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Evaluating cellular effects of PET microplastics in 2D/3D models: viability, oxidative stress, and comprehensive methodological considerations

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Abstract

Despite the widespread environmental contamination by micro- and nanoplastics (MNPs), the cellular toxicity of polyethylene terephthalate (PET) particles is poorly understood. Here, we assessed pristine PET-Micro- and nanoplastic particles (MNPs) (diameter: $1.24 \pm 0.52 \mu\text{m}$) and fluorescently labeled PET-MNPs (Fluo-PET, diameter: $1.25 \pm 0.97 \mu\text{m}$) in both 2D (monolayer cultures) and 3D models (multicellular spheroids) across different mammalian cell types. PET exposure caused dose- and cell type-dependent reductions in cell viability at concentrations of 2.5–5 $\mu\text{g/ml}$, with uptake rates of up to 16%. In 3D models, optical photothermal infrared spectroscopy (O-PTIR) enabled the detection and biodistribution analysis of non-labeled particles, highlighting its value as a polymer-specific, label-free technique. Short-term exposure (10 min – 120 min) induced rapid, concentration-dependent ROS generation across all cell types, while reactive nitrogen species (RNS) levels remained unchanged. Due to numerous potential interferences, particular attention was given to identifying artifacts caused by MNP interactions with conventional assays, including plastic-plate interferences due to MNP adsorption during spheroid formation, distortions in fluorescence-based assays due to light scattering, dye adsorption, or surfactant effects, all of which can yield false-positive or false-negative results if appropriate controls are not implemented. Our findings emphasize the importance of accounting for such artifacts and interferences, and the need to report and discuss these effects explicitly. In summary, PET particles induce significant cytotoxicity and oxidative stress, which is confirmed after correction for experimental artifacts.

Keywords Micro- and nanoplastic particles (MNPs), Polyethylene terephthalate (PET), Reactive oxygen species (ROS), In vitro, 3D-Spheroids, O-PTIR

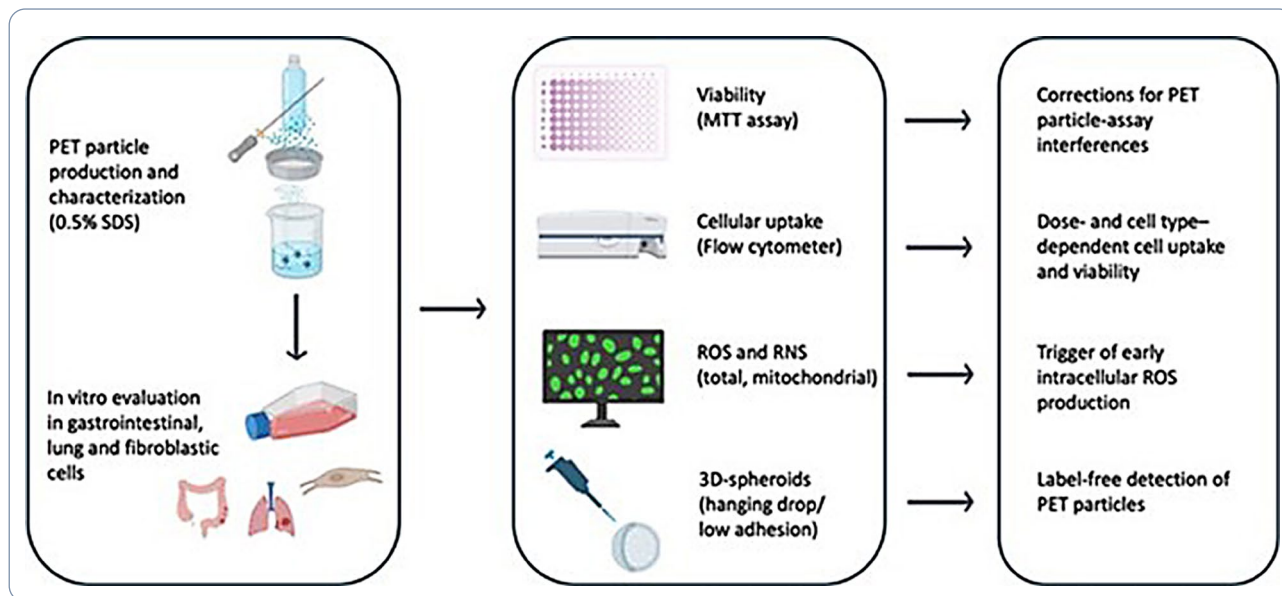
Graphical Abstract

Created in BioRender.com and adapted in MS Power Point

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Introduction

Increasing plastic pollution represents a global environmental problem with far-reaching ecological and health implications. Micro- and nanoplastic particles (MNPs)

originate either directly as primary particles, for example, in cosmetics or industrial products, or as secondary particles through the degradation of larger plastic items in the environment (Fig. 1) [2, 3]. In accordance with Annex

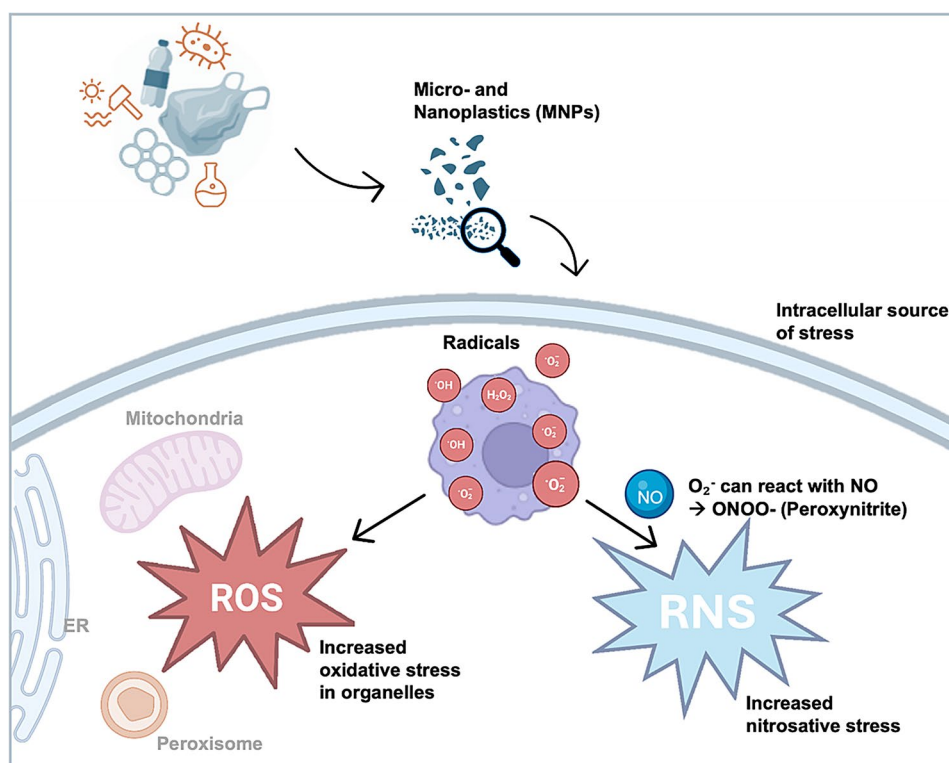


Fig. 1 Schematic overview of potential mechanisms by which MNPs may induce intracellular stress. MNPs are created by physical (e.g., friction, UV radiation), chemical (e.g., oxidation, hydrolysis) and biological (microbial enzymes) degradation processes of plastic. Upon entering cells, they are able to cause stress intracellularly. A distinction is made between reactive oxygen species (ROS) and reactive nitrogen species (RNS), which both may damage cellular structures. In addition to mitochondria, the endoplasmic reticulum and peroxisomes also contribute to ROS formation. Superoxide ($\text{O}_2^\bullet$), a central ROS, can react with nitric oxide (NO) to form peroxynitrite (ONOO^-) – a highly reactive RNS that can damage cellular structures as well [1], (BioRender content)

XVII, entry 78 of the REACH Regulation (ECHA, 2023/Annex XVII, 78), the EU has prohibited the placing on the market of primary microplastic particles (≤ 5 mm) since October 2023. MNPs are ubiquitously present in aquatic and terrestrial ecosystems and can enter the human body through the food chain [4, 5] or other routes. The most common absorption routes for MNPs include inhalation and oral uptake, with transdermal absorption also being a possible route [6]. After absorption, MNPs are potentially distributed via the bloodstream to multiple organs, including the lung, liver, kidney, and gut. [7–10]. This widespread dispersal contributes to diverse health risks, including immune dysfunction [11], intestinal barrier disruption [12], and systemic inflammatory or metabolic damage [13].

MNPs can elicit various biological effects at the cellular level, with impacts on cell viability varying depending on the cell type, particle type, and concentration [14, 15]. The main internalization mechanisms identified for MNPs of different chemical origins are based on various endocytic pathways. In mammalian cells, clathrin-mediated endocytosis is predominant [16]. Different internalization pathways may determine the extent and localization of particle-induced damage within the cell. 3D *in vitro* models, such as spheroids, better mimic *in vivo*-like cell-cell interactions and tissue-specific structures [17] and thus, can provide additional insights into intercellular particle transport. Among the mechanisms triggered by MNPs, the induction of oxidative stress has been identified as a key cellular response. This typically results from increased production of reactive oxygen species (ROS), which are natural byproducts of cellular metabolism, particularly during mitochondrial respiration. Mitochondrial ROS (mtROS), in particular, are generated *via* electron transport chain activity within the inner mitochondrial membrane [18]. Besides mitochondria, ROS can also be produced in other organelles such as the endoplasmic reticulum (ER) and peroxisomes [19]. At physiological levels, ROS play essential roles in cell signaling, host defense, and homeostasis. However, excessive ROS can trigger inflammatory responses and contribute to long-term cellular damage [20, 21]. Maintaining redox balance is especially critical in the gastrointestinal tract (GIT), where both reduced and elevated ROS levels are associated with inflammatory bowel diseases [22]. In addition to ROS, reactive nitrogen species (RNS) are central mediators of cellular stress responses. They are formed by the reaction of nitric oxide (NO) with oxygen radicals to generate peroxynitrite (ONOO⁻), which is one of the most reactive and biologically relevant RNS in biological systems. Due to its high reactivity, peroxynitrite can damage lipids, proteins, and nucleic acids, and is a decisive factor in inflammatory processes

as well as in the pathogenesis of diseases associated with oxidative and nitrosative stress [1] (Fig. 1).

