Increasing the versatility of an ex vivo model in nanosafety studies and fibrosis

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DOI:
10.33612/diss.119127385

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2020

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Download date: 01-11-2023
Chapter 2

Time-Resolved Quantification of Nanoparticle Uptake, Distribution and Impact in Precision-Cut Liver Slices

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Accepted in SMALL, January 2020, DOI: 10.1002/smll.201906523
ABSTRACT

Much effort within the nanosafety field is currently focused on the use of advanced in vitro models to reduce the gap between in vitro and in vivo studies. Within this context, precision-cut tissue slices are a unique ex vivo model to investigate nanoparticle impact using live tissue from laboratory animals and even humans. However, several aspects of the basic mechanisms of nanoparticle interactions with tissue have not yet been elucidated. To this end, we exposed liver slices to carboxylated and amino-modified polystyrene nanoparticles known to have different impact on cells. As observed in standard cell cultures, amino-modified polystyrene induce apoptosis, and their impact is affected by the corona forming on their surface in biological fluids. Subsequently, we performed a detailed time-resolved study of nanoparticle uptake and distribution in the tissue, combining fluorescence imaging and flow cytometry on cells recovered after tissue digestion. We found that, as observed in vivo, the Kupffer cells accumulate high nanoparticle amounts and, interestingly, they move within the tissue towards the slice borders. Similar observations were reproduced in liver slices from human tissue. Thus, tissue slices can be used to reproduce ex vivo important features of nanoparticle outcomes in the liver and study nanoparticle impact on real tissue.

KEYWORDS: liver slices; ex vivo model; primary Kupffer cells; nanosafety; fluorescence imaging; flow cytometry.
INTRODUCTION

Advanced models such as co-cultures, 3D models and organ-on-a-chip devices, are highly sought within the nanomedicine and nanosafety fields to bridge the gap between in vitro and in vivo testing[1–6]. They could contribute to speeding up the translation of nanomedicines to the clinic, and ideally allow screening of multiple nanomaterials, reducing the need for animal testing. Precision-cut tissue slices cultured ex vivo constitute an interesting alternative to fulfill several of these aspects and are already well-established as useful models for testing the mechanism and toxicity of small compounds and drugs[7–10].

Tissue slices of 5 mm diameter and around 250 µm thickness (roughly 10 cell layers) can be prepared from different organs (including diseased organs) and potentially any species, including from humans. They possess the complexity and architecture of real three-dimensional tissue and allow preserving ex vivo features essential for tissue function. In fact, they include all (primary) cells normally present in a tissue, with their natural abundance and organization (Supplementary Table S1 summarizes some of the key features of this established model)[7–9].

Several studies that use tissue slices with nanoparticles have been published[11–14]. In most cases, slices have been used to determine nanoparticle impact on the tissue, but also to test nanoparticle formulations for drug delivery, with many examples focused on the lungs,[11,13,15–17] but also intestine,[18] liver[14] and tumor tissue[19]. Most of these works used tissue from laboratory animals, with the exception of one study using human tissue from the lungs[17] and one from intestine[18]. Additionally, only in few cases the details of the cell types in which nanoparticle uptake was observed was included[14]. Similarly, detailed studies to quantify nanoparticle distribution within the tissue over time, and to determine uptake kinetics in the different cell types in which nanoparticle accumulate have not been reported yet.

More generally, a clear understanding of the basic mechanisms of nanoparticle interactions with tissue is missing. Thus, it is not always clear how to connect nanoparticle outcomes on tissue to what is observed in standard cell cultures or, indeed, in vivo. For instance, small molecules typically move relatively freely through tissue, and thus their distribution in vivo is largely captured by a tissue slice submerged in a solution of the compound of interest[20]. In contrast, nanoparticles are internalized and trafficked by cells using endogenous pathways and usually do not exit the cell again[21–23]. Thus, when applied in vivo, depending on the route of exposure or administration, they will
interact with specific cells, into which they may enter and are likely to remain unless they are able to transcytose or are exported. Penetration into tissue is, in fact, a barrier for nano-sized drug carriers[23–25]. Instead, when a tissue slice is submerged in a dispersion of nanoparticles, these can have access to any of the cells on the outer surface of the slice. This outer layer has a cell composition that depends on the way the slice has been cut. In other words, nanoparticles may directly get in contact with cells which in vivo they may never access. Because of this, it is important to determine whether tissue slices allow to reproduce ex vivo outcomes observed in in vivo nanoparticle distribution studies.

Another important aspect to consider is that liver slices are usually maintained in serum-free medium. However, nano-sized objects when applied in biological environments are modified by adsorption of biomolecules on their surface, and the resulting corona layer strongly affects the subsequent interactions with cells[26–28]. Thus, when testing nanoparticles, some biological fluid needs to be included to allow corona formation and to avoid unrealistic interactions with bare surfaces. However, not yet know how corona effects translate to tissue.

Overall, a better knowledge of nanoparticle behavior and outcomes at tissue level is needed in order to establish how tissue slices can be used to extract meaningful information for nanomedicine and nanosafety studies.

MATERIALS AND METHODS

Animals
Adult male Wistar rats (250-300 g) and male C57BL/6J mice aged 8–10 weeks were obtained from Harlan Laboratories B.V.. Rats and mice were kept in a temperature and humidity-controlled room with a 12 h light/dark cycle with food and water ad libitum. Animals were allowed to acclimatise for at least one week before starting the experiments. Rats and mice were sacrificed under isoflurane/O2 anesthesia and their livers were harvested. The organ was kept in ice-cold University of Wisconsin (UW) organ preservation solution (DuPont Critical Care) until the start of the slicing procedure. All experiments were approved by the Animal Ethical Committee of the University of Groningen.

Human Liver Tissue
The samples of human liver used in the present study were obtained anonymously from patients undergoing partial hepatectomy for the
removal of carcinoma (2 samples) or from liver tissue remaining as surgical waste after reduced-size liver transplantation (TX) (1 sample). The use of human tissue was approved by the Medical Ethical Committee of the University Medical Centre Groningen (UMCG), according to Dutch legislation and the Code of Conduct for dealing responsibly with human tissue in the context of health research (www.federa.org), refraining from the need of written consent for ‘further use’ of coded-anonymous human tissue. The procedures were carried out in accordance with the experimental protocols approved by the Medical Ethical Committee of the UMCG.

The samples were perfused with a cold UW organ preservation solution immediately after collection. Fewer conditions could be tested simultaneously for human liver samples in comparison to the experiments performed with rat liver because of the limited sample size available.

Liver slices preparation
Precision-cut liver slices were prepared as described previously. [7] Briefly, rat liver cores were extracted with a drill equipped with a 5 mm diameter cylindrical tip and mouse liver cores were made using a biopsy puncher with a diameter of 5 mm. Afterwards, cores were sliced with Krumdieck Tissue Slicer MD6000 (Alabama R&D) filled with ice-cold Krebs–Henseleit buffer supplemented with 25 mM d-glucose (Merck), 25 mM NaHCO$_3$ (Merck), 10 mM HEPES (MP Biomedicals) and saturated with a mixture of 95% oxygen and 5% CO$_2$. After the slicing procedure, liver slices of about 200-250 μm thickness and 5 mg weight were preserved in the UW organ preservation solution on ice until further use.

Pre-Incubation
The liver slices were transferred to a petri dish containing William’s Medium E + GlutaMAX (WME, with L-glutamine, Invitrogen) medium supplemented with 25 mM d-glucose and 50 μg ml$^{-1}$ gentamycin (Invitrogen) in order to remove the UW solution before the experiments (this medium is referred to as “WME medium” or simply “medium” in the rest of the manuscript). Then the liver slices were transferred to individual wells in a 12-well plate filled with 1.3 ml pre-warmed (37°C) serum-free WME medium or WME medium supplemented with 5% v/v Fetal Bovine Serum (FBS, Gibco from TermoFisher Scientific), and saturated with 80% O$_2$ / 5% CO$_2$. Finally, the slices were maintained in an incubator (Panasonic) at 37°C saturated with 80% O$_2$ / 5% CO$_2$ and gentle shaking for 3 h, prior to exposure to the nanoparticles. The 3 h
pre-incubation under these conditions allows the tissue to restore its function and decreases the presence of residual cell debris present on the edge of the slices after the cutting procedure, which could affect the subsequent exposure to nanoparticles.

**DLS measurement**

Far-red labelled 40 nm carboxylated polystyrene nanoparticles (FluoSpheres, PS-COOH, maximum excitation at 660 nm and emission at 680 nm) were purchased from Thermo Fisher Scientific and unlabelled 50 nm amino-modified polystyrene nanoparticles (PS-NH$_2$) were purchased from Bangs Laboratories. The nanoparticle dispersions in relevant buffers were characterised by Dynamic Light Scattering (DLS) using a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd). Briefly, PS-COOH and PS-NH$_2$ nanoparticles were dispersed in Milli-Q water, Phosphate-Buffered Saline (PBS), serum-free WME medium or WME medium supplemented with 5% v/v FBS by dilution of the nanoparticle stocks to a final concentration of 100 μg ml$^{-1}$ and measured immediately after dispersion. Additionally, dispersions in serum-free WME medium or WME medium supplemented with 5% v/v FBS were also measured after 24 h incubation in the conditions used for tissue maintenance (37°C saturated with 80% O$_2$ / 5% CO$_2$, and gentle shaking). The results are the average of 3 separate measurements, each containing 10 runs of 10 s.

