 mL of fresh cell culture media was applied to remove unbound radioactivity. The unbound amount of radioactivity as well as the bound fraction were measured in a calibrated dose calibrator.

Column equilibration, automatization and pressure limitations

For an automated setup the column was connected to a Merck/Hitachi pump. As mobile phase fully supplement cell culture media was used. The system was allowed to stabilize at different flow rates (0.5, 0.75, 1 mL/min) and the pressure was monitored over time. *Via* an escalation study the maximal applicable pressure for the involved plastic connectors were determined.

µPET measurement and post processing

Static PET imaging was acquired on a Siemens Inveon preclinical µPET/SPECT/CT system. The total PET acquisition time was 10 min. Differences between acquisition times are due to half-lives of the respective radionuclides (carbon-11: 20 min; gallium-68: 68 min, fluorine-18: 110 min). The CT raw data were reconstructed with a Feldkamp algorithm using a Ramp filter.

PET list mode data was sorted into three-dimensional sinograms and reconstructed using an OSEM3D/OP-MAP with scatter and attenuation correction. The CT data was corrected for beam-hardening. All relevant corrections (e.g. normalization, dead time, random artifacts, radioactive decay) for quantitative PET data were performed.

A calibration factor was applied to convert the activity information into absolute concentration units. Image data analysis was carried out using the Inveon Research Workplace (IRW; Siemens Medical Solutions, Knoxville, USA). Volumes of interest (VOIs), comprising the column compartments were outlined on the CT and transferred to the PET images.

Statistics

All experiments were performed at least in triplicates and independently repeated at least three times if not stated otherwise. All values are depicted as mean ± standard deviation. Statistical analysis was performed in MS Excel® 2013 or GraphPad Prism 6. The radiotracer accumulation was calculated as follows showing the background corrected percental accumulation normalized to the applied radioactivity dose (AD):

$$normalized\ accumulation\ \%AD = \frac{(mean\ of\ sample\ triplicates - background)}{(mean\ of\ applied\ dose - background)} x100$$

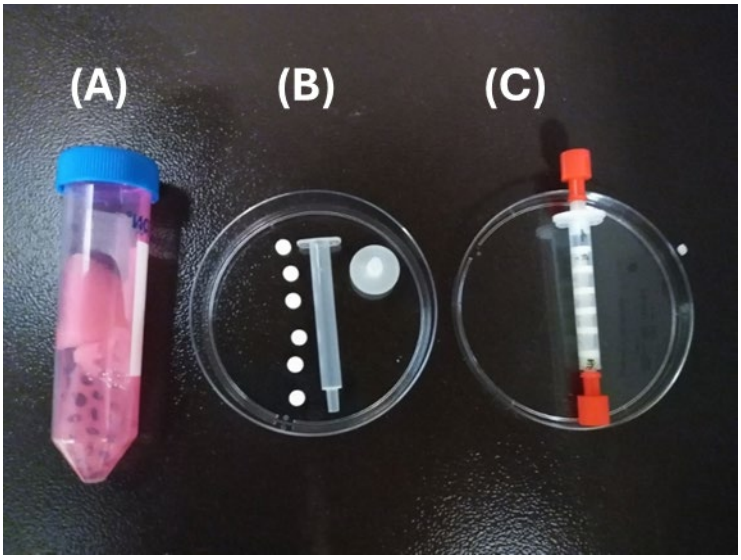
Accumulation experiments at different inhibitor concentrations were further processed as follows to determine the change of accumulation independent of the absolute radiotracer uptake:

$$ratio\ treated\ to\ non\ treated\ control = \frac{norm.accum.\%AD\ of\ treated\ sample}{norm.accum.\%AD\ of\ non-treated\ sample}$$