Two recent reviews have summarized how polystyrene (PS) particles induce oxidative stress in various *in vitro* models, using ROS assays such as DCFH-DA, DHE, CCK8, and MitoSOX™ [23, 24]. As inhalation and ingestion are primary routes of exposure to MNPs, most prior *in vitro* ROS studies so far have mainly explored used lung and intestinal cell lines, such as A549 (lung carcinoma) [25, 26] and Caco-2, and HT-29 (both intestinal carcinomas), in monoculture and co-culture settings [27–31]. HeLa cells (cervical cancer) [32, 33] and human dermal fibroblasts (HDFs) [34] have also been investigated. However, the applied particle concentrations varied considerably, typically ranging from 5 to 10 $\mu\text{g/ml}$ [35, 36] to 100 $\mu\text{g/ml}$ [37], with exceptional cases reporting exposures of up to 1000 $\mu\text{g/ml}$ [23, 38]. Such high concentrations are increasingly considered irrelevant in respect to human exposure levels, limiting the extrapolation of these findings to real-world exposure scenarios. Based on an analysis of 59 studies, Senathirajah et al. [39] estimated that humans worldwide ingest approximately 0.1 to 5 grams of MNPs per week through various exposure pathways, with most particles in the 0–1 mm size range [39, 40]. Although this number is impressive, it cannot be directly extrapolated into meaningful concentrations for cell culture experiments. The actual systemic absorption in the body is low [5], and a direct conversion to cell culture conditions is not possible due to biological, physical, and experimental differences. Importantly, across cell types and exposure ranges, most studies consistently reported convergent outcomes: MNPs can impair mitochondrial function, elevate intracellular ROS levels, and activate cellular antioxidant defense systems. The strength of these effects depends largely on properties such as particle size, charge, plastic type and the dose selected [3]. While previous studies have primarily focused on polystyrene (PS) or polyethylene (PE) particles, data on polyethylene terephthalate (PET) remain limited [41–43]. PET is commonly used in bottles, films, and textile fibers due to its corrosion resistance and mechanical strength [44]. Additionally, it is the second most common plastic found in stool samples [45, 46].

The present study evaluates cellular stress responses to PET-MNPs under intermediate-to-low exposure conditions ($< 5 \mu\text{g/ml}$) compared to most studies in this area ($< 1 \text{ mg/ml}$), with a particular focus on the importance of cell type and experimental model system. We assess not only cell viability and particle internalization, but also the impact of PET particles on 3D spheroid growth. Furthermore, we examine intracellular stress responses in detail by analyzing general ROS levels, mitochondrial ROS, and RNS (reactive nitrogen species). Particular attention is given to potential interferences of the MNPs with the

conventional assays. Together, these investigations provide a critical foundation for the toxicological evaluation of environmentally relevant plastic particles, such as PET.

Materials and methods

Equipment

A Malvern Panalytical Zetasizer Pro (Worcestershire, UK) was used for particle analysis. Deionized water was obtained using a LaboStar PRO water system (Evoqua Water Technologies GmbH, Germany). Fluorescence and absorbance measurements were performed using a microplate reader (Infinite® M200 Pro, Tecan, Männedorf, Switzerland and Synergy H1 Microplate Reader, BioTek). Flow cytometry was carried out with a Guava easyCyte™ 8HT (Merck Millipore, Burlington, MA, USA). Imaging was performed using a Nikon Eclipse TE2000-S fluorescence microscope.

Manufacturing of polyethyleneterephthalate (PET) particles

PET particles were produced according to the protocol published by our group [47], which is based on the method originally described by [48] using PET material derived from a commercial soft drink bottle [48]. In short, the plastic was filed/shredded with a metal file, and 250 mg were dissolved in 5 ml 90% TFA at 100 °C for multiple hours, then precipitated with 5 ml 20% TFA, washed 3 times with water, and resuspended in 30 ml 0.5% SDS. After sedimentation of the bigger aggregates, the supernatant was removed and characterized.

Fluorescein-isothiocyanate (FITC) labelled PET particles (Fluo-PET) were produced by washing 10 mg particles with 2×2 ml ethanol, drying under vacuum, and suspending in 2 ml DMF. Afterwards, adding 1 mg of KOtBu and after 30 min 1 mg of FITC, the reaction has been finished after 24 h at room temperature. After washing with 2×2 ml ethanol and 2 ml H₂O wash, the particles were resuspended in 0.5% SDS. Stability of fluorescence and alterations of the particle size were assessed prior to biological evaluation in phosphate-buffered saline (PBS) and in a 10% HCl solution (Fig. SI 1).

PET and Fluo-PET particle characterization

The PET particles were characterized using dynamic light scattering (DLS) and potential analysis. The equivalent

diameter was determined by ImageJ analysis of light microscope images at 40x magnification (Fig. SI 2), whereby the Abbe limit leads to a distortion in favor of larger particles. The diameter was calculated based on the particle surface area using the area equation of a circle ($A = \pi r^2$). In addition, the particle size (z-average) in suspension was determined using DLS. The ζ-potential was measured after centrifugation of the particles in 0.5% SDS (w/v) and subsequent resuspension in 1 mM KCl to evaluate the surface charge and electrostatic stability of the suspension. The ζ-potential measurements were performed under the following conditions for PET (conductivity 0.2019 mS/cm, pH7) and Fluo-PET (conductivity 0.2416 mS/cm, pH7). The particle concentration was determined gravimetrically after the particles were washed three times with ethanol and dried under vacuum. The results of the material characterization are shown in Table 1.

Cell culture

A total of five different cell lines were included in the study: mouse embryonic fibroblasts NIH-3T3 (ATCC CRL-1658™, USA), human colorectal adenocarcinoma cell line HT-29 (ATCC, HTB-38™, USA), human fibrosarcoma cell line HT-1080 (ATCC CCL-121™, USA), human colon cancer cell line HCT-116 (ATCC CCL-247™, USA) and human non-small cell lung cancer cell line NCI-H460 (ATCC HTB-177™, USA). However, depending on the experimental setup, only a selection of these cell lines was used. The cell lines used in each case are explicitly stated in the respective section. The human cells were cultured in Roswell Park Memorial Institute medium (RPMI-1640, Sigma-Aldrich, Merck) (containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S), and 1% L-glutamine) under standard cell culture conditions of 37 °C, 5% CO₂, and a controlled humid environment. NIH-3T3 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, Merck) with high glucose and supplemented with 10% FBS, 1% P/S, and 1% L-glutamine. All experiments were carried out within the Laminar Air Flow (LAF) cabinet to preserve aseptic conditions.

Table 1 Characteristics of the included PET particles: particles were analyzed by optical microscopy (using ImageJ) and DLS for their mean size and size distribution. Both samples showed variations in particle size, with the Fluo-PET sample having a higher Z-average, indicating a greater proportion of large particles

	c [µg/ml]	D _{eq} [µm]	D _{eq} -median [µm]	Z-average [µm]	ζ-potential [mV]	PDI
PET	300	1.24 ± 0.52	1.23	0.96 ± 0.097	− 45.73	0.17
Fluo-PET	4 000	1.25 ± 0.97	0.94	1.7 ± 0.26	− 37.74	0.55

c: concentration of stock suspensions; D_{eq}: mean particle size (equivalent diameter) ± standard deviation (SD); Z-average: mean diameter of particles; PDI: measure of particle size distribution, with higher values indicating greater polydispersity of the sample

MTT - cell viability assay

The cell lines NIH-3T3, HT-1080, HCT-116, and H460 were seeded in 96-well flat transparent plates (Corning Incorporated costar®) at a density of 2×10^3 cells/well. After a 24-hour settling period, cells were exposed for 72 h to 100 μ l of Fluo-PET particles at final concentrations ranging from 0.31 to 5 μ g/ml per well, which were prepared from a 4000 μ g/ml stock solution in 0.5% SDS. As a control, SDS dilutions were tested for potential interferences. Stock solutions of SDS were prepared by dissolving SDS powder in deionized water to form an 8% solution (% w/v). The dilution series ranged from 0.0005% to 0.008%. At a Fluo-PET concentration of 5 μ g/ml, the SDS content corresponded to 0.0006%. After 72 h of Fluo-PET and SDS treatment, a solution of 250 mg thiazolyl-blue-tetrazolium-bromide (MTT, Merck, St. Louis, Missouri, USA) in 50 ml PBS (pH 7.4) was prepared and stored in light-protected at 4 °C. The MTT stock solution was diluted 1:7 in non-supplemented RPMI culture medium. After removing the supernatant from the culture plates, 100 μ l of the diluted MTT solution was added to each well and incubated for 4 h under standard cell culture conditions of 37 °C, 5% CO₂, and a controlled humid environment. The MTT solution was aspirated, and 100 μ l of dimethyl sulfoxide (DMSO) was added to each well to dissolve the water-insoluble formazan crystals. The absorbance of the formazan solution was measured using a microplate reader at a measurement wavelength of 550 nm and a reference wavelength of 650 nm. The raw data were obtained using the iControl™ software and subsequently, statistical significance was determined using the t-test in Microsoft Excel. For each condition, the mean absorbance value was calculated from three biological replicates. These mean values were then normalized to the corresponding untreated control.

Flow cytometry

NIH-3T3, HT-1080 and HCT-116 were seeded at a density of 4×10^5 cells/well in flat transparent 24-well plates (Sarstedt AG & Co. KG) and incubated for 24 h. On the day of measurement, the confluent cells were treated with Fluo-PET concentrations ranging from 0.5–4 μ g/ml. After 2 h of treatment, the cells were washed three times with PBS and then trypsinized with 80 μ l/well trypsin. Trypsinisation was stopped by adding 2 ml medium to achieve an approximate of 200 cells/ μ l. Cellular uptake was measured using a flow cytometer and InCyte software, with GFP fluorescence excited at 488 nm and detected in the FITC channel (530/30 nm). A total of five thousand events were counted for each experiment. The data was analyzed using the open-source Floreada.io software (<https://floreada.io/>). Debris and cell doublets were excluded based on forward and side scatter (FSC/

SSC) parameters. Statistical significance was assessed using unpaired two-tailed t-tests in Microsoft Excel.

3D spheroid formation

The formation of spheroids was accomplished by using two different methods, which differed in terms of the timing and environment of spheroid formation. Method A was performed in agarose-coated U-bottom plates (low-adhesion plates) and method B *via* the hanging drop method published by Foty et al. [49]. Both methods will be described in more detail as follows:

Direct seeding in low-adhesion U-bottom plates (method A)

Non-cell culture treated, clear, U-bottom plates were pre-coated with a 1% (m/v) 2-hydroxyethyl agarose solution (Sigma-Aldrich Corporation) in deionized water to prevent cell adhesion. Cell suspensions of HT-29, HT-1080, and HCT-116 (2×10^3 cells per well) were prepared and mixed with Fluo-PET particles to a final concentration of 2 μ g/ml prior to seeding. Subsequently, 100 μ l of this mixture, or cell suspension without particles as a negative control, was transferred into agarose-coated U-bottom plates [50]. Thus, particles were present throughout spheroid formation. To ensure a homogeneous distribution, particle suspensions were thoroughly mixed before seeding and intermittently resuspended during the seeding process. The cells were allowed to settle for 72 h to enable the formation of the 3D models, followed by microscopical analysis.