**Exposure to nanoparticles**

After 3 h pre-incubation, the liver slices were exposed to the nanoparticles by transferring them to pre-warmed (37°C) and pre-saturated (80% O$_2$ / 5% CO$_2$) wells containing nanoparticle dispersions at different doses (0-100 μg ml$^{-1}$) in serum-free WME medium or WME medium supplemented with 5% v/v FBS, prepared as described above. Then the liver slices were maintained as described above for 24, 48 and 72 h exposure.

**Tissue viability**

The ATP content normalised by the total protein content was used as a measure of tissue viability after exposure to nanoparticles. For each experimental condition 3 slices were used as replicates. The slice ATP content was determined via an ATP assay as follows: after exposure to nanoparticles, the liver slices were washed twice with medium and once with PBS in order to remove debris and reduce the presence of nanoparticles adhering to the outer tissue layer. Each individual slice was then collected in 1 ml of sonication solution, containing 70% v/v ethanol
Materials and Methods

and 2 mM EDTA (pH 10.9), snap frozen in liquid nitrogen and stored at −80°C until further analysis. Then the ATP content was measured as described previously. Briefly, samples were thawed slowly on melting ice, homogenised for 45 s using a Mini-BeadBeater 24 (Biospec Products) and centrifuged at 16,100 rcf for 5 min at 4°C. The supernatant was diluted ten times in 0.1 M Tris HCl buffer (pH 7.8) containing 2 mM EDTA and the ATP content was determined using the ATP Bioluminescence Assay Kit CLS II (Roche), according to the manufacturer’s protocol. Samples were transferred into a black 96-well plate and luminescence was measured using a Lucyl luminometer (Anthos).

The pellet obtained after centrifugation of the homogenised tissue slices was used for determining the slice’s total protein content. The pellet was reconstituted in 200 μl 5 M NaOH for 30 min at 37°C. After dilution with 800 μl Milli-Q water, the protein content was measured by a Lowry assay using a Bio-Rad DC Protein Assay (Bio-Rad) following the manufacturer’s instructions. Serial dilutions of bovine serum albumin were used to make a calibration curve. Samples were transferred into a black 96-well plate, and after keeping the plate in the dark for 15 min, the absorbance at 650 nm was read using a Molecular Devices Thermo Max Microplate Reader.

Finally, for each slice the viability was obtained by normalising the ATP value (pmol) by the total protein amount (μg). For each condition 3 slices were used, and the average and standard deviation calculated. Supplementary Figures S2, S3 and S4 for rat liver slices, and Supplementary Figs. S13 and S14 for human liver slices, show the results obtained in 3 independent experiments (3 animals or 3 human liver samples). Figures 1 and 5 show the average and standard error of the mean of the results obtained in the same 3 independent experiments.

Caspase-Glo 3/7 Assay

Caspase 3/7 activity in the tissue slices was measured using the Caspase Glo 3/7 (Promega), following the procedure previously described to measure Caspase 9 activity. Briefly, slices were washed twice with serum-free WME medium or WME medium supplemented with 5% v/v FBS, as used for exposure to nanoparticles. For each condition, 3 samples (3 slices) were prepared and, after exposure to nanoparticles, the slices were collected together in a safe-lock vial containing 600 μl serum-free WME medium. The samples were homogenised immediately using a Mini-Bead Beater for 45 s and centrifuged for 2 min at 4°C, 16,100 rcf. Then, the supernatant was used to measure caspase activity in 3 separate wells as follows: 5 μl supernatant was transferred into one
of a 96-well plate (Costar, Corning) with 40 μl Caspase-Glo 3/7
Reagent and 55 μl serum-free WME medium. Subsequently, the plates
were gently shaken for 2 min, incubated for 30 min at room temperature
in darkness and the luminescence was measured using a luminescence
plate reader (Lumicount™). The average and standard deviation of
3 replicate wells were then calculated. Supplementary Fig. S5 shows
the average and standard error of the mean of the results obtained in 3
independent experiments. Figure 1 shows the same results after nor-
malisation of the values obtained in slices exposed to the nanoparticles
with the values obtained in untreated control slices.

Cryo- and paraffin-sections of liver slices

In order to prepare sections for fluorescence imaging and histochem-
istry, after exposure to the nanoparticles the slices were transferred
to individual wells of a 12-well plate filled with 1.3 ml pre-warmed
and pre-saturated nanoparticle-free WME medium or WME medium
supplemented with 5% v/v FBS, as during exposure. Then, slices were
maintained in the incubator for a further 3 h in order to reduce the po-
tential presence of nanoparticles adhering to the outside of the tissue,
which could confuse imaging results (Supplementary Fig. S6 shows
images of slices prepared for microscopy directly after exposure or af-
ter 3 h in medium without nanoparticles to illustrate this). After 3 h in
medium without nanoparticles, slices were washed with PBS/0.2% (v/v)
triton X-100 (Sigma-Aldrich) and once with PBS. To prepare cryoblocks, liver slices were embedded
in KP-cryocompound (Klinipath) and frozen in 2-methylbutane (Sigma-
Aldrich) on dry ice. Afterwards, transversal sections of the tissue
slices with a thickness of 4 μm were prepared using a Cryostar NX70
cryostat (Thermo Fisher Scientific) perpendicular to the surface of the
slice. For paraffin embedding, slices were fixed in 4% formaldehyde
in PBS for 24 h at 4°C and stored in 70% ethanol at 4°C until analysis.

Immunofluorescence staining of cryo-sections

Cryo-sections with a thickness of 4 μm were cut and stained the same
day. Sections were dried for 30 min at room temperature, and per-
fused with PBS/4% formaldehyde (Klinpath) for 5 min at room temperature and per-
mefilised with 0.2% triton X-100 (Sigma-Aldrich) for 5 min. Then,
sections were incubated with 50 μl of primary antibodies for 60 min at
room temperature, followed by incubation with secondary antibo-
for further 60 min at room temperature in darkness. Antibodies were diluted in 5% serum of the same species (rat, mouse, or human) in PBS in order to block non-specific binding. The primary antibodies used for rat liver slices were: a goat anti-CD163 rat antibody (also known as ED2, AbD SeroTec, 1:50 dilution) to stain Kupffer cells, a mouse anti-CD31 rat monoclonal antibody (BD Biosciences 1:100 dilution) as a general marker for vascular and lymphatic endothelial cells, and mouse anti-SE-1 rat monoclonal antibody (Novusbio, 1:100 dilution) as a more specific marker for hepatic sinusoidal endothelial cells. A mouse anti-CD68 human monoclonal antibody (DAKO, 1:50 dilution) and a rat anti-CD68 mouse monoclonal antibody (BIO-RAD, 1:50) were used to stain Kupffer cells in the human and mouse liver slices, respectively. The secondary antibodies used were a mouse anti-goat Alexa Fluor 555 (Thermo Fisher Scientific, 1:200 dilution), a donkey anti-mouse Alexa Fluor 555 (Thermo Fisher Scientific, 1:200) and a goat anti-rat Alexa 555 (Thermo Fisher Scientific, 1:200).

Cell nuclei were stained by incubation for 5 min in 4’,6-diamidino-2-phenylindole (DAPI, 1 μg ml\(^{-1}\)). Finally, the slides were mounted with glass cover slips using MOWIOL 4-88 (Sigma-Aldrich). Images were acquired using a Leica SP8 confocal microscope, using a 40X and 63X objectives, with a 405 nm laser for DAPI excitation, a 552 nm laser for Alexa Fluor 555, and a 638 nm laser for the far-red nanoparticles. For the images shown in Figure 3 and Supplementary Figure S20, imaging settings for the nanoparticle and ED2 channels were kept constant in order to allow comparison of the intensity of images of samples at different exposure times.

In order to obtain images of the entire slice section, multiple adjacent images were acquired in the same z-plane using the same settings after which individual TIFF files were merged together using the 2D stitching plugin of the Fiji-ImageJ software.

**Tissue digestion and flow cytometry**

The mouse liver dissociation kit from Miltenyi Biotec was used for the enzymatic digestion of murine liver slices. For each condition, 12 slices were prepared and, after exposure to nanoparticles, were washed for 3 h with WME medium supplemented with 5% v/v FBS. Then, the slices were pulled together in a 50 ml tube containing the dissociation mix, which was prepared as follow: 100 μl Enzyme D solution, 50 μl Enzyme R solution, and 10 μl Enzyme A solution in 5 ml WME medium supplemented with 5% v/v FBS. The samples were incubated for 15 min at 37°C in a water bath with shaking and every 5 min samples were gently
resuspended. After digestion, the liver tissue was passed through a 70 μm nylon strainer (BD Bioscience) to obtain single cell suspensions and the filters were washed with extra 5 ml of medium. Approximately 1x10⁶ cells per sample were recovered. Cells were centrifuged and resuspended twice in sterile PBS. Then, in order to discriminate live and dead cells, samples were incubated with Fixable Viability Dye eFluo 450 (eBioscience, 1:2000 dilution) in serum/protein-free PBS for 30 min on ice in the dark. After that, cells were washed twice with a solution of 2% v/v FBS, 5 mM EDTA in PBS (PFE buffer) and incubated with Fix/Perm buffer (eBioscience) for 30 min on ice. Next, cells were washed with Perm-buffer (eBioscience) once. Afterwards, the isolated cells were incubated with a PE / Cy7 anti-mouse CD68 antibody (Biolegend, 1:100) for 30 min on ice in the dark in order to stain the Kupffer cells. Finally, cells were washed twice with Perm-buffer, resuspended in PFE buffer, and immediately measured using a Cytoflex Flow Cytometry (Beckman Coulter) with 405 nm (for live/dead staining), 488 nm (for Kupffer cells) and 630 nm (for nanoparticles) lasers. Data were analysed using Flowjo software (Flowjo, LLC). Dead cells were excluded from the analysis by setting gates in side scattering versus FL5:PB450 double scatter plots. Cell doublets were excluded by setting gates in the forward scattering area versus forward scattering height double scatter plots. The gating strategy is illustrated in Supplementary Fig. S21. For each sample, 20,000-70,000 cells were acquired. Figures 4a–c show the average and standard deviation of the results obtained in 3 independent experiments (with the exception of the 16 h sample which was included only in 2 experiments).