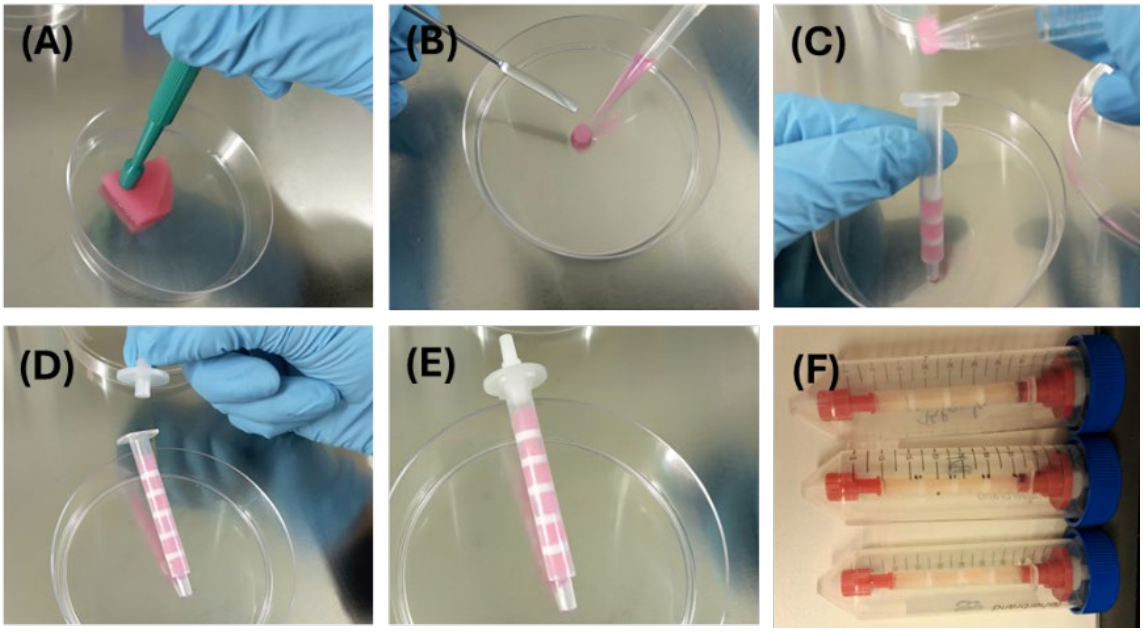
Statistical significance was calculated for the accumulation experiments at different temperatures *via* an unpaired t-test and for effects caused by inhibitors *via* two-way ANOVA by means of GraphPad Prism 10. P-values are defined as follows: ns – not significant, * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001.

All experiments were repeated at least three times, and all values are shown as mean ± standard deviation if not stated otherwise.