Hanging drops with petri dishes (method B)

HT-29, HT-1080, and HCT-116 cells in a density of 1×10^3 cells per drop (10 μ l) were mixed with Fluo-PET (final concentration 5 μ g/ml). The Spheroids were formed by pipetting 10 μ l on the lid of a Petri dish, and the dish was filled with PBS to allow a highly humidified atmosphere [51]. Cells without particles were used as a negative control. The lid was reversed, and the spheroids were allowed to settle for 72 h with as minimal mechanical movement as possible. After the spheroids were formed, they were transferred to the previously described agarose-coated U-bottom plates for further cultivation in 150 μ l/well of fresh medium.

For both methods, spheroids were imaged on days 3, 4, 6, and 7 without media exchange using a fluorescence microscope. All spheroids were analyzed upon their size and morphology by image analysis applying ImageJ/Fiji. Therefore, the images were converted to 8-bit (256 grayscale levels) and thresholded. The spatial scale was calibrated to either 400 μ m or 1000 μ m, depending on the image resolution. After binarization, spheroid areas were measured using ImageJ's 'Analyze Particles' function; if binarization was not feasible, areas were manually delineated using the line or freehand selection tool.

Cryosections of spheroid cultures

Spheroids were embedded in O.C.T.[™] compound (Tissue-Tek[®]) on day 7 and then frozen at -80°C . The frozen spheroids were sectioned ($7.5\text{ }\mu\text{m}$ thickness) using a cryostat (Leica CM3050 S) and mounted on Superfrost[™] microscope slides (Eprelia). The images of the sections were taken with a fluorescence microscope using a FITC filter. Afterwards, the sections were dehydrated through an ethanol series with 50, 70, 80, 95, 100, 100, and 100% for 10 min each.

Optical photothermal infrared spectroscopy (O-PTIR)

For analysis and more precise distribution of the Fluoro-PET particles within the spheroids, chemical images of the spheroid sections were recorded and compared to fluorescence-based microscopy. Chemical images of the cryosliced spheroid samples were obtained with the Optical Photothermal Infrared Spectroscopy (O-PTIR) on a mIRage+R chemical imaging microscope. (Photothermal Spectroscopy Corp., Santa Barbara, CA, USA). This technique is largely immune to typical artifacts known from Fourier transform infrared (FTIR) spectroscopy in reflection geometry. Such measurements are often affected by optical scattering [52], baseline shifts [53], band shifts, and particle geometry effects [52]. The method achieves submicro-spatial resolution, which is 20 to 30 times higher than that of conventional infrared spectroscopy. The technique operates on a non-contact, reflection-based pump-probe principle. It employs a tunable mid-IR quantum cascade laser to selectively excite vibrational modes within the sample, resulting in localized photothermal effects. This resulting molecular-specific response is detected by a co-aligned, visible laser at 532 nm. Signal enhancement and noise suppression are achieved *via* lock-in amplification synchronized with the modulation of the IR pump laser. The system enables pointwise acquisition of IR spectra with a minimum step size of 100 nm and a high diffraction-limited spatial resolution of approximately 500 nm, defined only by the visible probe beam. This method significantly surpasses the diffraction limit of conventional IR techniques. The spectrally tunable IR excitation beam facilitates broadband vibrational excitation in the range of $3001\text{--}2677\text{ cm}^{-1}$ and $1853\text{--}933\text{ cm}^{-1}$. Each reported spectrum represents the average of three consecutive acquisitions. Although the system allows for simultaneous Raman acquisition at the same coordinates, that functionality was not utilized due to fluorescence interference and impractically long acquisition times under the given conditions [54, 55].

Invitrogen[®] Total reactive oxygen species (ROS) assay kit 520 nm

Cells (NIH-3T3, HT-29, HT-1080, HCT-116, and H460) were seeded in a concentration of 2×10^3 /well/100 μl

into black 96-well plates with transparent bottom (Corning[®]). A 500X stock solution of ROS stain (Thermo Fisher Scientific Inc., Waltham, MA, USA, Ref. 88–5930) was prepared by dissolving 40 μl in DMSO, and then further diluted in cell culture medium to 0.2X. After 24 hours, the cells were incubated with 100 μl /well of freshly prepared ROS stain 0.2X. The 500X stock solution can be stored at $\leq -20^{\circ}\text{C}$ protected from light until further usage. After 60 minutes incubation at 37°C and 5% CO_2 , the cells were treated with 100 μl of the respective PET particle concentrations ranging from 0.31–5 $\mu\text{g}/\text{ml}$ per well. Cells without particles were used as a negative control, and a treatment with 15% hydrogen peroxide (H_2O_2) as a positive control. Additional controls included the particles alone (without cells) as well as cells with SDS only. The treated cells were measured and analyzed at two time points (after 10 min and 120 min) at an ex/em: 480/530 nm fluorescence wavelength.

To check for particle interference and provide additional control, the human prostate carcinoma epithelial cell line 22RV1, which showed minimal ROS levels, was used with a ROS Stain concentration of 1X. The cells were simultaneously exposed to particles and H_2O_2 in increasing concentrations, ranging between 0.15–7.5%, and the ROS assay was carried out as described before. In order to analyze potential absorbance of the ROS dye, only particles were also incubated with the dye and analyzed by microscopy (Fig SI 3).

MitoSOX Red (MedChem Express[®])

NIH-3T3, HT-1080, HCT-116 and H460 cells were seeded in a concentration of 1×10^4 cells/well into black 96-well plates with transparent bottom for the mitochondrial ROS assay (MitoSOX Red, MedChem Express[®]). The assay was conducted as described in the vendors instructions. A MitoSOX Red stock solution (5 mM) was prepared by dissolving the required amount of MitoSOX Red (50 μg) in 13 μl of DMSO and stored at -20°C . The stock solution was diluted with PBS to a working solution (5 μM) shortly before use. The cells were allowed to settle for 24 h, the medium was removed, and the cells were treated with 100 μl of PET particles solution (0.31–5 $\mu\text{g}/\text{ml}$ per well). The negative control consisted of untreated cells, and as positive control 30% H_2O_2 was used. As additional control SDS was used. From this step onwards, phenol red-free medium (Sigma-Aldrich RPMI-1640 Medium, modified, with L-glutamine, without phenol red and sodium bicarbonate) was used. After 1 h incubation, the medium was removed and 100 μl /well MitoSOX Red working solution was added, and the cells were further incubated at room temperature for 20 min in the dark. The cells were washed 1 $\times$ with PBS, then refilled with PBS, and fluorescence was measured with the plate reader at 510/580 nm (ex/em) fluorescence

wavelength. Images were acquired using a fluorescence microscope with the Texas Red Filter (EVOS™ LED Cube, TX Red®) and subsequently quantified using ImageJ software. For automated image analysis, a macro was created for ImageJ and applied to all captured images ($n=3-4$, per concentration). The macro analyzed the total fluorescence in cells (cells + particle), the adsorbed fluorescence dye on the particles (particle alone), and then subtracted particle fluorescence intensity from the overall intensity, allowing the analysis of a corrected, semi-quantitative signal (Fig. SI 4). For optimal quantification of fluorescence, the corrected total cellular fluorescence (CTCF) was calculated. Additional controls include PET particles stained by MitoSOX Red without cells to check for potential interferences.

OxiSelect™ intracellular nitric oxide (NO) assay kit

NIH-3T3, HT-1080, HCT-116, and H460 cells were seeded in black 96-well plates with clear bottom at a density of 2×10^4 cells/well per 100 μ l and incubated overnight at 37 °C and 5% CO₂. After media removal, the cells were treated with 100 μ l of the PET particle solutions in a similar concentration range (0.3–5 μ g/ml) as used before. As negative control, non-treated cells were used, as positive control, a solution of sodium nitroprusside (SNP) (25 g, CAS Nr. 13755–38-9, Carl ROTH®) (50 mM) in deionized water was prepared and then diluted to a working concentration of 700 μ M in cell culture medium [56]. Additional controls included SDS, in concentrations as described above as well as PET particles without cells. After a total incubation time of 1 hour, the cells were incubated with the nitric oxide fluorometric probe (Cell Biolabs, Inc.) for 2 h according to the vendor's protocol. Cells are then treated with 1X cell lysis buffer for 5 minutes and fluorescence was measured using a plate reader at 480/530 nm (ex/em). Since a lysis buffer was used for sample preparation, fluorescence microscopy imaging was not possible.

Statistical analysis

All experiments were performed in technical triplicate and at least three independent biological repetitions ($n=3$), if not stated otherwise. The results are presented as mean $\pm$ standard deviation ($\pm$ SD) if not indicated otherwise. The measured values were normalized to the control (0 μ g/ml) for the MTT, ROS, MitoSOX, and NO assay. All experiments were statistically analyzed by GraphPad Prism 10.6.1, via 2-way ANOVA (multiple comparisons). A p -value of <0.05 was considered statistically significant. Statistical significance was indicated as follows: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. For each test series, an untreated control (0 μ g/ml) without particles was included as a blank control. In addition, positive controls were applied in each experiment to ensure assay

reliability and accuracy of the results, as stated in their respective sections.

Results

Characterization of PET and Fluo-PET particles

PET and Fluo-PET particles were produced using a precipitation protocol and thoroughly characterized as published before [47]. The two samples showed comparable average D_{eq} values of PET and Fluo-PET of $1.24 \pm 0.52 \mu$ m and $1.25 \pm 0.97 \mu$ m, respectively (Table 1). A high absolute ζ -potential value indicates strong repulsion between the particles, which prevents aggregation and promotes stability, while low values indicate an increased risk of particle agglomeration. A comparison of the Z-means showed that the PET sample ($0.96 \pm 0.097 \mu$ m) contained a higher proportion of smaller particles than Fluo-PET ($1.7 \pm 0.26 \mu$ m). The Fluo-PET particles exhibited a greater poly dispersity index (PDI) of 0.55 than PET (0.17), which indicates a more homogeneously composed PET sample.

For stability testing, particles were incubated for 20 h at 37 °C in PBS buffer or 0.1 M HCl (Fig. SI 1). ζ -potentials of -45.73 and -37.74 mV indicate high stability of PET and Fluo-PET-MNPs, respectively. Fluorescence was retained in both samples. The HCl sample appeared less concentrated due to aggregation at low pH. The HCl sample was neutralized before imaging as FITC fluorescence is pH dependent. Images were taken in the GFP channel at 50% excitation intensity.