**Click-iT Plus TUNEL assay**

Cryo-sections of 4 μm thickness were cut and used for TUNEL staining on the same day to detect apoptosis with the Click-iT Plus TUNEL Assay for In Situ Apoptosis Detection, Alexa Fluor 488 dye (Thermo Fisher Scientific). Sections were dried for 30 min at room temperature, fixed with 4% formaldehyde for 15 min at room temperature and permeabilised with 0.2% Triton X-100 (Sigma-Aldrich) for 15 min. Afterwards, samples were washed twice in PBS for 5 min and rinsed in deionised water. Then, 100 μl of the kit’s TdT reaction buffer was added for 10 min at 37°C. Meanwhile the TdT reaction mixture was prepared according to the kit manual and 50 μl was added to the samples for 60 min at 37°C. A humidified chamber was used to protect against evaporation. Next, the sections were washed with 3% BSA and 0.1% Triton X-100 in PBS for 5 min, rinsed in PBS and incubated with 50 μl of the Click-iT Plus
TUNEL reaction cocktail for 30 min at 37°C while protected from light. The sections were washed with 3% BSA in PBS for 5 min and rinsed in PBS. The samples were then incubated with CD163 antibody, as described above, or directly stained with DAPI (1 μg ml⁻¹) for 15 min at room temperature and protected from light. Finally, the slides were mounted with glass coverslips using MOWIOL 4-88 (Sigma-Aldrich). Images were acquired using a Leica SP8 confocal microscope, using a 40X objective, with a 405 nm laser for DAPI excitation, a 488 nm laser for TUNEL-Alexa Fluor 488, and a 552 nm laser for Alexa Fluor 555-CD163. Fiji-ImageJ software was used to merge multiple images of the same section as described above.

Morphological assessment on paraffin-sections
Paraffin sections were stained with hematoxylin and eosin (Klinipath) (H&E) as described previously. Briefly, the paraffin sections were deparaffinised and rehydrated with decreasing strengths of ethanol (100% to 50%). Then, sections were immersed in hematoxylin (Klinipath) for 10 min. Afterwards, sections were dehydrated in baths of increasing strengths of ethanol (50% to 100%) and immersed for 2 min in eosin (Klinipath). Finally, slides were mounted with glass cover slips using DePeX (Serva).

Quantification of Kupffer cell movement
To characterise the distance of the Kupffer cells to the slice border we used fluorescence confocal microscopy images of slices. A polygon estimating the outline of the slice was drawn and the location of all Kupffer cells within the slice was determined from the ED2 fluorescence. A few Kupffer cells close to the image border were discarded, as their closest distance to the slice border was not necessarily within the field of view. Then the closest distance from each Kupffer cells to the polygon was calculated. The distances presented in Fig. 3d are pooled from several slices under the same conditions. To exclude effects due to different sizes of the imaged slices, the distances expected due to chance were estimated by Monte Carlo simulations. Thus, for each slice a position within the image was selected at random. If the position did not fall within the polygon outlining the slice, then it was rejected; otherwise, its distance to the border was calculated. This was repeated for 100,000 positions per image to allow a good estimate of the distances expected due to chance. The proportion of Kupffer cells within 20 μm quantified experimentally was then normalized (divided by) the proportion estimated to be due to chance. Number of slices /
number of Kupffer cells per condition: 24 h ctrl: 5 slices/305 Kupffer cells; 48 h ctrl: 2/219; 72 h ctrl: 6/781; 24 h treated: 5/283; 48 h treated: 2/208; 72 h treated: 6/790.

**Statistical analysis**
Normal distributions could not be assumed due to small sample size (n ≤ 8). Therefore, statistical differences between two groups were assessed using nonparametric Mann-Whitney U for unpaired data or Wilcoxon for paired data. For the comparison of multiple groups, we used Kruskal Wallis for unpaired data or Friedman for paired data. p < 0.05 was considered significant. The data were analysed using GraphPad Prism 8, except for Fig. 3e where scipy version 0.19.1 was used.

**RESULTS AND DISCUSSION**

As a first step, we aimed at comparing the response of the tissue to the outcomes observed in vitro using standard cell cultures and using the same nanomaterials. To this end, we exposed rat liver tissue slices to carboxylate- and amino-modified polystyrene nanoparticles (fluorescently labelled 40 nm PS-COOH and unlabeled 50 nm PS-NH$_2$). The positively charged PS-NH$_2$ nanoparticles have been shown to induce cell death by apoptosis, while the PS-COOH nanoparticles usually persist within the cells without any evident toxicity\[22,29–31\]. Thus, these materials were selected as well-characterized models in order to test how mechanisms of toxicity observed on cells translate to tissue. Additionally, to determine potential corona effects in tissue, as a first approximation for liver testing, nanoparticles dispersed in medium with 5% fetal bovine serum (FBS) were tested, together with [artificial] serum-free dispersions. Prior to exposure to the slices, the dispersions of the nanoparticles in media with and without serum were characterized (Supplementary Figure S1 and Table S2). For the PS-COOH nanoparticles, dynamic light scattering confirmed formation of stable dispersions in both media, with some agglomeration after 24 h in serum-free conditions. In contrast, for the PS-NH$_2$ nanoparticles, good dispersions were obtained in serum-free medium, while agglomeration was observed in 5% FBS, suggesting that these nanoparticles are less stable in the presence of serum.

Then, rat liver slices were exposed for up to 72 h to the two nanoparticles in the two media (Figure 1). Different nanoparticle concentrations were used to enable direct comparison of the outcomes with in
Results and discussion

In vitro studies on cells exposed to similar concentrations of the same nanomaterials[30, 31]. The ATP and protein levels were used to assess tissue viability (see Experimental section for details). For slices exposed to the PS-NH₂ nanoparticles, a strong reduction of slice viability was detected both in the absence or presence of a corona in serum (up to 80% reduction in viability after exposure for 72 h). However, earlier effects were observed for bare nanoparticles in serum-free conditions, where a statistically significant reduction of viability of around 60% was determined after only 24 h exposure to the highest nanoparticle concentration tested (Figure 1a–c and Supplementary Figures S2–4).

Morphological analysis of tissue sections after hematoxylin and eosin staining confirmed the viability results and showed loss of tissue structure and strong toxicity in slices exposed to the PS-NH₂ nanoparticles both in the presence and absence of a corona, already after 24 h exposure (Figure 1a). In contrast, consistent with in vitro studies, no significant effects on viability were observed for slices exposed to the PS-COOH nanoparticles under all conditions tested (Figure 1d–f and Supplementary Figures S2–4).[29]

We next characterized the mechanism of cell death. Cell death by apoptosis can be detected by measuring the activity of the proteases caspase 3 and 7. Similarly, DNA fragmentation, another hallmark of apoptosis, can be imaged by TUNEL assay (see Experimental section for details). For PS-COOH nanoparticles, activation of caspases was observed, more evident after 72 h exposure in medium with FBS, by which time it roughly doubled in comparison to untreated slices (Figure 1g–i and Supplementary Figure S5). However, the TUNEL assay did not show apoptosis activation (Supplementary Figure S6). For the PS-NH₂ nanoparticles, instead, around 2.5 fold increase in caspase activation was observed already after 24 h exposure, both in 5% FBS (for which no decrease of slice viability had been detected yet) and in serum-free medium (Figure 1g). TUNEL imaging confirmed the presence of many apoptotic cells (Figure 1g). Apoptosis activation was less evident for increasing exposure times, probably due to the strong reduction in viability under these conditions (Figure 1h–i). Similar outcomes were also observed with murine liver slices exposed to the two nanoparticles (Supplementary Figure S7).

Overall, these results indicate that the tissue slices responded to the selected model nanoparticles in similar ways as reported for different cell cultures in in vitro studies.[30, 31] However, the activation of caspases in slices exposed to the PS-COOH nanoparticles may be a sign of a different response of the tissue in comparison to what is observed
on cells with the same material, for which no sign of apoptosis has been reported. [29] Similarly, in the case of the PS-NH₂ polystyrene, usually, bare positive nanoparticles exhibit much stronger toxic effects on cells than corona-coated ones, [31] while here the difference was rather small. This is likely a specific effect due to tissue characteristics in comparison to cell cultures. Thus, while in a standard cell culture every cell comes immediately into contact with the nanoparticle dispersion, in the tissue slice only the outer layer of cells (initially) interacts with the nanoparticles and hence one would expect a less evident difference in the impact of the bare and corona-coated nanoparticles. Nevertheless, it is clear that the effect of a corona should be considered also in tissue and, especially when using liver slices, artificial serum free conditions should be avoided.