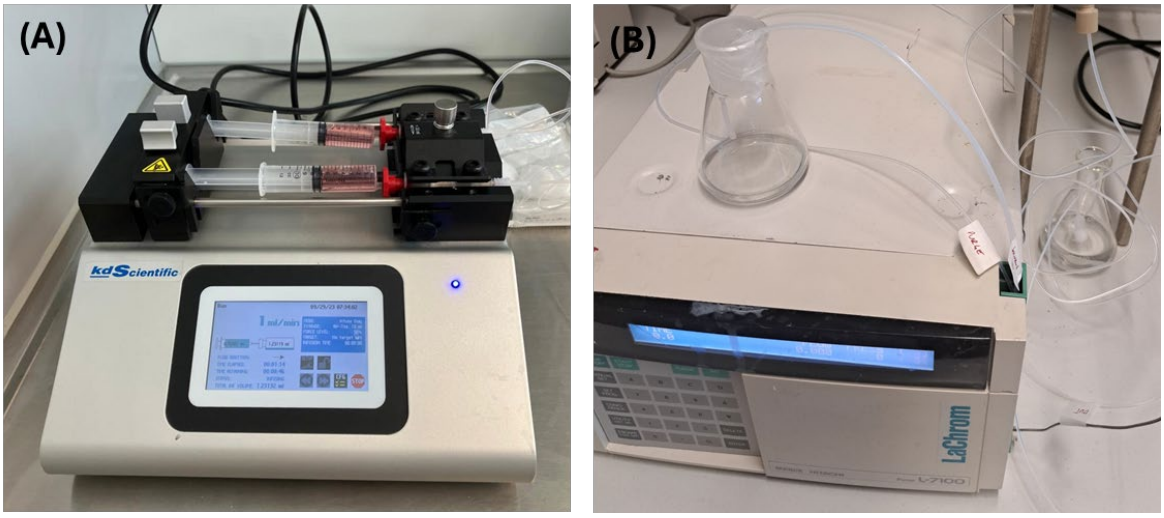
Figures



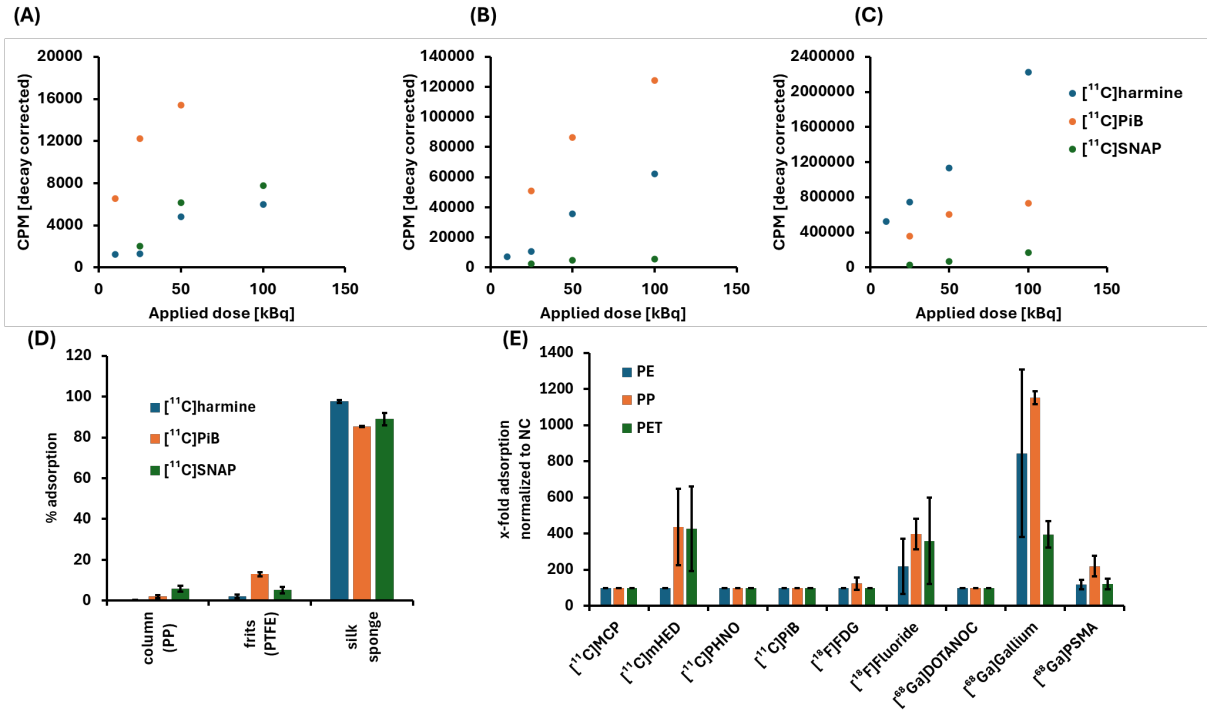
Supplemental Figure 1: Composition of the developed column system including (A) self-produced silk sponges as a surrogate for extracellular matrix and the built-up of a 3D-tissue construct, (B) plastic parts required for column built-up including the column body, frits to separate the compartments and the connector part enabling connection to tubings, and (C) the assembled system.