Influence of PET micro- and nanoplastics on cell viability

Cell viability was examined in five cell lines to analyze concentration-dependent effects of Fluo-PET particles in the range of 0.3 to 5 μ g/ml (Fig. 2A) in aqueous suspension, including SDS as particle surfactant to avoid particle agglomeration. All cell lines responded in a concentration-dependent and cell type-specific manner. At the highest concentration of 5 μ g/ml, fibroblasts (NIH-3T3) showed the strongest decrease in cell viability to 0.40 ± 0.03 (normalized to control), followed by H460 cells, which showed a significant reduction to 0.52 ± 0.06 . These two cell types proved to be the most sensitive and showed the most pronounced response to exposure to the selected Fluo-PET particle concentrations. HT-1080 and HCT-116 cells were the least sensitive, falling to 0.7 ± 0.03 of their viability at the highest concentration tested (5 μ g/ml). As a result, an EC_{50} was only reached in NIH-3T3 cells, and therefore EC_{50} calculations were not performed. As SDS was used as a surfactant, SDS was tested without particles to allow for corrections and to avoid misinterpretation of the results. The detergent was found to be cytotoxic in all tested cell lines in concentrations higher than 0.0063%, with HCT-116 being the most sensitive cell line (Fig. 2B). The highest concentration of

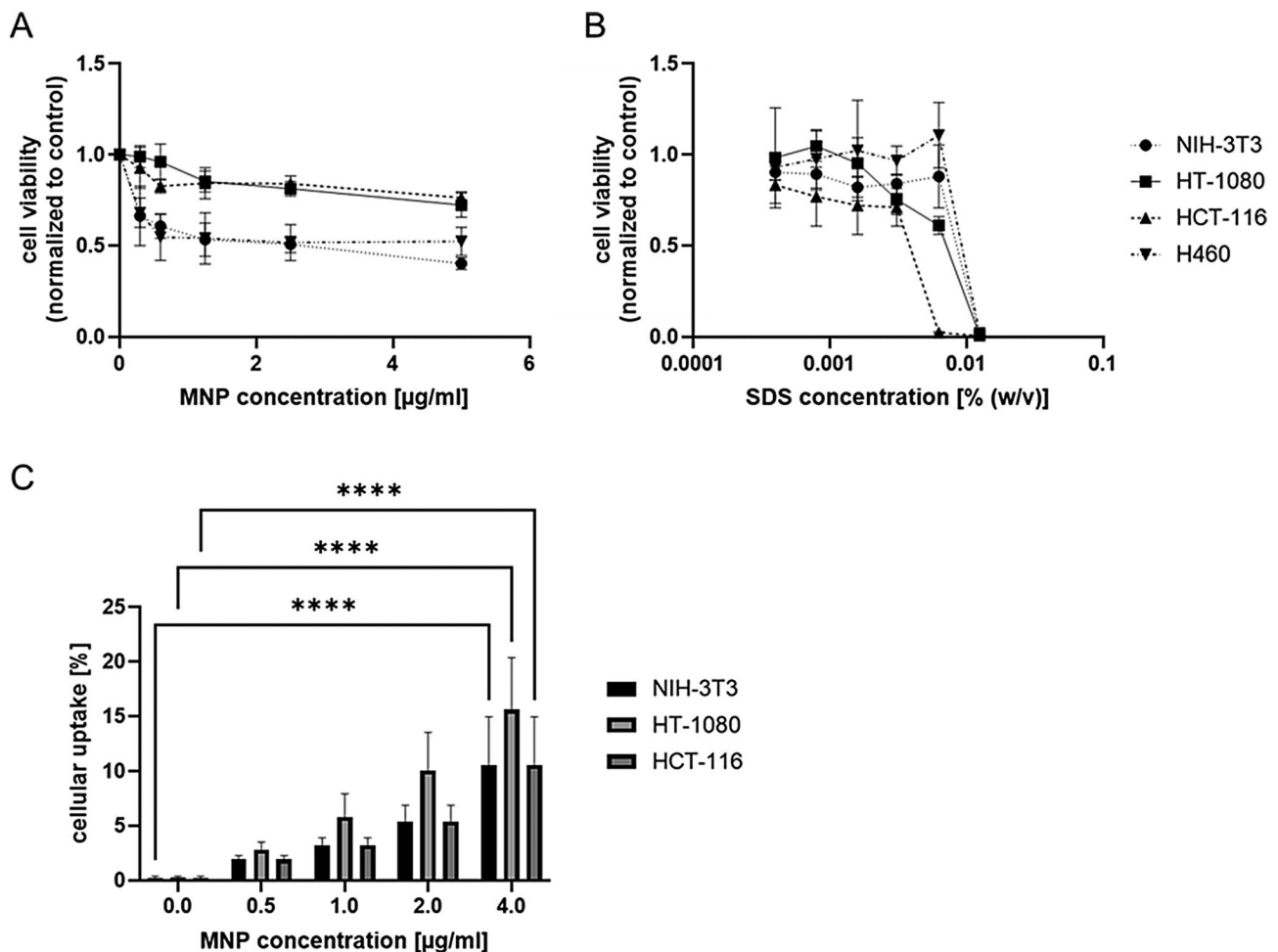


Fig. 2 Cell viability assessment and particle uptake. **A)** Fluo-PET particles affected the cell viability in a concentration and cell-dependent manner for NIH-3T3, H460, HCT-116 and HT-1080. **B)** SDS concentrations in % (w/v) with MTT tests in NIH-3T3, HT-1080, HCT-116, and H460; the highest concentration of SDS in the MNPs treated cells is 0.000625% SDS for the 5 $\mu\text{g/ml}$ concentration. In **A)** and **B)**, data are shown normalized to the negative control $\pm$ SD. Statistical analysis is not shown. **C)** cellular uptake of Fluo-PET particles in three cell lines in % (in mean $\pm$ SD). Two-way ANOVA was performed by Graph-Pad prism with a p -value of $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$; $n = 3$

Fluo-PET particles was 5 $\mu\text{g/ml}$, prepared from a stock solution of 4000 $\mu\text{g/ml}$ in 0.5% SDS, which corresponded to an SDS concentration of about 0.0006% (Fig. 2B). At this level, SDS alone did not affect cell viability, allowing the assessment of Fluo-PET's impact on cell viability without interference from the surfactant.

Cellular uptake of Fluo-PET particles by flow cytometry

Flow cytometry was used to quantify the percentage of Fluo-PET particles taken up in three cell lines (NIH-3T3, HT-1080, and HCT-116) (Fig. 2C). Fluo-PET particles were used to facilitate the quantification of the particles internalized into the cell. A significant, concentration-dependent increase in particle uptake was observed in all three cell lines. There was a clear difference between the cell lines in the concentration range $> 1 \mu\text{g/ml}$, which is presumably due to their different tissue-specific origins. HT-1080 cells showed the highest uptake, with $16 \pm 3.68\%$

at the highest concentration (4 $\mu\text{g/ml}$), followed by NIH-3T3 cells with $11 \pm 3.57\%$, and the lowest, but still significant uptake was in HCT-116 at $8 \pm 2.83\%$. No particles were present in the control group (0 $\mu\text{g/ml}$).

Spheroid growth dynamics after Fluo-PET particle exposure and comparative method analysis

Besides intracellular uptake of the Fluo-PET particles as tested in 2D, their behavior in a more physiologically relevant system was tested to study intratissue distribution and create a spiked tissue material for O-PTIR measurement validation. Here, 3D spheroid models were used to investigate potential particle interaction and distribution within a tissue-like environment. Unlike 2D cultures, spheroids better mimic tissue architecture, which may influence particle distribution and cellular exposure in a tissue-like setting. HT-1080, HCT-116, and HT-29 cells were mixed with Fluo-PET particles to allow their

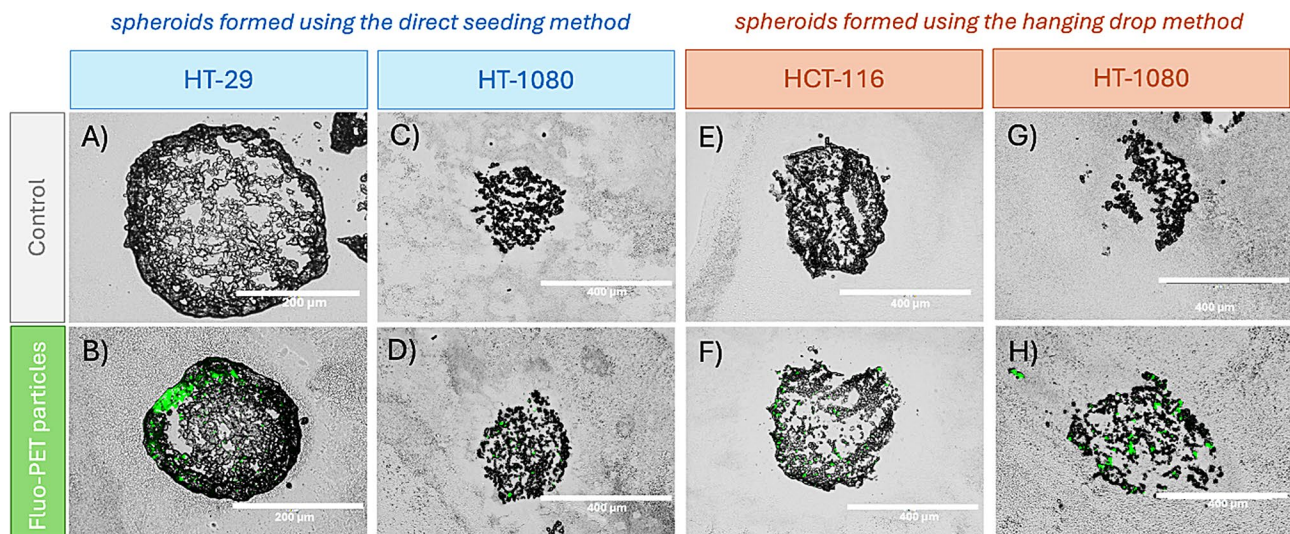


Fig. 3 Spheroid formation with method a and method b: cryosections of spheroids generated using the low-adhesion or hanging drop method from different cell lines after 7 days of growth; low-adhesion: **A)** HT-29 spheroid section without Fluo-PET; **B)** HT-29 spheroid section treated with Fluo-PET particles; **C)** HT-1080 spheroid section without Fluo-PET; **D)** HT-1080 spheroid section treated with Fluo-PET particles; hanging drop method: **E)** HCT-116 spheroid section without Fluo-PET particles; **F)** HCT-116 spheroid section treated with Fluo-PET; **G)** HT-1080 spheroid section without Fluo-PET particles; **H)** HT-1080 spheroid section treated with Fluo-PET particles; scale bars: 200–400 μm

incorporation into the spheroid during formation and to monitor their spatial distribution over time. As model systems, HT-1080, HCT-116 and HT-29 were mixed with the Fluo-PET particles to allow homogeneous distribution within the spheroid and to follow their allocation over the growth period.

First, spheroid methodology must be optimized and tested to see if the Fluo-PET particles interfere with spheroid formation. We used our lab-established ultra-low attachment plates (method a) to generate spheroids without Matrigel or other additives, as previously done for PS particles [51]. HT-29 and HCT-116 formed more compact and stable spheroids, whereas HT-1080 spheroids tended to be loosely packed, which is a cell-line dependent factor if cells prefer close cell-to-cell contacts or not. The introduction of Fluo-PET particles to the cell solution prior to formation of the spheroids did not interfere with spheroid formation. However, initial analysis of the spheroids *via* fluorescence microscopy showed a heterogeneous distribution of the particles, mainly in the areas where the spheroid was in proximity to the u-bottom plate, indicating that the Fluo-PET particles seem to be drawn/adsorbed by the plastic plate. Consequently, we tested this hypothesis by creating the spheroids *via* the hanging-drop method (method b). As hypothesized, initial investigations showed a more homogeneous distribution. To complement fluorescence microscopy and visualize the interior of the spheroid, cryosections were prepared (Fig. 3), enabling more detailed analysis of particle distribution in cross-sections. The cryosections were prepared at day 7 with a thickness of 7.5 μm .