As a next step, it will be important to perform similar studies exposing tissue to nanoparticle concentrations comparable to those to which the liver is exposed in vivo. These are hard to determine and will vary depending on the nanoparticle and application investigated. As an example, in order to obtain some preliminary information on likely concentrations to which the liver may be exposed, we have compared our conditions with the results reported by Ogawara et al[32] with

Figure 1. Viability and apoptosis in rat liver slices exposed to PS-NH₂ and PS-COOH nanoparticles. Liver slices were exposed for 24 h to increasing doses of (a) PS-NH₂ and (d) far-red PS-COOH nanoparticles in serum-free medium (WME) or medium supplemented with 5% FBS (WME + 5% FBS). Additionally, slices were exposed to 100 µg ml⁻¹ nanoparticles in the two media for 48 and 72 h (b-c for PS-COOH, and e-f for PS-NH₂, respectively). Viability is expressed as the ATP content (pmol) normalized by total protein amount (µg). Tissue morphology after 24 h exposure to 100 µg ml⁻¹ nanoparticles was assessed by histochemistry (a and d, for cross-sections of slices exposed to PS-COOH and PS-NH₂ nanoparticles, respectively). Scale bar 200 µm. Caspase 3/7 activity was also measured in slices exposed for (g) 24 (h) 48 and (i) 72 h to 100 µg ml⁻¹ nanoparticles in the two media. The viability and caspase data show the mean and standard error of the mean (SEM) of the results obtained from three independent experiments. Every dot represents the result of an independent experiment. For each experiment, the results of the treated slices are compared to the results of untreated control slices from the same animal (0 µg/ml). For each condition three slices of the same animal were used, and the average and SEM were calculated (the individual viability experiments are shown in Supplementary Figures S2–4 and the raw caspase results in Supplementary Figure S5). Confocal fluorescence images of TUNEL assay on cross-sections of untreated control slices cultured for 24 h and slices exposed for 24 h to 100 µg ml⁻¹ PS-NH₂ in serum-free medium are also included to identify potential apoptotic cells (g). Blue: DAPI-stained nuclei. Green: TUNEL-positive nuclei of apoptotic cells. Scale bar: 100 µm. Exposure to PS-NH₂ nanoparticles leads to decreased viability in both media and activation of apoptosis. A Friedman test with Dunn’s correction was performed when comparing multiple groups, and Wilcoxon test when comparing two groups. p ≤ 0.05 was considered significant. *= p ≤ 0.05.
Figure 2. Confocal fluorescence imaging of rat liver slices exposed to PS-COOH and PS-NH2 nanoparticles. Cross-sections acquired by confocal fluorescence microscopy of liver slices exposed to 10 μg ml⁻¹ far-red PS-COOH nanoparticles in 5% FBS medium for (a) 24, (b) 48 and (c) 72 h and (d–f) to 50 μg ml⁻¹ (unlabeled) PS-NH₂ nanoparticles in 5% FBS medium for 24 h. a–c: Indicated areas are shown at increased magnification to the right. Scale bars, from the left: 100, 40 and 10 μm. Blue: DAPI-stained nuclei. Red: nanoparticles. Green: ED2-labelled Kupffer cells. The results confirm nanoparticle uptake into the tissue and, interestingly, high nanoparticle uptake by Kupffer cells, mainly at the edge of the tissue slices, as evidenced by the overlap...
similar polystyrene nanoparticles. In their work, 50 nm polystyrene similar to those used in our study were injected IV in rats at a dose of 12.5 mg/kg and the authors found that around 50% of the injected dose accumulated in the liver. This roughly corresponds to an initial blood concentration of 200 µg/ml nanoparticles, comparable to the concentrations used here.

Next, in order to understand more in detail the observed impact at tissue level (Figure 1), we turned to resolving whether nanoparticles were internalized by cells in the tissue ex vivo. More specifically, we aimed at determining the cell types involved both in uptake and – as a consequence of uptake – in the toxic responses observed in slices exposed to the PS-NH₂ nanoparticles. After optimization to overcome the natural autofluorescence of the tissue and reduce the interference of extracellular nanoparticles adhering onto the outer surface of the slice (Supplementary Figure S8), confocal fluorescence imaging of rat liver slices exposed to fluorescently labelled PS-COOH nanoparticles clearly confirmed nanoparticle uptake into cells of the tissue (Figure 2 and Supplementary Figure S9). Images of transversal sections of the slices showed that, as expected, the nanoparticles were mainly internalized by cells located in the outer layer, though some nanoparticles were observed in cells deeper into the tissue as well. One could expect nanoparticles to be internalized by any of the cell types present in the outer layer of the slice, with the hepatocytes (roughly 70% of all liver cells)[33,34] presumably being highly prevalent. Instead, imaging showed that some cells accumulated more nanoparticles. Immunostaining clearly indicated that these were the liver Kupffer cells (Figure 2a–c; overlap of red and green signal).[32,35] Uptake in CD-31 stained vascular and lymphatic endothelial cells or SE-1 stained liver sinusoidal cells was observed only in rare cases, in slices where these cell types were present in the outer cell layers (Supplementary Figures S10–11). A preferential uptake in Kupffer cells was also observed on murine liver slices exposed to the same nanoparticles (Supplementary Figure S12).

between the nanoparticle (red) and Kupffer cell label (green) signal. d–f: A TUNEL assay was performed on slices exposed to PS-NH₂ nanoparticles in order to stain apoptotic cells (see Experimental section for the details). Blue: DAPI-stained nuclei. Red: ED2-labelled Kupffer cells. Green: apoptotic (TUNEL positive) cells. The indicated region in panel d is shown at increased magnification in panel e, and similarly for panels e–f. Scale bar: 100 (d), 50 (e) and 10 (f) µm. Imaging showed that most of the apoptotic cells (green) were also ED2-labelled (red) and there were not many apoptotic cells that were not ED-2 labelled. This all together suggests that in the slices exposed to the nanoparticles most apoptotic cells were Kupffer cells (as indicated by the substantial overlap of the apoptotic (green) and Kupffer cell (red) signals) accumulated at the slice borders.
It is important to compare these observations with in vivo intrahepatic distribution studies. As an example, using polystyrene microsphere of 50 and 500 nm, Ogawara et al.[32] found that after IV injection, within the liver 60-70% of the particles were taken up by Kupffer cells. However, uptake in parenchymal and endothelial cells was also present (28% and 13%, respectively, for the smaller particles and 5% and 24% for the larger ones). Similar results were obtained on mice by Park et al.[36] with PLGA particles, which were taken up by Kupffer cells with highest efficiency, although uptake was present also in liver sinusoidal cells and hepatic stellate cells, and – to much lower extent – in hepatocytes. Similar results were reported by Dragoni et al.[14] after injection of gold nanoparticles in rat. Lee et al.[37] compared the distribution of silica nanoparticles with different surface functionalization within the liver after IP injection in mice. They also found the highest accumulation in the Kupffer cells. However, uptake in liver sinusoidal cells was also comparable. On the contrary, Sadauskas et al.[38] studied the distribution of gold nanoparticles within the liver in mice after IV and IP injection and found uptake only in Kupffer cells.

Overall, these studies indicate that within the liver in most cases the Kupffer cells show higher accumulation of nanoparticles. Importantly, our results show that, even though nanoparticles enter the tissue in a very different way in comparison to arrival from the blood vessels, tissue slices mimic this key feature of in vivo distribution studies, namely a preferential nanoparticle accumulation in Kupffer cells.

We also noted a higher intensity of ED2 staining in comparison to slices not exposed to the nanoparticles (Figure 2, also visible in the single channel images of Figure 3). ED2, also known as CD163, is a surface glycoprotein, whose expression increases in the context of resolution of inflammation and tissue repair.[39,40] The higher expression of this marker suggests that the Kupffer cells maintain their key functions in the tissue slice and respond to nanoparticle exposure.

As a next step, we attempted to connect the response to the PS-NH$_2$ nanoparticles at tissue level (the activation of apoptosis and decreased tissue viability, as shown in Figure 1) with the observed nanoparticle uptake and distribution within the tissue. To this end, we combined a TUNEL assay to detect apoptotic cells with immunostaining by ED2 to identify the Kupffer cells (Figure 2d). Kupffer cells engulfing apoptotic cells to clear them – if present – can also be stained by TUNEL assay [41]. However, imaging clearly showed that in the slices exposed to the PS-NH$_2$ nanoparticles, most of the TUNEL positive cells were ED2-positive. These results suggest that the apoptotic cells were mainly
Kupffer cells. Thus, tissue slices can be used to connect the response at tissue level to the effect nanoparticles induce in the specific cell types in which they accumulate.