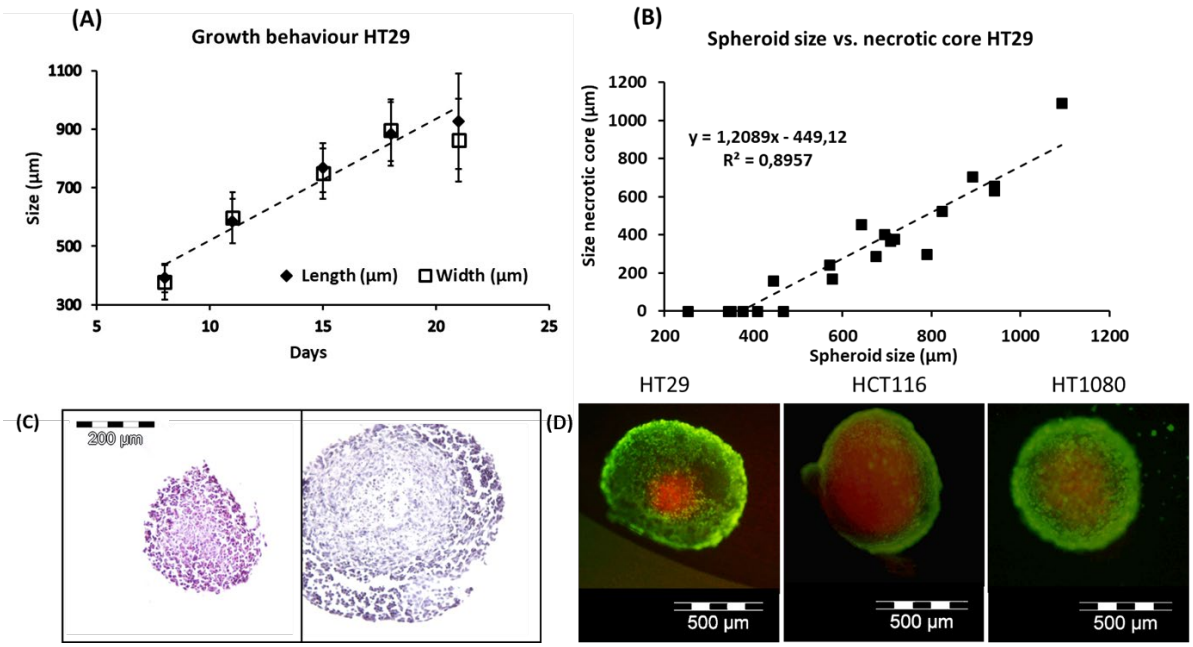
Supplemental Figure 2: Steps for column assembly. (A) Punching of the silk sponge to provide the shapes and size; **(B)** seeding of the respective cells on the sponge, and incubation to let the cells grow on the sponge for at least 4 h; **(C)** careful assembling of the column and **(D)** closure of the system with the respective adapter. **(E)** The final assembled column and **(F)** closed columns for short-term transport to the pump system.



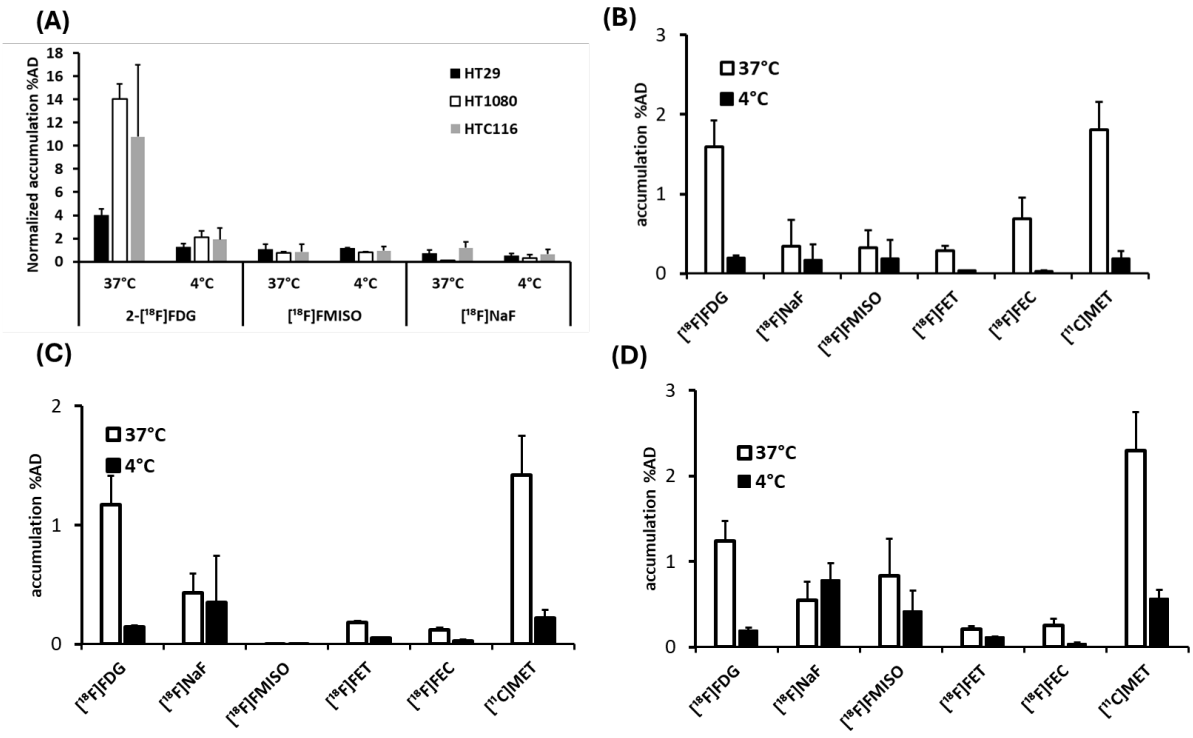
Supplemental Figure 3: Tested pump systems for automatized dynamic flow. (A) Syringe pump used for application of the radiotracer and short-term experimentation due to limited applicable volumes in the syringe. **(B)** Repurposed HPLC pump for long-term dynamic experiments.