The analysis confirmed that Fluo-PET particles did not distribute evenly in the HT-29 spheroids (Fig. 3B) when using method a, whereas the particles in HT-1080 cryosections were more homogeneously distributed (Fig. 3D). Overall, the distribution of the Fluo-PET particles was more homogenous for all cell lines (exemplarily shown for HCT-116 and HT-1080, Fig. 3F and H). As already described for the spheroid formation, HT-1080 cryosections showed reduced structural integrity of the spheroid section compared to the other cell lines in both methods, which manifested in lower compactness and a less coherent tissue architecture (Fig. 3D and H).

Importantly, the Fluo-PET particles did not accumulate in the hypoxic or necrotic core, but predominantly in the lateral region of the spheroid, as described for PS particles [51].

In the next step, the impact of the Fluo-PET particles on spheroid growth, morphology, and distribution was analyzed. Overall, exposure of Fluo-PET particles to spheroids of HT-29, HT-1080, and HCT-116 at concentrations of 2 $\mu\text{g}/\text{ml}$ and 5 $\mu\text{g}/\text{ml}$ did not result in any significant changes in spheroid growth and area compared to the non-treated control groups under either culture condition (method a and method b) (Figs. 4A, 4C, Fig. SI 5A, 5C).

Analysis of spheroid growth over a period of seven days showed differences between the two culture methods, mainly attributed to the different seeding densities applied to create spheroids. For the low-adhesion method (method a), HT-1080 cells had a spheroid area of $2.98 \times 10^5 \mu\text{m}^2$ on day 3, which was about 2.5 times larger than the spheroid area in the hanging drop

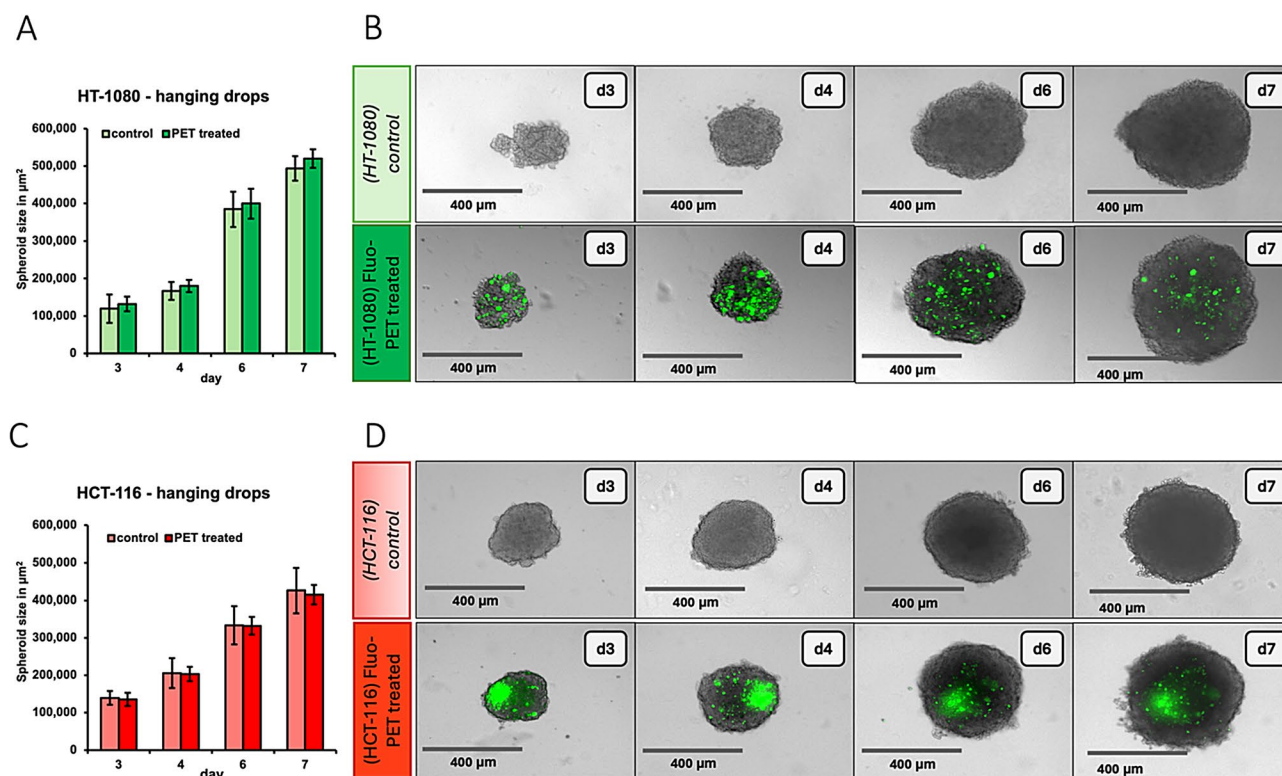


Fig. 4 Spheroid generation with the hanging drop method: **A**) quantitative measurement of spheroid growth of HT-1080 cells with no significant influence after Fluo-PET (conc. 5 µg/ml) exposure; **B**) representative images of the distribution with and without green fluorescence marked PET particles in HT-1080 spheroids at different time points (day 3, 4, 6 and 7); **C**) No significant influence on HCT-116-spheroid growth after Fluo-PET exposure; **D**) representative images of distribution with and without green-labelled Fluo-PET particles in HCT-116 spheroids at different times; $n = 3$; scale bars: 400 µm and 1000 µm

method ($1.20 \times 10^5 \mu\text{m}^2$). Over the course of days 4 and 6, HT-1080 cells showed comparable spheroid areas for both methods, which was characterized by continuous growth. Both approaches ultimately led to a spheroid area of approximately $5.00 \times 10^5 \mu\text{m}^2$ on day 6. The colon carcinoma cells (HT-29 and HCT-116) also showed similar growth behavior despite the different methods. On day 3, the spheroids reached a size of approximately $1.68 \times 10^5 \mu\text{m}^2$ and grew to a size of approximately $4.00 \times 10^5 \mu\text{m}^2$ by the end of the observation period.

Fluo-PET particle distribution over time showed cell line-dependent behavior. HT-1080 had a homogenous particle distribution (Fig. SI 5B), whereas in HT-29 spheroids, Fluo-PET particles appeared to preferentially accumulate in the inner spheroid region (Fig. SI 5D). In HCT-116 spheroids, the accumulation in the center of the spheroid was most pronounced (Fig. 4D). To clarify and specify these differences in distribution, O-PTIR measurements were performed.

O-PTIR-based detection of Fluo-PET particles in 3D cell culture model sections

In addition to fluorescence-labelling, the O-PTIR technique, a recently introduced label-free, non-destructive

method, was employed to localize and identify PS particles in spheroid sections [57]. This approach was applied to Fluo-PET particles and it was demonstrated for different spheroids originating from HCT-116, HT-1080, and HT-29, with and without incorporated Fluo-PET particles. The fluorescence signals (cyan), overlaid with the O-PTIR signals for the PET-specific IR absorption band (magenta), facilitate direct comparison (Fig. 5). For this, a characteristic band for PET (1727 cm^{-1}) was selected and normalized to a tissue/cell-specific band (amide I band, 1660 cm^{-1}) to correct for the surrounding spheroid matrix. The spheroid was scanned using these two wavenumbers with a resolution of $0.15 \mu\text{m}$ and a scanning speed of $300 \mu\text{m}/\text{ms}$. These images were then used to generate a ratio plot ($1727 \text{ cm}^{-1}/1660 \text{ cm}^{-1}$) per pixel, which provided sufficient contrast between the Fluo-PET particles and the cell matrix. At positions where this ratio plot indicated the presence of Fluo-PET particles, full O-PTIR spectra were recorded to unambiguously confirm the identity of Fluo-PET by its characteristic fingerprint spectrum.

The grayscale background images represent the bright-field visible view of the investigated spheroid sections, providing a structural context (Fig. 5). Cyan colored