We then followed nanoparticle uptake and distribution within the tissue for up to 72 h (Figures 2–3 and Supplementary Figures S13–15). With increasing exposure time, naturally, nanoparticle uptake increased (Figure 2). Interestingly, we observed accumulation of Kupffer cells at the slice borders, both for control and nanoparticle-exposed slices (Figure 3a–c). It is known that Kupffer cells have the capacity to move within the tissue: for instance infiltration of Kupffer cells was observed in liver in animals exposed to titania nanoparticles.[42] Similar effects were also observed in other studies with liver slices.[43]

In order to investigate if this effect was due to the nanoparticles, we quantified it by calculating the (closest) distance of each Kupffer cell from the slice border. Thus, Figure 3d shows the fraction of Kupffer cells identified in each slice as a function of their distance from the slice borders, including data from all slices under the same conditions. This gives the distribution of Kupffer cells in untreated and PS-COOH treated slices, but is confounded at larger distances by the unequal sizes of the imaged slices. It may be observed that already after 24 h exposure to PS-COOH nanoparticles, a substantial fraction of the Kupffer cells had moved to the slice border (within 20 μm from it) (Figure 3d). This accumulation is clear also after 48 and 72 h (also in Figure 3d). There is some accumulation at the border also for the Kupffer cells in control slices not exposed to the nanoparticles (Figure 3d), in comparison to what is observed in a fresh slice of liver tissue (Supplementary Figure S16). This can be connected to the initial response of the tissue to the damage caused by the cutting procedure. However, the effect is clearly stronger for slices exposed to the nanoparticles. Comparison of the distributions for individual slices shows that accumulation at the border is prevalent in some slices, but not all slices exhibit this behavior (Supplementary Figures S17–19). This is likely a consequence of the high heterogeneity in Kupffer cell distribution within the liver[44] and variability across animals as well as tissue slices. As a further assessment of the effect, we quantified the proportion of Kupffer cells within the first 20 μm, also normalizing by the proportion expected due to chance for each slice (see Experimental section for details). The proportion of cells within the first 20 μm was higher for the slices exposed to PS-COOH nanoparticles at all times (Figure 3e). Accumulation of Kupffer cells towards the slice borders was observed also with unlabeled PS-COOH nanoparticles and the toxic unlabeled PS-NH₂ model (Supplementary
Figure 3. Quantification of Kupffer cell distribution within rat liver slices at increasing exposure times. Liver slices were exposed to 10 μg ml⁻¹ far-red PS-COOH nanoparticles in 5% FBS medium for (a) 24, (b) 48 and (c) 72 h prior to confocal imaging and quantification of Kupffer cell distribution within the tissue, performed as described in the Experimental section. a–c: Representative cross-sections acquired by confocal fluorescence microscopy of liver slices with ED2-stained Kupffer cells. Untreated control slices cultured for the same time in medium with 5% FBS without nanoparticles (untreated) and slices exposed to the nanoparticles (PS-COOH treated) are both shown. Scale bar: 200 μm. The signal of the Kupffer cell marker (ED2) is shown, while the corresponding nanoparticle and nuclei signals are shown in Supplementary Figures S13-15. d: Kupffer cell distribution within the slice, expressed as the fraction of Kupffer cells as a function
Results and discussion

of the distance from the slice borders (see Experimental section for details), for untreated tissue slices (top panels, blue bars) and slices exposed to PS-COOH nanoparticles (bottom panels, red bars). The results show accumulation of Kupffer cells towards the slice borders under nanoparticle-exposed conditions, with some accumulation toward the borders also for control slices. Furthermore, cells exposed to the nanoparticles seem to have higher ED2 expression, suggesting Kupffer cell activation. Counted Kupffer cells for the 24 h control: 305; 48 h control: 219; 72 h control: 781; 24 h treated: 283; 48 h treated: 208; 72 h treated: 790 Kupffer cells.

The proportion of Kupffer cells investigated, normalized by that expected due to chance (see Experimental section for details). The results for untreated control slices are shown in blue and those for slices exposed to the nanoparticles in red with data points indicating individual slices, the bar the mean over slices, and error bars the standard error of the mean. The results show that at all exposure times, the proportion of cells within the first 20 μm was higher for the slices exposed to PS-COOH nanoparticles and the effect was statistically significant after 72 h (testing each time separately). *= p ≤ 0.05.
Results and discussion

Figure S20), thus excluding artefacts due to nanoparticle fluorescence spillover. Overall, these results suggest that, as observed in vivo,[42] the primary Kupffer cells in the tissue slices retain their capacity to respond to signals and accumulate towards the site of exposure to – in this case – the nanoparticles.

Next, as a further confirmation of what was observed by confocal microscopy and in order to quantify nanoparticle uptake kinetics in the Kupffer cells and all other cells, murine liver slices exposed to PS-COOH nanoparticles were digested enzymatically (see Experimental section for details). Enzymatic digestion opens up the possibility to recover mixtures of all cells from the tissue after exposure to the nanoparticles, and perform further analysis and quantitative studies at individual cell level.

Following enzymatic digestion, we used flow cytometry to measure nanoparticle uptake in several thousand individual cells recovered from the tissue over time. Combined with immunostaining to identify the Kupffer cells (Supplementary Figure S21), this allowed us to determine nanoparticle uptake kinetics in these and all other cells (Figure 4). The

Figure 4. Flow cytometry analysis of nanoparticle uptake by Kupffer cells recovered from murine liver slices exposed to PS-COOH nanoparticles. Liver slices were exposed to 25 μg ml⁻¹ far-red PS-COOH nanoparticles in 5% FBS medium for different times up to 48 h prior to tissue digestion, performed as described in the Experimental section. The isolated cells were analyzed by flow cytometry. a: Fraction of cells with nanoparticles, determined as the number of cells with nanoparticles normalized by the total number of cells measured. b: Fraction of Kupffer cells with nanoparticles, determined as the number of CD68 positive cells with nanoparticles normalized by the total number of CD68 positive cells. The fraction of cells with nanoparticles increases with increasing exposure time. The high percentage of Kupffer cells with nanoparticles is consistent with the accumulation of Kupffer cells towards the slice borders observed by microscopy. c: Mean cell fluorescence intensity due to nanoparticle uptake of the fraction of cells with nanoparticles. The mean fluorescence intensity over time of the Kupffer cells (CD68 positive cells) with nanoparticles and all other cells (CD68 negative cells) with nanoparticles are shown. In a-c the average and standard deviation of the results obtained in 3 independent experiments are shown (with the exception of the 16 h sample performed only in two experiments). For each condition 20,000–70,000 individual cells were acquired (see Experimental section for details). Uptake increases over time in both Kupffer cells and all other cells with nanoparticles, but at all times the average intensity of Kupffer cells with nanoparticles is higher than that of all other cells with nanoparticles. This suggests that Kupffer cells are the cells with highest uptake efficiency. d: Representative double scatter plots of cell fluorescence intensity in the nanoparticle channel (nanoparticle uptake, PS-COOH) versus CD68 staining. The Kupffer cells (CD68 positive) are shown in red and all other cells (CD68 negative) are shown in green. The results show that as time increases more cells internalize nanoparticles (as indicated by the increasing number of dots inside the rectangular gate) and a population of cells with much higher nanoparticle uptake becomes visible (delimitated by a round gate). CD68 staining confirms that most of these cells are Kupffer cells (red CD68 positive dots, see also Supplementary Figure S21 for further analysis).
Figure 5. Nanoparticle exposure in human liver slices. Human liver slices (prepared as described in the Experimental section) were exposed for 24 h to increasing doses of (a) PS-NH₂ nanoparticles or (c) 100 µg ml⁻¹ PS-COOH nanoparticles in serum-free medium (WME) or medium supplemented with 5% FBS (WME + 5% FBS). Additionally, slices were exposed to 100 µg ml⁻¹ PS-NH₂ and PS-COOH nanoparticles in the two media for 48 h (b and d, respectively). Viability is expressed as the ATP content (pmol) normalized by total protein amount (µg). The viability data show the mean and standard error of the mean (SEM) of the results obtained from three independent experiments. Every dot represents the result of an independent experiment. For each experiment, the results of the treated slices are compared to the results of untreated control slices from the same animal (0 µg/ml). For each condition three slices of the same animal were used, and the average and SEM were calculated (the individual viability experiments are shown in Supplementary Figures S22–23). Exposure to PS-NH₂ nanoparticles leads to decreased viability in both media. Confocal fluorescence images of cross-sections of slices exposed to 10 µg ml⁻¹ far-red PS-COOH in 5% FBS medium for (e) 24 and (f) 48 h are also included. Details of the same area at increasing magnification are shown (scale bars: 100, 20 and 10 µm, respectively). Blue: DAPI-stained nuclei. Red:
results clearly showed that with increasing exposure time the fraction of cells containing nanoparticles increased (Figure 4a), and, interestingly, after 48 h 80% of the Kupffer cells contained nanoparticles (Figure 4b). This is consistent with the accumulation of Kupffer cells towards the slice borders observed by fluorescence microscopy (Figures 2–3).

Furthermore, as suggested by imaging, flow cytometry analysis confirmed that in all cells with nanoparticles uptake increased over time, but at all times the Kupffer cells were the cells with the highest nanoparticle fluorescence (Figure 4c–d). These results confirmed once more that tissue slices allow to resemble ex vivo key features of what is observed in vivo in the liver, and, more specifically, a preferential accumulation of nanoparticles by Kupffer cells, as well as the capacity of these specialized cells to respond to stimuli and accumulate towards the sites of exposure.