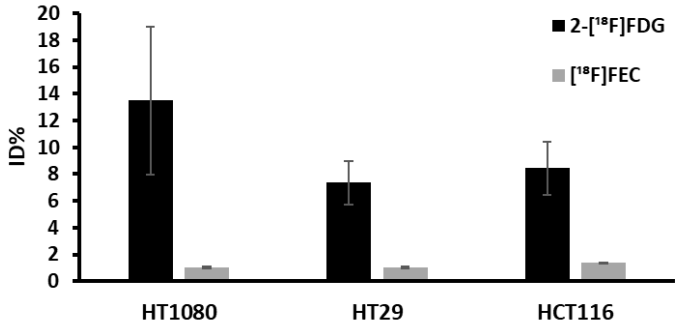
Supplemental Figure 4: Testing of non-displaceable binding of different radiotracers to the most common plastic parts used for the column-system. Dose-dependent adsorption of [^{11}C]harmine, [^{11}C]PiB, and [^{11}C]SNAP on the column parts (n = 1): **(A)** column-body (PP), **(B)** frits (PTFE), **(C)** silk-sponge (silk fibroin). **(D)** Proportional amounts of non-displaceable binding to the respective parts calculated as percentage, showing the high interaction with the natural protein fibroin. **(E)** Adsorption of nine other radiotracers on different microplastic particles to estimate the plastic type with the lowest adsorption properties.



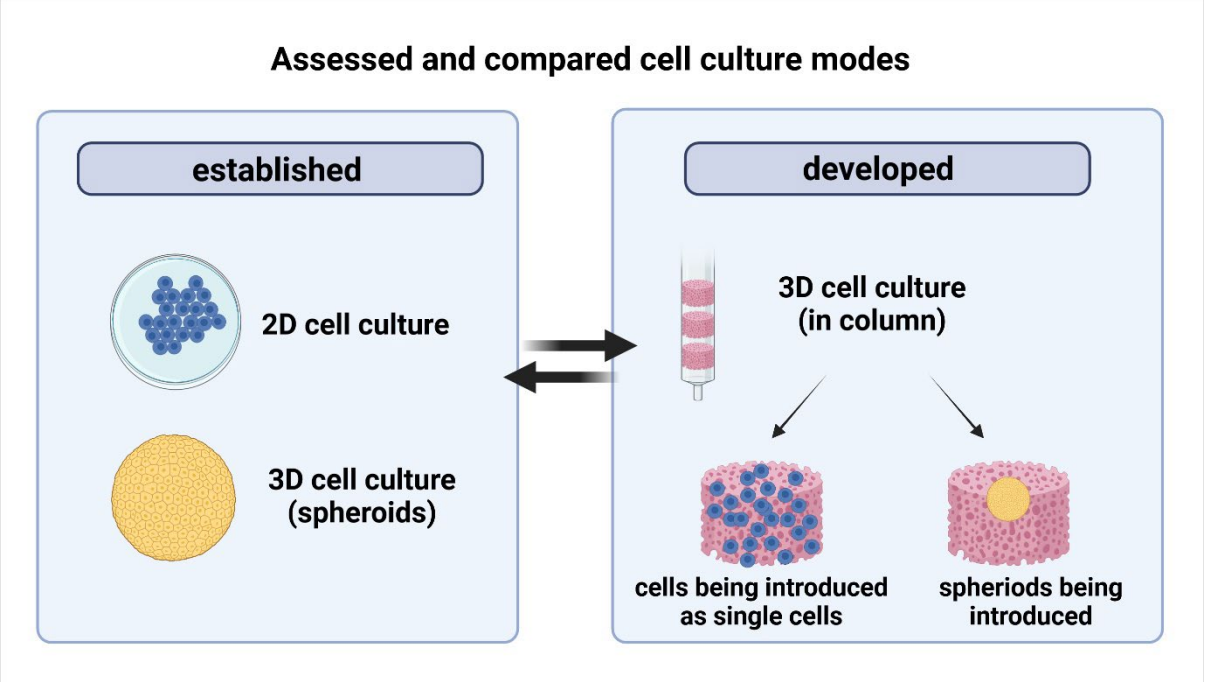
Supplemental Figure 5: Characterization of multicellular tumor spheroids: (A) growth behavior over a period of 21 days for HT29 cells; **(B)** formation of a necrotic core in dependence of the spheroid size for HT29 spheroids; **(C)** H&E staining of cryoslices of spheroids with a size around 200 µm (left) and 500 µm (right). **(D)** Live-dead fluorescence staining (calcein-AM and propidium iodide) of MTS derived from HT29, HCT116, HT1080 and A431 cell lines. The green fluorescence shows the living cells on the outer sphere, whereas the red regions indicate the presence of a necrotic core in the inside of the spheroids.