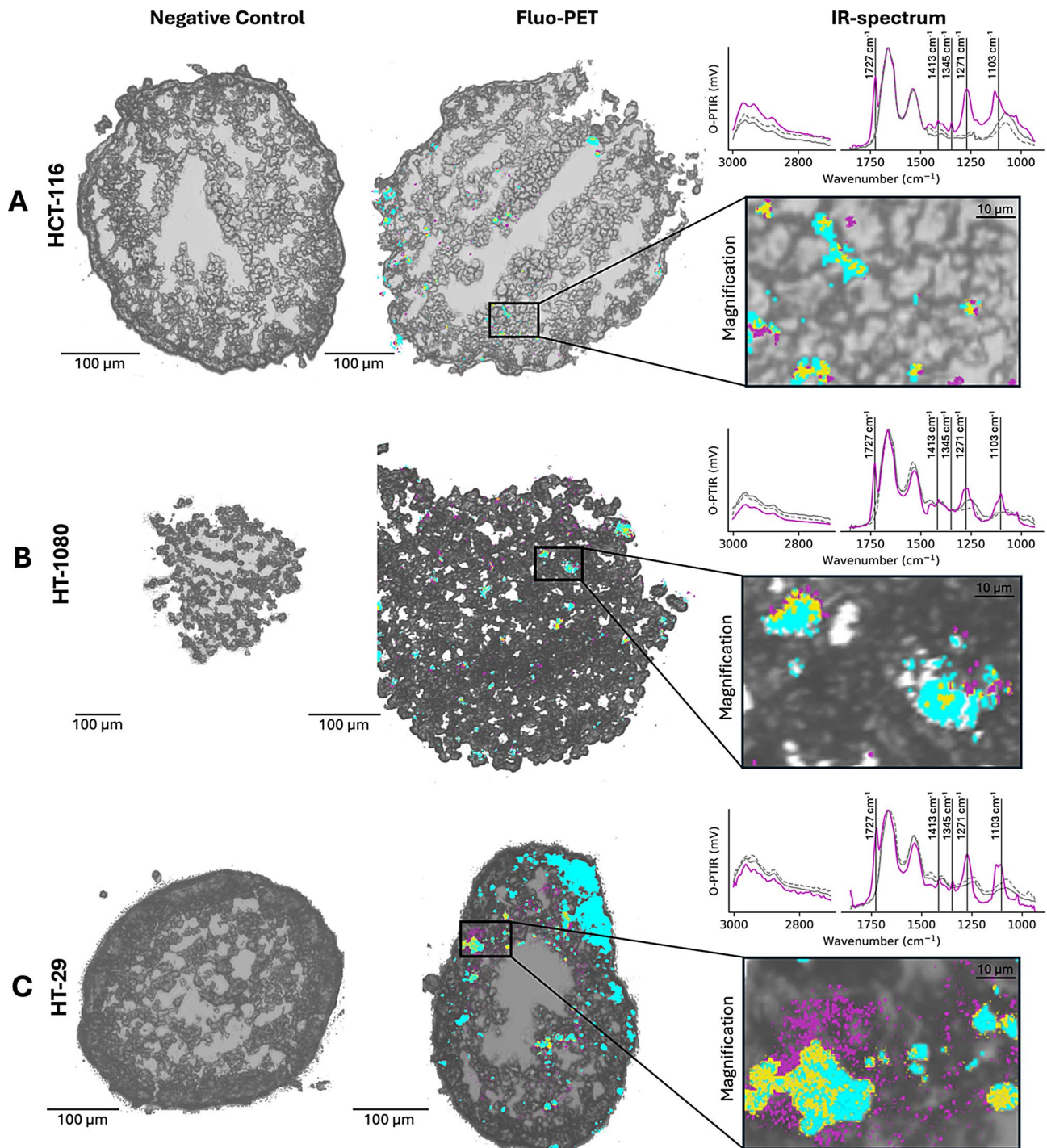


Fig. 5 O-PTIR-based detection of Fluo-PET particles in spheroids: **A)** on the left, a cross-section of the non-spiked control HCT-116 spheroid is shown. The middle panel (Fluo-PET spiked) displays a cross-section of an HCT-116 spheroid grown in medium containing Fluo-PET particles. Cyan indicates the fluorescence signal, magenta represents the PET-specific O-PTIR signal, and yellow highlights their spatial overlap. The region marked in black is shown magnified in the right panel to enhance visibility. In the top-right corner, three representative full spectra are displayed. The grey dashed line indicates a spectrum of the negative control. The grey solid line represents an O-PTIR spectrum from a region in the spheroid where no PET particles are present. The magenta spectrum corresponds to a PET particle within the spheroid. **B)** spheroids were derived from the HT-1080 cell line under the same conditions. **C)** spheroids were derived from the HT-29 cell line under the same conditions

regions represent the fluorescence signal from Fluo-PET, while the magenta colored regions correspond to the label-free O-PTIR signal specific for PET. Regions where both signals show an overlap are indicated by yellow color. In the top-right corner of each panel, representative O-PTIR spectra are displayed: the dashed grey line shows the spectrum of a negative control (not exposed to Fluo-PET); the solid grey line represents a PET-negative region within a Fluo-PET-exposed spheroid; the magenta line corresponds to a PET-positive region. Vertical black lines indicate selected IR bands characteristic of PET, thereby enabling its precise chemical identification.

It should be noted that fluorescence images were acquired before removing the Tissue-Tek medium, which was necessary to prevent interference in the O-PTIR measurements due to Tissue-Tek's own absorption spectrum. This removal step may have slightly shifted particle positions. For HT-29 specifically, portions of the spheroid's edge were lost during this process.

All three images (Fig. 5) confirmed the absence of a PET signal in the negative control, both in the fluorescence channel and in the O-PTIR measurements. In the spiked samples, the fluorescence and O-PTIR signals were overlaid to visualize the co-localization of Fluo-PET particles. These overlaps are particularly clear in the magnified views. Notably, for the HCT-116 spheroid, some particles were visible only through the O-PTIR signal (magenta), which may be attributed to background levels of PET particles or incomplete fluorescence labeling. These signals were analyzed and confirmed to be PET based on their IR spectral signature. In general, every signal detected by O-PTIR was verified using a full IR spectrum to confirm its chemical identity as PET. The overlaid spectra in the upper corner of each figure show representative examples used to distinguish PET particles from the surrounding cells. For the HT-1080 spheroid, both signals were clearly visible. Notably, some particles show a stronger signal concentrated along their edges. In the Fluo-PET spiked HT-29 spheroid, a larger portion of the spheroid's outer regions was lost during the preparation for O-PTIR measurements as described before. While some of the observed O-PTIR signals align with the fluorescence signal, they could not be conclusively identified as PET based on their IR spectra, unlike the PET particles in the HCT-116 and HT-1080 samples, which were clearly confirmed through spectral analysis.

Overall, the O-PTIR analysis of the spheroid cryosections confirmed that there is a heterogeneous distribution of the particles within the spheroid in a cell line-dependent manner. For HCT-116 and HT-29 spheroid, a less tight center of the spheroid was observed, indicating a necrotic core, which is not seen in the HT-1080 spheroid. As a result, the Fluo-PET particles for HCT-116 and HT-29 spheroids were mainly found in the proliferating

outer area, whereas a more homogenous distribution was observed for the HT-1080 spheroid without necrotic areas.

Impact of PET particles on intracellular ROS generation after 10 min and 2 h

To minimize interferences with the assay's fluorescence, and as the Fluo-PET signal was not required anymore for particle identification, all further experiments were performed with the respective PET particle suspension. To evaluate if PET particles influence the general, intracellular ROS production, a ROS assay *via* DCFH-DA was used to quantify the amount of total reactive oxygen species. For this purpose, the cell lines NIH-3T3, HT-29, HT-1080, HCT-116, and H460 were used in a 2D format and were exposed to PET particle concentrations ranging from 0.3 to 5 µg/ml. The measurement was carried out directly after treatment (10 min) and after a further incubation period of 120 min. The results showed that exposure to PET particles caused a dose-dependent increase in intracellular ROS production and revealed cell type-specific differences (Fig. 6). In NIH-3T3 cells (Fig. 6A), a significant increase in ROS activity was already visible after 10 min at a concentration of 1.25 µg/ml (1.17 ± 0.04) normalized to control (0 µg/ml). The ROS values increased exponentially with increasing concentration and reached an approx. 4-fold fluorescence intensity compared to control at the highest concentration. After 120 min, a similar pattern and a maximum in fluorescence intensity was observed of around 6-fold. HT-29 cells were less sensitive and showed an initial significant increase in ROS values (2.23 ± 0.57) at 2.5 µg/ml PET, which was about twice as high as the control. At the highest tested concentration of 5 µg/ml, ROS production increased further to 4-fold compared to the control (4.28 ± 0.83) and stabilized around 5.20 ± 1.54 even after 2 h of incubation (Fig. 6B). In contrast, HT-1080 fibrosarcoma cells exhibited greater sensitivity, with significant ROS induction (2.97 ± 0.80 -fold) already at 2.5 µg/ml. This response intensified over time, peaking at 7.07 ± 2.60 -fold after 2-hour exposure to 5 µg/ml PET. Similar patterns emerged in HCT-116 and H460 cell lines. Both lines showed rapid responses, with significant ROS increases (2.39 ± 0.61 and 2.79 ± 0.50 -fold, respectively) after just 10 min at 2.5 µg/ml. The most dramatic effects appeared in H460 cells, where 2-hour exposure to 5 µg/ml PET triggered a remarkable 10.15 ± 1.75 -fold ROS formation - the strongest response observed across all cell lines.

Concluding, all cell lines showed a consistent pattern: at low concentrations of 0.3 and 0.6 µg/ml, no ROS was observed, with even values below the control level. ROS was significantly observed in all cell lines at concentrations > 2.5 µg/ml and already after a short time of approximately 10 min of incubation. In terms of controls applied

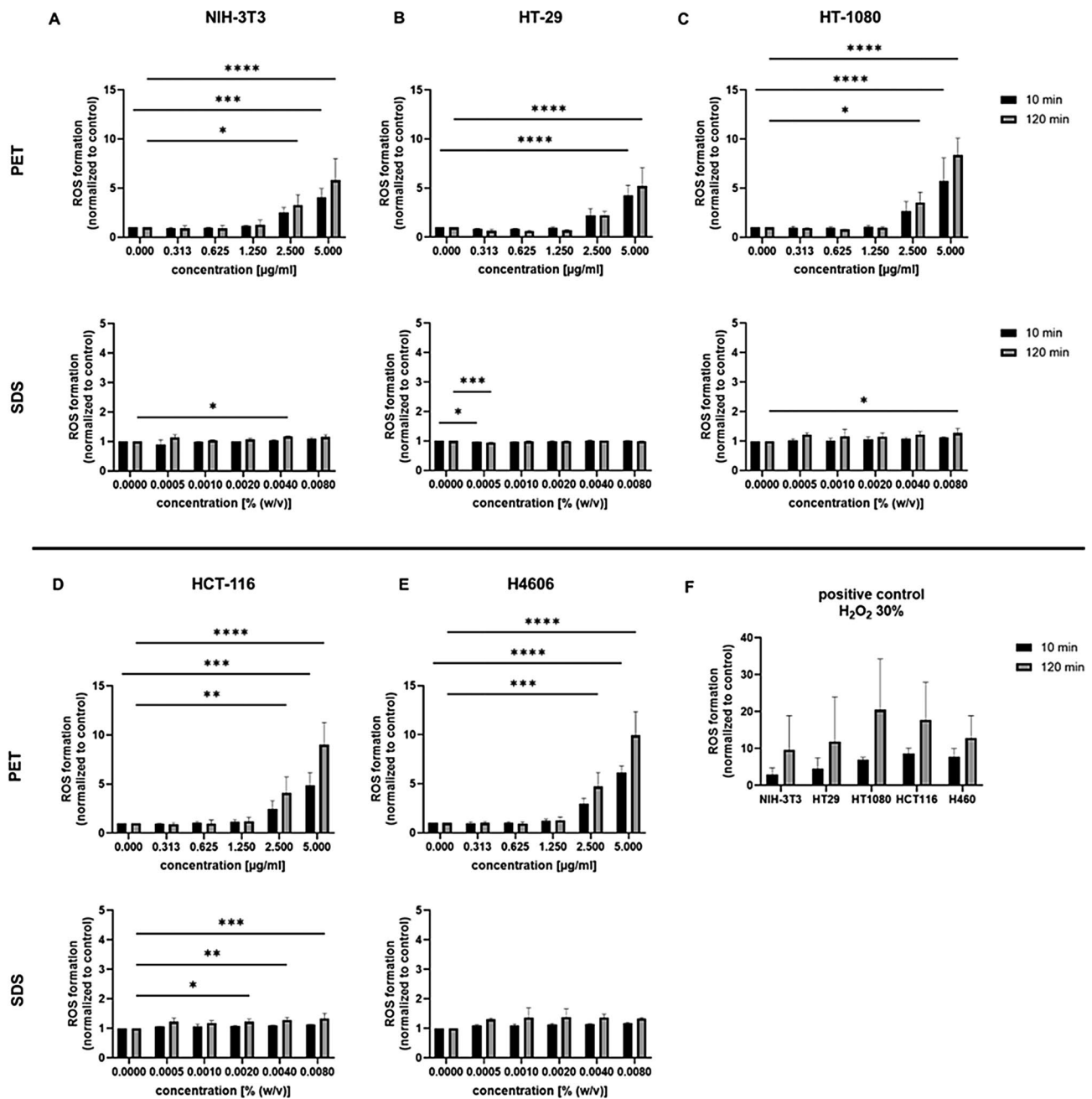


Fig. 6 ROS was measured using DCFH-DA in five different cell lines **A**) NIH-3T3, **B**) HT-29, **C**) HT-1080, **D**) HCT-116 and **E**) H460 for PET particles and SDS as well as **F**) positive control of 15% H₂O₂. The bars represent the values after 10 min and 120 min of treatment in the similar cells (kinetic mode) normalized to control of the respective time point. In the control group, ROS production remained very low in all cell lines and served as a baseline. All values were normalized relative to the control (0 µg/ml). Two-way ANOVA was performed by GraphPad prism with a *p*-value of **p* < 0.05, ***p* < 0.01, ****p* < 0.001, and *****p* < 0.0001; *n* = 3–4

for the ROS assay, we first investigated the SDS solution to correct for potential interference with the assay. As described before, the SDS solution was tested with a defined and comparable concentration range in order to control potential ROS formation caused by the surfactant (Fig. 6, column SDS). In general, significant amount of ROS was also produced by SDS alone in NIH-3T3, HT-29, and H460. No significant changes were observed

in HCT-116 and HT-1080 cell lines. However, ROS production was significantly higher when treated with PET particles, indicating that the main effect was caused by the particles and not the surfactant. The positive control (15% H₂O₂) confirmed the functionality of the assay (Fig. 6, column positive control).