Finally, similar studies were performed using human (as opposed to rat and murine) liver tissue slices isolated from surgical waste material. To the best of our knowledge this is the first study using live human liver tissue to test nanoparticle uptake and impact. The results (Figure 5 and Supplementary Figures S22–23) show that similar outcomes could be observed also in human liver tissue. Thus, exposure to the PS-NH\textsubscript{2} nanoparticles led to a decrease in viability (Figure 5a–b) and, also in human liver slices, PS-COOH nanoparticles were taken up preferentially by Kupffer cells at the slice borders (Figure 5c–d). This opens up the possibility to use this model to study nanoparticle uptake and impact on primary human Kupffer cells still embedded in their normal tissue environment.

CONCLUSIONS

In summary, in this work we investigated the basic mechanisms of nanoparticle interactions with liver tissue slices in order to understand how the outcomes observed in standard cell cultures and in vivo translate to tissue. To this end, we used slices from rat and murine livers and – for the first time for nanoparticle studies – also from human liver.
We combined time resolved confocal microscopy and flow cytometry on individual cells recovered from the tissue to perform a quantitative study of nanoparticle distribution and uptake in the different cell types in which they accumulate within the tissue. As a first step, tissue slices were exposed to doses comparable to in vitro studies performed on standard cell cultures with the same nanomaterials, thus allowing a direct comparison of the outcomes in the tissue slices to those obtained on simpler cell culture systems. Comparable outcomes were, indeed, observed in tissue slices, including effects related to the presence or absence of a corona on the nanoparticles. As a future step, it will be important to repeat similar studies with doses comparable to those to which the liver may be exposed in vivo. This is hard to determine and of course will depend on each specific nanoparticle and condition investigated. For instance, in the context of nanosafety, the dose that arrives to the liver will depend (among many other factors) on exposure, while in the context of nanomedicine one may refer to administered doses and clinically relevant doses. Nevertheless, most nanoparticle distribution studies show preferential particle accumulation in the liver and thus there is little doubt on the likely exposure of the liver to nanoparticles.

While the advantages of using real tissue, including from humans, are clear, tissue slices, like every model, also present some limits. One is the relatively short viability, currently optimized to up to around 5 days after excision. [43] Further optimization could include, for instance, the addition of a microfluidic system, which could also be used to connect slices from different organs. [45] Perhaps the major limitation in the use of tissue slices is related to the way nanoparticles are exposed to the tissue and come into contact with the different cell types within this organ in comparison to exposure from the blood in vivo. For instance, uptake in liver sinusoidal cells was observed mainly when these cells were present in the outer cell layer of the tissue slices. Thus, the model is likely to underestimate specific effects on liver sinusoidal cells for nanoparticles which show substantial uptake in this cell type in vivo[36,37]. Nevertheless, in most cases intrahepatic distribution studies have shown that the cells with highest uptake efficiency within the liver are the Kupffer cells[14,35–38] and our results clearly show that liver tissue slices do resemble ex vivo this key feature. Furthermore, by maintaining them in their tissue environment, these primary macrophages seem to preserve intercellular communication and cell signaling, as suggested by the observed capacity to migrate towards the sites of exposure, to – in this case – the nanoparticles. This indicates
that even after tissue extraction, these primary cells maintained some of the key features for their functions and possibly were able to respond to extracellular signals. Thus, after exposing the complete tissue to nanoparticles, fluorescence microscopy and other imaging methods can be used to visualize and characterize Kupffer cells still embedded in their environment, while tissue digestion can be used to recover them from the tissue after exposure. In this way, high throughput quantitative methods, such as flow cytometry used here, can be utilised for a more in depth study at individual cell level. Additionally, by using human samples, tissue slices can be used to gain important insights on possible outcomes of nanoparticles in humans. Overall, this makes tissue slices an attractive ex vivo model for studying the response induced by nanoparticles in the liver and, more specifically, effects induced in primary Kupffer cells, crucial players in nanosafety and nanomedicine outcomes.

ACKNOWLEDGMENTS

The authors would like to thank M.H. de Jager for technical help with organ extraction and preparation, and E. Gore and G.H.H. Prins for assistance in the preparation of the human tissue. A.S. and Y.L.B. kindly acknowledge the University of Groningen for funding (Rosalind Franklin Fellowship). A.S. also kindly acknowledges funding from the European Research Council (ERC) under the European Union’s Horizon 2020 research and innovation programme under grant agreement no. 637614 (NanoPaths).

AUTHOR CONTRIBUTIONS

R.B. designed and performed all experiments, analyzed and interpreted the data and drafted the manuscript. C.Å. performed the quantification of Kupffer cell distribution and revised the manuscript. B.N.M. contributed to the optimization of tissue digestion and flow cytometry sample preparation and data analysis, and revised the manuscript. Y.L.B. and P.O. designed the experiments, interpreted the data and revised the manuscript. A.S. designed the experiments, interpreted the data and wrote the manuscript.
REFERENCES


SUPPLEMENTARY INFORMATION

Time-Resolved Quantification of Nanoparticle Uptake, Distribution and Impact in Precision-Cut Liver Slices
Roberta Bartucci, Christoffer Åberg, Barbro N. Melgert, Ykelien L. Boersma, Peter Olinga, Anna Salvati*

Supplementary Table S1. Key features of liver tissue slices as an established ex vivo model for drug activity and toxicity studies.

<table>
<thead>
<tr>
<th>Key features</th>
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<tbody>
<tr>
<td>Incubation system</td>
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<tr>
<td>Tissue slices maintained in multi-well plates in oxygenated incubator. High oxygen and shaking recommended [1–9].</td>
</tr>
<tr>
<td>Viability</td>
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<tr>
<td>Tissue slices viable up to 120 hours, as demonstrated by both ATP content and tissue morphology [1,6–10].</td>
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<tr>
<td>Cell composition</td>
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<tr>
<td>Kupffer cells are viable and their function is preserved [2,11,12].</td>
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<tr>
<td>Endothelial cells are viable and their function is preserved [9].</td>
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<tr>
<td>Stellate cells are viable and their function is preserved [7,8,10,13,14].</td>
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<tr>
<td>Metabolic capacity</td>
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<tr>
<td>Hepatocytes in the tissue slices remain capable to reproduce phase 1 and phase 2 drug metabolism [15].</td>
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<tr>
<td>Toxicity studies</td>
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<tr>
<td>Validated as a model to study</td>
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<tr>
<td>- apoptosis</td>
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<tr>
<td>- necrosis [5,16]</td>
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<tr>
<td>- cholestasis [16,17]</td>
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<tr>
<td>- fibrosis [8,10,14,17,18]</td>
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Supplementary Table S2. Nanoparticle dispersion characterization by Dynamic Light Scattering. The nanoparticle dispersions (100 µg ml⁻¹) in Milli-Q water, PBS, serum-free medium (WME) and medium supplemented with 5% FBS (WME + 5% FBS) were characterized by Dynamic Light Scattering (DLS), immediately after dispersion or after 24 h incubation under the same conditions used for tissue maintenance (37°C saturated with 80% O₂ / 5% CO₂ and gentle shaking). The results are the average of 3 separate measurements from a representative experiment. Both nanoparticles formed homogenous dispersions in all media, except for the PS-NH₂ nanoparticles in medium supplemented with 5% FBS and the 24 h dispersion of PS-COOH in serum free medium which showed some agglomeration (see Supplementary Figure S1 for corresponding distributions). The corresponding distributions by CONTIN analysis are shown in Supplementary Figure S1.