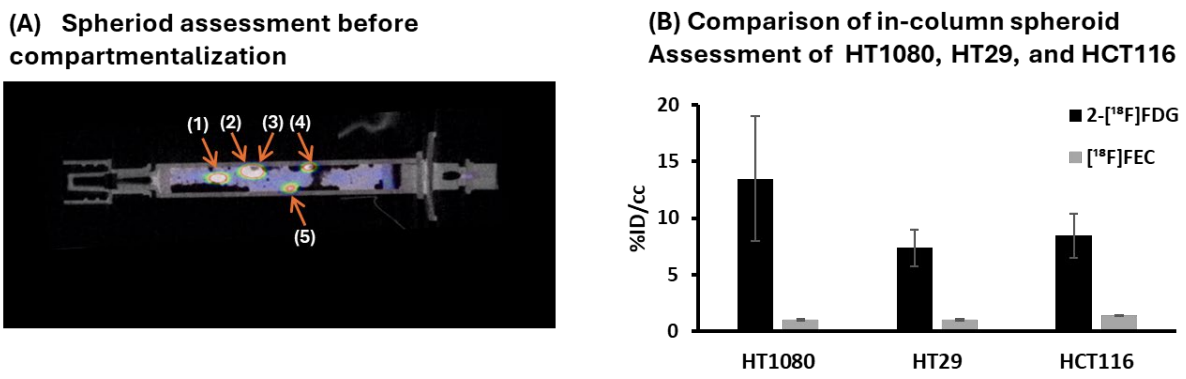
Supplemental Figure 6: Radiopharmaceutical accumulation experiments conducted at 37°C and 4°C using a conventional (**A**) 2D cells and 3D cell culture assay (conventional assay, not-in column) of (**B**) HT29; (**C**) HT1080, and (**D**) HT116 for studying the discrimination of active and passive transport mechanisms.



Supplemental Figure 7: Radiopharmaceutical accumulation experiments of 2-[^{18}F]FDG and [^{18}F]FEC conducted at 37°C for HT1080, HT29 and HCT116 cells, where the cells were grown on silk-scaffold, introduced in the column and the respective radiotracer were applied in a dynamic mode.



Supplemental Figure 8: Overview of assessed models for comparison. Four different settings were assessed and compared including conventional 2D cell culture, multicellular tumor spheroids for tissue-like structure, as well as cells grown on silk scaffold as artificial extracellular matrix and tumor spheroids grown on silk scaffolds, both within the column-based system. Created by biorender.com



Supplemental Figure 9: Radiopharmaceutical accumulation experiments conducted at 37°C for spheroid culture implemented within the in-column system. (A) Spheroid analysis before compartmentalization of the column including five HT29 spheroids of a size range of 500-700 µm, whereof numbers 1, 4, and 5 have been resolved, whereas 2, and 3 were not distinguishable (for compartmentalized image see Fig. 2F). (**B**) 2-[^{18}F]FDG and [^{18}F]FDG accumulation of spheroids within the column system for comparison with conventional analysis (compare Fig SI6).