Additionally, as in the HT-29 cell lines, showing the lowest ROS production, ROS levels below the control

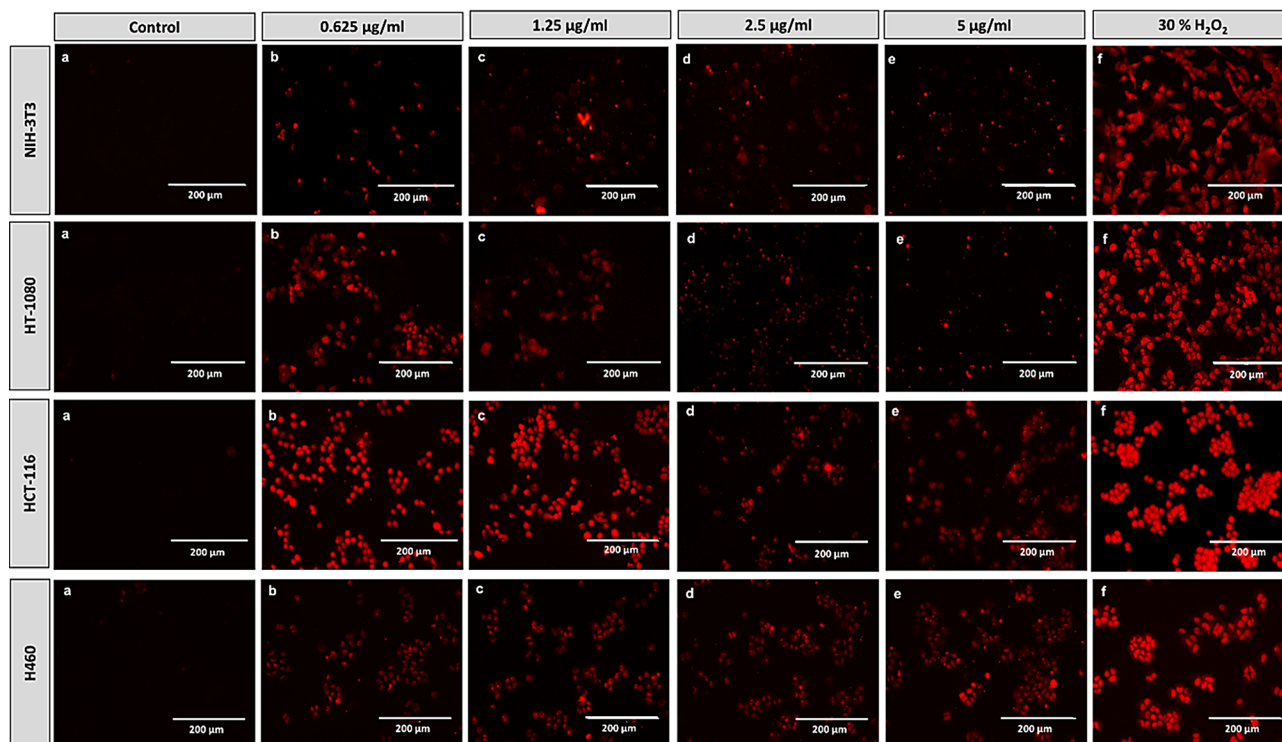


Fig. 7 Mitochondrial oxidative stress levels: fluorescence images of NIH-3T3, HT-1080, HCT-116 and H460 cells stained with MitoSOX Red® to detect specifically mitochondrial ROS. Cells were treated with different concentrations of PET particles. Scale bar: 200 µm. Positive control: 30% H₂O₂. Quantitative analysis of mitochondrial ROS in NIH-3T3, HT-1080, HCT-116, and H460 cells after MitoSOX Red® staining can be found in fig. SI 9

level were observed. Tests were performed to confirm the validity of the assay. In an additional cell line, 22RV1, which was included as a control due to its lack of ROS response to the PET particles, similar effects were observed, with ROS levels found to be below the control level (Fig. SI 6). In a concentration-dependent manner, and by the addition of H₂O₂ in different concentrations, it was observed that increasing PET particle concentration shielding or masked the signal to a significant extent. Therefore, the impact of any tested particles on fluorescence signal and assay validity should be tested beforehand. We verified that the dye does not adhere to the PET particles by performing a control using only DCFH-DA and PET particles (Fig. SI 3).

MitoSOX® red stain for analysis of the mitochondrial oxidative stress level

The application of MitoSOX Red aimed to further investigate the participation of mitochondria in the overall PET-induced ROS production. Therefore, the cells were exposed to similar PET concentrations as before (0.625–5 µg/ml), and the investigation was limited to those four cell lines (NIH-3T3, HT-1080, HCT-116, and H460) with the highest total ROS production. This method enables the specific detection of mitochondrial superoxides to clarify whether the previously observed

ROS levels originated intracellularly from mitochondrial superoxides or possibly from other organelles.

The cells were stained with MitoSOX Red and assessed with both a plate reader (Fig. SI 7) as well as a fluorescence microscopy (Fig. 7). For the plate reader measurements, fluorescence intensities, normalized to the control (0 µg/ml), are plotted on the Y-axis, and the different treatment conditions on the X-axis (Fig. SI 7). However, there was no significant increase in mitochondrial ROS levels by PET (Fig. SI 7A), or SDS-treated cell (Fig. SI 7C), while H₂O₂ as positive control displayed a significant signal (Fig. SI 7B). To validate the sensitivity of the plate reader, the cells were investigated under the fluorescence microscope.

Fluorescence microscopy revealed a ROS signal in all treated cells, as well as in the particles themselves. To prove if the particles interact with the stain, the staining protocol was performed with particles and without cells. PET particles alone also showed fluorescence when stained with MitoSOX Red (Fig. SI 8), therefore, a method to separate the signal from the cells and particles was established *via* ImageJ. Here, the overall fluorescence intensity per cell was corrected to the fluorescence intensity originating from the PET particles, to allow semi-quantitative ROS signals per cell. In all cell lines, ROS generation was observed and visible in the microscopy. In NIH-3T3 cells, ROS generation at lower

concentrations (0.625 µg/ml) was more pronounced than in higher concentrations, indicating a potential cellular behavior change due to oxidative stress. A similar pattern was found for HT-1080 and HCT-116 cells where the strongest signal was already observed at the lowest concentration. For H460, increasing concentrations of up to 5 µg/ml created a higher ROS signal. The positive control with 30% H₂O₂ showed strong fluorescence in all cell lines, confirming the functionality of the assay, whereas the negative untreated control was non-fluorescent. The semi-quantitative evaluations using ImageJ (Fig. 7B) corresponded to the observed fluorescence patterns and are presented as bar charts with mean CTFC values and ± SD in the Supporting Information (Fig. SI 9).

Overall, these results indicate that our plate reader is not sensitive enough to quantitatively assess the effect, whereas fluorescence microscopy proved the involvement of mitochondrial stress caused by ROS production, and an interference of the dye with the particles itself.

Nitric oxide (NO) production as a marker for reactive nitrogen species (RNS) after PET exposure

The NO assay was performed to measure the contribution of NO as another reactive species to intracellular stress and thus to better differentiate the overall response to PET exposure. Four cell lines (NIH-3T3, HT-1080, HCT-116, and H460), tested with the same concentrations (0.3–5 µg/ml) were used. The results for OxiSelect™ intracellular NO assay showed no significant effects in any of the cell lines at any of the applied concentrations (Fig. SI 10A). As expected, the positive control with 700 µM SNP showed significant NO production in all tested cell lines, confirming the functionality of the assay. In comparison, treatment with SDS also did not lead to a significant increase in NO production either (Fig. SI 10B).

Discussion

This study aimed to characterize the potential cellular effects of PET-MNPs in vitro, with focus on oxidative stress. Due to assay interference from surfactants and PET particles, the results are presented in three parts: first, an assessment of potential false positives caused by additives and laboratory consumables; second, an analysis of methodological challenges and assay interference, an often overlooked but critical issue in the literature; and third, an evaluation of the cellular effects of PET-MNPs, adjusted for the identified interferences. We explicitly want to emphasize that, although these interferences are well known, they are often overlooked in recent MNP studies, underscoring the need to address and discuss them in detail in our work.

Reagent and consumable interferences can lead to false positive results

The usage of commercial particles, or in our case, self-produced reference particles, require additives like surfactants to keep a stable, non-aggregated solution over time. Despite surfactants being well described in literature, the association with MNPs suspension is barely mentioned or not classified. If the MNP particles contain surfactants, additional controls are needed. Our recent research describes a PET nanoparticles production protocol for detergent-free formulations [47]. As this study was performed in parallel, we included additional controls to correct synergistic effects between SDS and the particles. At our highest Fluo-PET particle concentration (0.0006% SDS at 5 µg/ml particles), no SDS effect on cell viability was observed. However, at this low SDS concentration, we still had ROS formation, showing that a positive control for each assay is required, as effects cannot be ruled out by cytotoxicity testing. This observation is consistent with previous studies that also described concentration- and time-dependent cytotoxicity of SDS [58, 59]. Wang et al. also observed weak ROS formation in primary mouse hepatocytes at SDS concentrations between 0.1 µM and 0.1 mM over 12 h [60]. These findings are consistent with our results, in which measurable ROS induction was also observed after shorter exposure (120 min). However, the low ROS levels in the SDS control illustrate that the oxidative stress observed in the PET-exposed cells is primarily due to the particles themselves and not to the detergent used.