<table>
<thead>
<tr>
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<th>Medium</th>
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<th>PDIᵃ</th>
<th>Diameter (nm)ᵇ</th>
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<td></td>
<td>PBS</td>
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<tr>
<td></td>
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<tr>
<td></td>
<td>WME + 5% FBS</td>
<td>*</td>
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<td></td>
<td>WME (24 h)</td>
<td>74</td>
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<td></td>
<td>WME + 5% FBS (24 h)</td>
<td>*</td>
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<tr>
<td>PS-COOH</td>
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<tr>
<td></td>
<td>WME + 5% FBS</td>
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<td>103</td>
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<td>WME (24 h)</td>
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<td></td>
<td>WME + 5% FBS (24 h)</td>
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<td>95</td>
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ᵃAgglomeration
ᵇAverage hydrodynamic diameter (z-average) and polydispersity index (PDI) of nanoparticle dispersions in relevant media obtained from cumulant fitting of Dynamic Light Scattering data.
ᵇAverage hydrodynamic diameter determined from CONTIN size distribution.
Supplementary Figure S1. Nanoparticle dispersion characterization. Size distributions of nanoparticle dispersions in relevant media obtained from CONTIN analysis of Dynamic Light Scattering (DLS) data. The nanoparticle dispersions (100 µg ml\(^{-1}\)) in Milli-Q water, PBS, serum-free medium (WME) and medium supplemented with 5% FBS (WME + 5% FBS) were characterized by DLS, immediately after dispersion or after 24 h incubation under the same conditions used for tissue maintenance (37°C saturated with 80% O\(_2\) / 5% CO\(_2\) and gentle shaking). Three separate measurements from a representative experiment are shown in each graph. The results show that homogenous dispersions were formed under all conditions, with the exception of the PS-NH\(_2\) nanoparticles in medium supplemented with 5% FBS and the 24 h dispersion of PS-COOH in serum-free medium which showed agglomeration.
Supplementary Figure S2. Viability of rat liver slices after 24 h exposure to nanoparticles. Liver slices were exposed for 24 h to increasing doses of PS-NH$_2$ [a, c, e] and far-red PS-COOH [b, d, f] nanoparticles in serum-free medium (WME) or medium supplemented with 5% FBS (WME + 5% FBS), after which their viability was measured. The results obtained in 3 independent animal experiments are shown (here denoted as “one”, “two”, “three”) and their average is shown in Figures 1a and 1d. In a and b, the viability is expressed as the ATP content (pmol) normalized by total protein amount (µg). The results are the average and standard deviation of 3 replicate slices treated in the same way. c–f: The raw ATP content [c, d] and amount of total protein [e, f] of one representative experiment are also shown as an example of the original data from which viability is calculated as described in the Experimental section.
Supplementary Figure S3. Viability of rat liver slices after 48 h exposure to nanoparticles. Liver slices were exposed for 48 h to 100 µg ml⁻¹ of PS-NH₂ (a, c, e) and far-red PS-COOH (b, d, f) nanoparticles in serum-free medium (WME) or medium supplemented with 5% FBS (WME + 5% FBS), after which their viability was measured. The results obtained in 3 independent animal experiments are shown (here denoted as “one”, “two”, “three”) and their average is shown in Figures 1b and 1e. In a and b, the viability is expressed as the ATP content (pmol) normalized by total protein amount (µg). The results are the average and standard deviation of 3 replicate slices treated in the same way. c–f: The raw ATP content (c, d) and amount of total protein (e, f) of one representative experiment are also shown, as an example of the original data from which viability is calculated as described in the Experimental section.
Supplementary Figure S4. Viability of rat liver slices after 72 h exposure to nanoparticles. Liver slices were exposed for 72 h to 100 µg ml⁻¹ of PS-NH₂ (a, c, e) and far-red PS-COOH (b, d, f) nanoparticles in serum-free medium (WME) or medium supplemented with 5% FBS (WME + 5% FBS), after which their viability was measured. The results obtained in 3 independent animal experiments are shown (here denoted as “one”, “two”, “three”) and their average is shown in Figures 1c and 1f. In a and b, the viability is expressed as the ATP content (pmol) normalized by total protein amount (µg). The results are the average and standard deviation of 3 replicate slices treated in the same way. c-f: The raw ATP content (c, d) and amount of total protein (e, f) of one representative experiment are also shown, as an example of the original data from which viability is calculated as described in the Experimental section.

Supplementary Figure S5. Caspase 3/7 activity in rat liver slices exposed to nanoparticles. Liver slices were exposed for 24, 48 and 72 h to 100 µg ml⁻¹ far-red PS-COOH and PS-NH₂ nanoparticles in serum-free medium (WME) or medium supplemented with 5% FBS (WME + 5% FBS), prior to assessment of caspase 3/7 activity, performed as described in the Experimental section. The results are the mean of 3 separate animal experiments and error bars are the corresponding standard error of the mean (see Experimental section for details). The raw caspase activity results are shown and the same results normalized for the values obtained in untreated control slices are given in Figures 1g–i.
Supplementary Figure S6. Imaging of apoptotic cells in rat liver slices exposed to PS-COOH nanoparticles. a: Cross-sections acquired by confocal fluorescence microscopy of liver slices exposed to 100 μg ml⁻¹ PS-COOH nanoparticles in 5% FBS medium for 72 h. b: Cross sections of untreated control slices not exposed to the nanoparticles and cultured for the same time are also shown for comparison. Blue: DAPI stained nuclei. Green: apoptotic (TUNEL positive) cells. Scale bar: 200 μm. A TUNEL assay was performed on the slices in order to stain apoptotic cells (see Experimental section for the details). No substantial differences in the number of apoptotic TUNEL positive cells could be detected in slices exposed to these nanoparticles.
Supplementary Figure S7. Viability of murine liver slices exposed to PS-NH$_2$ and PS-COOH nanoparticles. Murine liver slices were exposed for 48 h to 25 and 50 µg ml$^{-1}$ PS-NH$_2$ and far-red PS-COOH nanoparticles in medium supplemented with 5% FBS. The viability is expressed as the ATP content (pmol) normalized by total protein amount (µg) and for each experiment the results obtained in slices exposed to the nanoparticles are normalized by the viability in untreated control slices from the same animal cultured for the same time (%). The results are the mean and standard error of the mean of the results obtained in 3 and 6 independent experiments, for slices exposed to, respectively, 25 and 50 µg ml$^{-1}$ nanoparticles. As observed in rat liver slices, exposure to PS-NH$_2$ nanoparticles leads to decreased viability.

Supplementary Figure S8. Optimization of experimental conditions for fluorescence imaging of tissue slices exposed to nanoparticles. Cross-sections acquired by confocal fluorescence microscopy of two liver slices exposed for 24 h to 10 µg ml$^{-1}$ far-red PS-COOH nanoparticles in 5% FBS medium. In the top panel (0 h chase), the first slice was immediately prepared for confocal microscopy as described in the Experimental section. In the bottom panel (3 h chase), after removal of the extracellular nanoparticle dispersion, the second slice was maintained in refreshed medium without nanoparticles for a further 3 h (chase). Scale bars: 200 µm. Blue: DAPI stained nuclei. Red: nanoparticles. The images clearly show that after exposure, a large amount of nanoparticles adheres to the edge of the slice. By maintaining the slice for a further 3 h in medium without nanoparticles, the amount of nanoparticles adhering to the slice borders drastically decreases, thus allowing easier visualization of the intracellular nanoparticle distribution within the tissue (Note that for these nanoparticles cellular export or degradation have not been observed in in vitro studies$^{[19]}$). Based on these results, in all imaging experiments performed in this work, after exposure to the nanoparticles the liver slices were maintained for 3 h in medium without nanoparticles, prior to preparation for confocal fluorescence microscopy.
Supplementary Figure S9. Cross-section acquired by confocal fluorescence microscopy of liver slice exposed to 10 μg ml⁻¹ far-red PS-COOH nanoparticles in 5% FBS medium for 24 h. The result confirms nanoparticles uptake into the tissue. From the left: scale bars 50, 20 and 10 μm, respectively. Blue: DAPI-stained nuclei. Red: nanoparticles.

Supplementary Figure S10. Analysis of nanoparticle uptake by endothelial cells in rat liver slices. Cross-section acquired by confocal fluorescence microscopy of liver slices exposed for 24 h to 10 μg ml⁻¹ far-red PS-COOH nanoparticles in 5% FBS medium. Blue: DAPI stained nuclei. Red: nanoparticles. Green: CD31-labelled vascular and lymphatic endothelial cells. Scale bar: 200 μm. Details of the same area at increased magnification are shown in the lower panels, including the corresponding individual channels (Scale bar: 100 μm). Some uptake of nanoparticles by endothelial cells was observed in the indicated area, where a cross-section of what looks like a blood vessel, lined by endothelial cells (green) is visible. These images suggest that nanoparticle uptake in endothelial cells was present only when the tissue slice was extracted from the liver in an orientation that allowed these cells to be exposed to the nanoparticles.
Supplementary Figure S11. Analysis of nanoparticle uptake by liver sinusoidal endothelial cells in rat liver slices. Cross-section acquired by confocal fluorescence microscopy of liver slices exposed for 24 h to 10 μg ml⁻¹ far-red PS-COOH nanoparticles in 5% FBS medium. Blue: DAPI stained nuclei. Red: nanoparticles. Green: SE-1-labelled liver sinusoidal cells. Scale bar: 200 μm. Details of the same area at increased magnification are shown in the lower panels, including the corresponding individual channels (Scale bar: 100 μm). Uptake of nanoparticles in liver sinusoidal cells was rarely observed.
Supplementary Figure S12. Nanoparticle uptake in murine liver slices and accumulation in Kupffer cells. Cross-sections acquired by confocal fluorescence microscopy of murine liver slices exposed for 48 h to 10 μg ml⁻¹ far-red PS-COOH nanoparticles in 5% FBS medium. Scale bar: 200 μm. Blue: DAPI stained nuclei. Red: nanoparticles. Green: CD68 labelled Kupffer cells. Details of the indicated area at increased magnification are shown in the lower panels, including the corresponding individual channels (Scale bar: 100 μm). The results confirm nanoparticle uptake into the tissue and, as observed in rat liver slices, high nanoparticle uptake by Kupffer cells, mainly at the edge of the tissue slices, as evidenced by the overlap between the nanoparticle (red) and Kupffer cell (green) signals.
Supplementary Figure S13. Analysis of nanoparticle uptake and Kupffer cell distribution in rat liver slices after 24 h exposure. Representative cross-sections acquired by confocal fluorescence microscopy of liver slices exposed for 24 h to 10 μg ml⁻¹ far-red PS-COOH nanoparticles in 5% FBS medium (b) and untreated control slices cultured for the same time (24 h) without nanoparticles (a). Blue: DAPI stained nuclei. Red: nanoparticles. Green: ED2-labelled Kupffer cells. Scale bar: 200 μm. On the right, the individual channels are shown (gray intensity scale). The ED2 channel of the same image is shown in Figure 3a, and has been used together with other similar images for the quantification of Kupffer cell distribution shown in Figures 3d–e.
Supplementary Figure S14. Analysis of nanoparticle uptake and Kupffer cell distribution in rat liver slices after 48 h exposure. Representative cross-sections acquired by confocal fluorescence microscopy of liver slices exposed for 48 h to 10 μg ml⁻¹ far-red PS-COOH nanoparticles in 5% FBS medium (b) and untreated control slices cultured for the same time (48 h) without nanoparticles (a). Blue: DAPI stained nuclei. Red: nanoparticles. Green: ED2-labelled Kupffer cells. Scale bar: 200 μm. On the right, the individual channels are shown (gray intensity scale). The ED2 channel of the same image is shown in Figure 3b, and has been used together with other similar images for the quantification of Kupffer cell distribution shown in Figures 3d–e.
Supplementary Figure S15. Analysis of nanoparticle uptake and Kupffer cell distribution in rat liver slices after 72 h exposure. Representative cross-sections acquired by confocal fluorescence microscopy of liver slices exposed for 72 h to 10 μg ml⁻¹ far-red PS-COOH nanoparticles in 5% FBS medium (b) and untreated control slices cultured for the same time (72 h) without nanoparticles (a). Blue: DAPI stained nuclei. Red: nanoparticles. Green: ED2-labelled Kupffer cells. Scale bar: 200 μm. On the right, the individual channels are shown (gray intensity scale). The ED2 channel of the same image is shown in Figure 3c, and has been used together with other similar images for the quantification of Kupffer cell distribution shown in Figures 3d–e.
Supplementary Figure S16. Quantification of Kupffer cell distribution within rat liver slices. Liver slices were sectioned and imaged as described in the Experimental section just after preparation in order to determine the distribution of Kupffer cells in fresh liver tissue (0 h incubation). **a:** Representative cross-section acquired by confocal fluorescence microscopy of a fresh liver slice. Blue: DAPI stained nuclei. Green: ED2-labelled Kupffer cells. Scale bar: 200 µm. **b:** The distribution of Kupffer cells is quantified from images such as those shown in panel a in terms of the distribution of the (closest) distance between each cell and the slice border and is presented for each slice separately. The y axes show the normalized frequency (number of Kupffer cells within the stated distances divided by the total number of Kupffer cells within that slice).
Supplementary Figure S17. Quantification of Kupffer cell distribution within rat liver slices after 24 h exposure. Left column: untreated control slices cultured for 24 h without nanoparticles; right column: slices exposed for 24 h to 10 μg ml⁻¹ far-red PS-COOH nanoparticles in 5% FBS medium. The distribution of Kupffer cells is quantified in terms of the distribution of the (closest) distance between each cell and the slice border and is presented for each slice separately; the data is combined in Figures 3d–e. The y axes show the normalised frequency, i.e. the number of Kupffer cells within the stated distances divided by the total number of Kupffer cells within that slice.

Supplementary Figure S18. Quantification of Kupffer cell distribution within rat liver slices after 48 h exposure. Left column: untreated control slices cultured for 48 h without nanoparticles; right column: slices exposed for 48 h to 10 μg ml⁻¹ far-red PS-COOH nanoparticles in 5% FBS medium. The distribution of Kupffer cells is quantified in terms of the distribution of the (closest) distance between each cell and the slice border and is presented for each slice separately; the data is combined in Figures 3d–e. The y axes show the normalized frequency, i.e. the number of Kupffer cells within the stated distances divided by the total number of Kupffer cells within that slice.
Supplementary Figure S19. Quantification of Kupffer cell distribution within rat liver slices after 72 h exposure. Left column: untreated control slices cultured for 72 h without nanoparticles; right column: slices exposed for 72 h to 10 μg ml⁻¹ far-red PS-COOH nanoparticles in 5% FBS medium. The distribution of Kupffer cells is quantified in terms of the distribution of the (closest) distance between each cell and the slice border and is presented for each slice separately; the data is combined in Figures 3d–e. The y axes show the normalized frequency, i.e. the number of Kupffer cells within the stated distances divided by the total number of Kupffer cells within that slice.
Supplementary Figure S20. Analysis of Kupffer cell distribution in rat liver slices after 72 h exposure to unlabeled nanoparticles. Representative cross-sections acquired by confocal fluorescence microscopy of liver slices exposed for 72 h to (a) 10 μg ml⁻¹ unlabeled PS-COOH (50 nm, from Polysciences) and (b) PS-NH₂ in 5% FBS medium. Blue: DAPI stained nuclei. Green: ED2-labelled Kupffer cells. Scale bar: 200 μm. On the right, the individual channels of the same image are shown (gray intensity scale). Accumulation of Kupffer cells towards the slice borders was also observed when slices were exposed to unlabeled PS-COOH (a). This allowed us to exclude that the observed colocalization of nanoparticles and Kupffer cells (Figure 2) was simply due to spillover of nanoparticle fluorescence in the Kupffer cell channel. Furthermore, a similar accumulation of Kupffer cells towards the slice borders was also observed when slices were exposed to PS-NH₂ nanoparticles (b), suggesting that Kupffer cells were also accumulating these nanoparticles.

Supplementary Figure S21. Flow cytometry analysis of cells recovered from murine liver slices exposed to nanoparticles. Liver slices were exposed to 25 μg ml⁻¹ far-red PS-COOH nanoparticles in 5% FBS medium for up to 48 h prior to tissue digestion, performed as described in the Experimental section. The isolated cells were analyzed by flow cytometry. An example to illustrate the gating strategy is shown. a: Double scatter plot of side scatter area versus live/dead staining. This plot is used to exclude the dead cells (high live/dead staining) and select the live cells (polygonal gate in the image). Enzymatic digestion of liver tissue is known to cause strong cell death, as also visible in this plot. Because of this, many slices were pulled together for the procedure, as described in the Experimental section. b: Double scatter plot of forward scatter height versus forward scatter area of live cells. This plot is used to exclude from the live cells
(selected as shown in a) eventual cell doublets and select single cells (polygonal gate in the image). c: An example of the double scatter plot of side scatter area versus forward scatter area of single live cells is included for illustration. d–e: Double scatter plot of forward scatter area versus CD68 fluorescence of all recovered live single cells (d) unstained or (e) after staining the Kupffer cells with CD68-PE antibody (see Experimental section for details). The unstained sample is used to set a gate as shown in d in order to select the CD68-stained Kupffer cells in panel e. As indicated in panel e, the Kupffer cells were 4–11% of all cells recovered, consistent with the natural abundance of Kupffer cells in the liver. f: Double scatter plot of nanoparticle fluorescence (PS-COOH) versus CD68 fluorescence. Only the Kupffer cells are shown (CD68 positive cells, selected as shown in panel e). The same results for all cells are shown in Figure 4d (in which the Kupffer cells are shown in red and all other cells, here not included, are shown in green). A gate is set in a sample of untreated cells not exposed to the nanoparticles (0 h exposure, rectangular gate) in order to determine the percentage of Kupffer cells accumulating nanoparticles (the results are included in Figure 4b). The results show that at increasing exposure time, nanoparticle uptake increases and more Kupffer cells accumulate nanoparticles. g: Double scatter plots of forward scatter area versus nanoparticle fluorescence (PS-COOH). The Kupffer cells (CD68 positive) are shown in red and all other cells (CD68 negative) are shown in green. Plotting the data in this way helps to visualize once more that the Kupffer cells are the cells with higher nanoparticle uptake. The population of cells at highest nanoparticle uptake contains also some cells that are not Kupffer cells (in green), which possibly were cells on the slice borders, however clearly the majority of these cells are Kupffer cells (in red). These results are consistent with what was observed by confocal microscopy.
Supplementary Figure S22. Viability of human liver slices after 24 h exposure to nanoparticles. Human liver slices were exposed for 24 h to increasing doses of PS-NH₂ (a, c, e) and 100 µg ml⁻¹ far-red PS-COOH (b, d, f) nanoparticles in serum-free medium (WME) or medium supplemented with 5% FBS (WME + 5% FBS), after which their viability was measured. The results obtained with 3 different biopsies are shown (here denoted as “one”, “two”, “three”) and their average is shown in Figures 5a and 5c. In panel a and b, the viability is expressed as the ATP content (pmol) normalized by amount of total protein (µg). The results are the average and standard deviation of 3 replicate slices treated in the same way. c-f: The raw ATP content (c, d) and total protein amount (e, f) of one representative experiment are also shown, as an example of the original data from which viability is calculated as described in the Experimental section.
Supplementary Figure S23. Viability of human liver slices after 48 h exposure to nanoparticles. Human liver slices were exposed for 48 h to 100 µg ml\(^{-1}\) of PS-NH\(_2\) (a, c, e) and far-red PS-COOH (b, d, f) nanoparticles in serum-free medium (WME) or medium supplemented with 5% FBS (WME + 5% FBS), after which their viability was measured. The results obtained with 3 different biopsies are shown (here denoted as “one”, “two”, “three”) and their average is shown in Figures 5b and 5d. In panel a and b, the viability is expressed as the ATP content (pmol) normalized by amount of total protein(µg). The results are the average and standard deviation of 3 replicate slices treated in the same way. c–f: The raw ATP content (c, d) and total protein amount (e, f) of one representative experiment are also shown, as an example of the original data from which viability is calculated as described in the Experimental section.
ADDITIONAL REFERENCES