As PET particles can be considered highly lipophilic and have a higher density than water, there is a physicochemical tendency of the particles to sediment and separate from the aqueous cell culture medium [61]. This is even more pronounced when cells are in direct contact with plastic consumables used in the lab, as the particles are potentially attracted to the more lipophilic plate surface. When investigating the PET particle distribution over time in tissue-like constructs, like spheroids, we experienced a direct impact of the seeding method on the result. A similar effect was already observed for PS particles [51]. Overall, the particles accumulated in the area of contact between spheroid and U-bottom plate. However, a statistical distribution of the particles within the spheroid, which allowed the analysis of a growth-dependent redistribution of the particles, was only possible when applying the hanging-drop method, where there is no close contact between particles and plate. The high variability of the data based on just a seeding method can lead to wrong conclusions. A validation of all applied assays to rule out confounding variables is required.

Sample preparation in the subsequent process can further introduce and increase PET particle background and contamination [62]. Therefore, method cross-validation,

reproducibility and consistency checks are advised to be added. In our case, we analyzed the whole spheroids and spheroid cryosections with fluorescence microscopy, and the cryosections additionally with O-PTIR. Our findings show that, especially when sample preparation prevents displacement of embedded particles, O-PTIR enables clear, high-reliable identification of PET based on its distinct IR spectral signature. In reference to the fluorescence method, similar detection patterns can be reproduced, allowing meaningful comparison between both techniques. In general, particle identification *via* O-PTIR remains possible even if particles might have shifted during sample preparation. However, in such cases, it is no longer possible to directly align the O-PTIR chemical images with the fluorescence images, which limits comparative analysis between the two methods. Moreover, it allows the detection of particles that are unlabeled or have undergone fluorescence fading, thus overcoming a key limitation of dye-based techniques. In some instances, PET particles were detected solely by O-PTIR, without any corresponding fluorescence signal, likely due to incomplete labelling, photobleaching, or background levels. These particles were confirmed *via* their spectral features, underlining the importance of chemical verification. Furthermore, fluorescence signals often appeared larger than their O-PTIR counterparts, an effect attributed to dye diffusion and potential non-specific binding [63]. In contrast, O-PTIR provides a polymer-specific signal, directly linked to the material's vibrational characteristics. The method proved to be a robust alternative, eliminating the need for prior fluorescence labelling, thus paving the way to applications in real-life samples that incorporate unlabeled MNPs.

Spectroscopic limitations and scattering impact potential false negatives or positives

For O-PTIR, which is based on IR spectroscopy, the main methodological limitation occurs in overlapping bands. Such spectral overlaps can be addressed through multivariate or machine learning-based spectral unmixing methods. These can be corrected, for example, using machine learning tools. For other spectroscopic methods such as UV-Vis, fluorescence, and luminescence spectroscopy, the main interferences result from scattering and diffraction effects. Since particles inherently scatter and diffract light, as is also the principle used for particle size analysis in DLS, these effects are unavoidable when working with particle-based systems. Relying solely on plate readers can lead to false positive or unexpected results, such as apparently lower levels of ROS, which may be falsely interpreted as an antioxidative effect. Therefore, cross-validation using FACS or fluorescence microscopy is essential to verify effects observed through light-based spectroscopy. Baseline corrections of controls

with particles must be included, which can also be potentially automated *via* machine learning [64].

As extensively described in the literature, one of the most common methods to label MNPs is based on the adsorption of fluorescent dyes onto the particle surface. This approach is associated with potential dye leaching, which can be avoided by using covalently bound dyes, as applied in our study. Nevertheless, the opposite effect may also occur. A dye intended for the analysis of biochemical processes, such as ROS formation, DNA intercalation, or membrane stability, may adsorb onto the MNP surface unintentionally. This can lead to an increased signal not due to the actual biochemical process but a dye-corona formation. To avoid such artifacts, the compatibility of each dye must be verified through additional controls, including pre-incubation with the pure particles before conducting biological assessments. Dye-corona formation can interfere with all light-based measurement techniques, including plate readers, flow cytometry, and fluorescence microscopy. However, microscopy-based image analysis allows the distinction between concentrated signals localized on particles and those associated with identifiable cellular structures. Taking such precautions is essential to avoid false positives and to ensure the reliability of spectroscopic data.

PET particles were internalized, and similarly created intracellular ROS as PS

Once all interferences were identified and corrected for, the final question of what effects were caused by PET particles was ready to be answered. In our study, a maximum dose of 5 µg/ml was used for 1 µm large PET particles. The selected concentrations are considerably lower than those commonly applied in many *in vitro* and *in vivo* studies, which frequently exceed 0.1 mg/ml and can reach up to 1 mg/ml for smaller (40–500 nm) PS particles [65, 66]. Due to the absence of reliable and robust data on human exposure, which are currently critically discussed, the concentrations in this study were selected in an intermediate to low-dose condition (<5 µg/ml). Notably, a study by Goodman et al. also employed concentration ranges of around 5 µg/ml and reported measurable biological effects [26]. PS nanoparticles are commonly used in gastrointestinal models at higher concentrations [23], whereas PET, though environmentally also relevant, has been studied scarcely [42]. While the interest in PET and other plastic types is growing, systematic research remains limited due to the low availability of well-characterized reference particles, especially in the nanometer range [67]. Our PET particles averaged $1.24 \pm 0.52 \mu\text{m}$ in size, which is in a size range that is relevant for cellular toxicity and uptake. Fluo-PET particles indeed caused a concentration-dependent decrease in cell viability across all tested cell lines. H460 lung cancer cells were

particularly sensitive, likely due to lung cells' known susceptibility to environmental stressors, such as low antioxidant capacity or increased particle uptake [68]. NIH-3T3 fibroblasts also showed higher sensitivity, while HT-1080 fibrosarcoma cells were more resistant—potentially due to the greater adaptability and metabolic activity of malignant cells [69]. These findings align with previous reports showing variable cytotoxicity of PE and PS particles in similar cell lines [14, 51].

A concentration- and time-dependent increase in the uptake of Fluo-PET particles was observed in all tested cell lines, confirming their ability to cross cell membrane. Cell type-specific factors such as membrane structure, endocytosis activity, and receptor expression may influence particle internalization (dos [70]). Previous studies have shown that parameters such as particle size, shape, and surface charge can significantly influence the internalization efficiency [18, 71]. The duration of exposure was also found to be an important factor in particle uptake, which has already been observed in comparable studies with microplastic particles of different polymer composition [72].

Not only cell internalization but also tissue re-distribution is an important component in understanding the migration of particles within tissue. Spheroid cultures offer a more physiologically relevant model for studying PET particle distribution and intercellular migration [73, 74].

When starting spheroid formation with distributed PET particles within a 7-day period, fluorescence microscopy indicated a redistribution to the center of the spheroid. However, cross-sections of cryopreserved spheroids showed a re-distribution of the PET particles with a clear accumulation in the lateral area at the endpoint. This confirms that PET particles do not tend to be static in tissue-like construction and that significant migration and movement take place not only intracellularly, but also intercellularly.

Even on short-term exposure (10 min and 120 min), the investigated PET particles induce a concentration-dependent increase in ROS levels in the tested cell lines, consistent with previous findings for other plastic particles [24, 75–77]. Tumor cells (HT-29, HT-1080, HCT-116, H460) were more sensitive than NIH-3T3 fibroblasts, likely due to their altered redox balance and higher basal ROS levels [78]. Most MNP-ROS studies focus on later time points (24–48 h) [75], however, our results show that ROS is an early onset cellular response to PET particle exposure. Similar early ROS responses were reported in HT-29 cells exposed to 3 µm PS-MNPs [79] and in HCT-116 cells exposed to PS nanoparticles [37]. These findings indicate that ROS production is mainly driven by the presence and properties of particulate matter, as reportedly PE, and also modified PS lead to ROS formation [15, 80, 81].

To understand the origin of ROS formation, mitochondrial superoxide was investigated, and although several assay interferences were observed, mitochondrial-dependent ROS was detected in fluorescence microscopy. Semi-quantitative analysis of the fluorescence images proved to be crucial in this regard, as the plate reader used was not sensitive enough to reliably detect the effects. This observation underscores the need for combined methodological approaches in order to validly assess particle-induced ROS formation. Saenen et al. have already noted that particles can produce a fluorescent signal when incubated with the stain [82].

Overall, our study confirms that PET-MNPs rapidly trigger oxidative stress in a particle-, dose-, and cell-dependent manner, in a similar way as described for other MNPs. These results confirm existing knowledge and expand it to PET particles, showing that ROS response seems to be based on the particulate itself and not the chemical origin.

Conclusion

This study demonstrates that PET-based MNPs can induce oxidative stress *in vitro*, with effects depending on particle concentration, cell type, and exposure time. A major focus was placed on identifying and correcting for assay interferences caused by surfactants, additives, and particle behavior, which can lead to both false positive and false negative results. Methodological controls, including cross-validation with imaging techniques and baseline correction, proved essential for reliable data interpretation. Although these interferences are well-known, a lack of inclusion of corrections is shown in recent MNP literature; therefore, we see the need to include this discussion in our study.

Our findings demonstrate that PET particles are internalized by cells and trigger rapid ROS formation, especially at potentially relevant intermediate-to-low dose concentrations. While fluorescence methods provided insight into cellular uptake and oxidative stress, O-PTIR offered a robust, label-free alternative for particle identification, particularly in complex 3D models. Differences in particle distribution were strongly influenced by culture methods and sample preparation, further underlining the importance of protocol selection.

Overall, this study supports existing knowledge on MNP-induced oxidative stress and extends it to PET particles. The results confirm that the observed cellular responses are primarily particle-driven, independent of chemical origin or labeling. Careful experimental design, validation of spectroscopic data, and a critical assessment of potential artifacts remain essential to ensure reliable conclusions in micro- and nanoplastic toxicology [83, 84].

Supplementary information

The online version contains supplementary material available at <https://doi.org/10.1186/s43591-026-00195-4>.

Supplementary Material 1

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Authors contribution

Lisa Aichinger: Methodology, Investigation, Formal analysis, Writing – original draft; Kristina Duswald: Methodology, Investigation, Formal analysis, Writing – original draft; Doris Pakusic: Methodology, Investigation, Writing – review & editing; Lukas Kogler: Investigation, Writing – review & editing; Sarah Stellnberger: Investigation, Writing – review & editing; Verena Karl: Methodology, Writing – review & editing; Matea Stojanovic: Methodology, Writing – review & editing; Dominik Wenisch: Methodology, Writing – review & editing; Michael Jakupc: Supervision, Resources, Writing – review & editing; Cécile Philippe: Supervision, Resources, Writing – review & editing; Lukas Kenner: Supervision, Resources, Writing – review & editing; Markus Brandstetter: Supervision, Resources, Writing – original draft; Verena Pichler: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Supervision, Resources, Funding.

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Data availability

The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files. Should any raw data files be needed in another format they are available from the corresponding author upon reasonable request.

Declarations

Ethics approval declaration

Not applicable.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used ChatGPT in order to improve the readability and language of the manuscript only. After using this tool/service, the author(s) reviewed and edited the content as needed and take full responsibility for the content of the published article.

Competing interest

The authors declare no competing interests.

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