Supporting information

Hitchhiking Probiotic Vectors to Deliver Ultra-Small Hafnia for 'Color' Gastrointestinal Tract

Photon Counting X-ray Imaging

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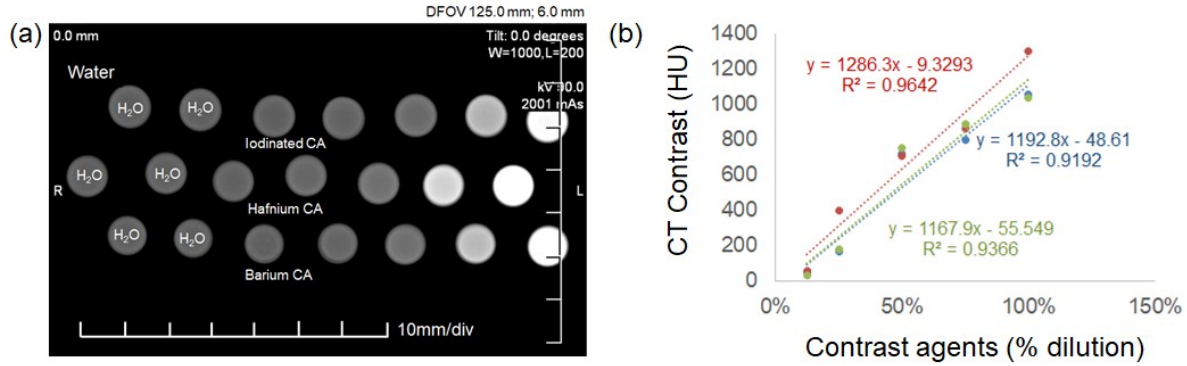

Figure S1. Conventional CT imaging in suspension: (a) CT opacity as a function of concentration generated from centrifuge tubes (Internal Diameter: I.D. 10 mm, Outer Diameter: O.D. 13 mm, vol. 1 cc) filled with iodinated (top), hafnium (middle) and barium (bottom) contrast agents, with varying concentration of metal, and water; cross sectional CT images of serially diluted equimolar contrast agents (0.01mmole/ml) ; (b) variation of CT signal (HU) with contrast agent (red: hafnium; green: barium and blue: iodine) concentration (100, 75, 50, 25, 12.5%).

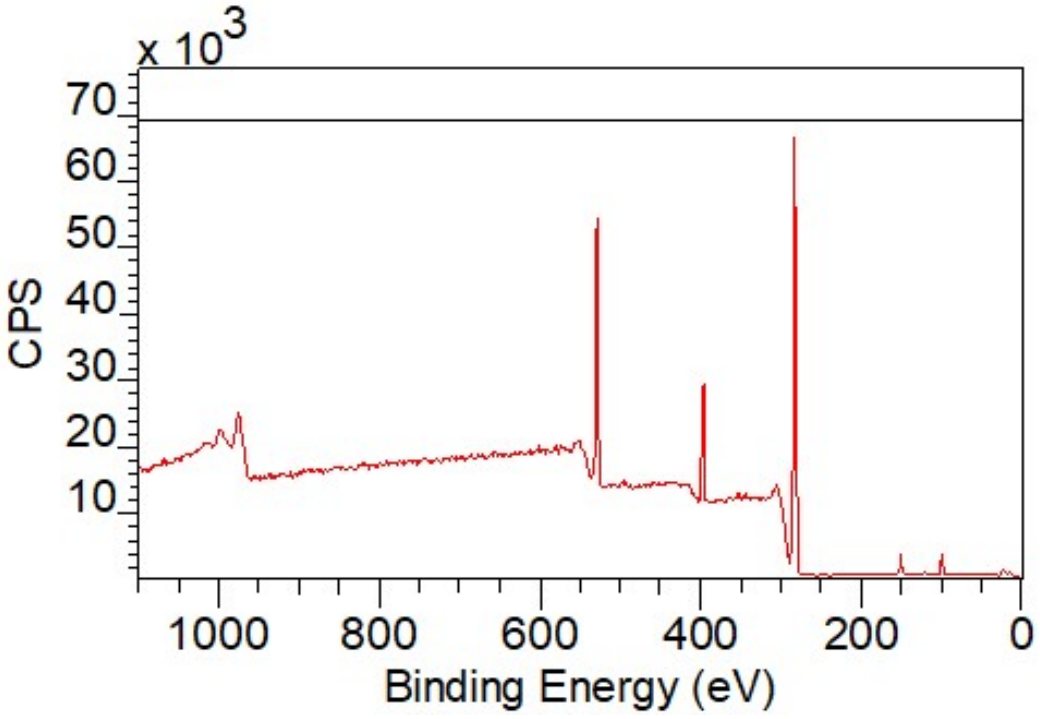

Figure S2. XPS of PVP in survey mode.

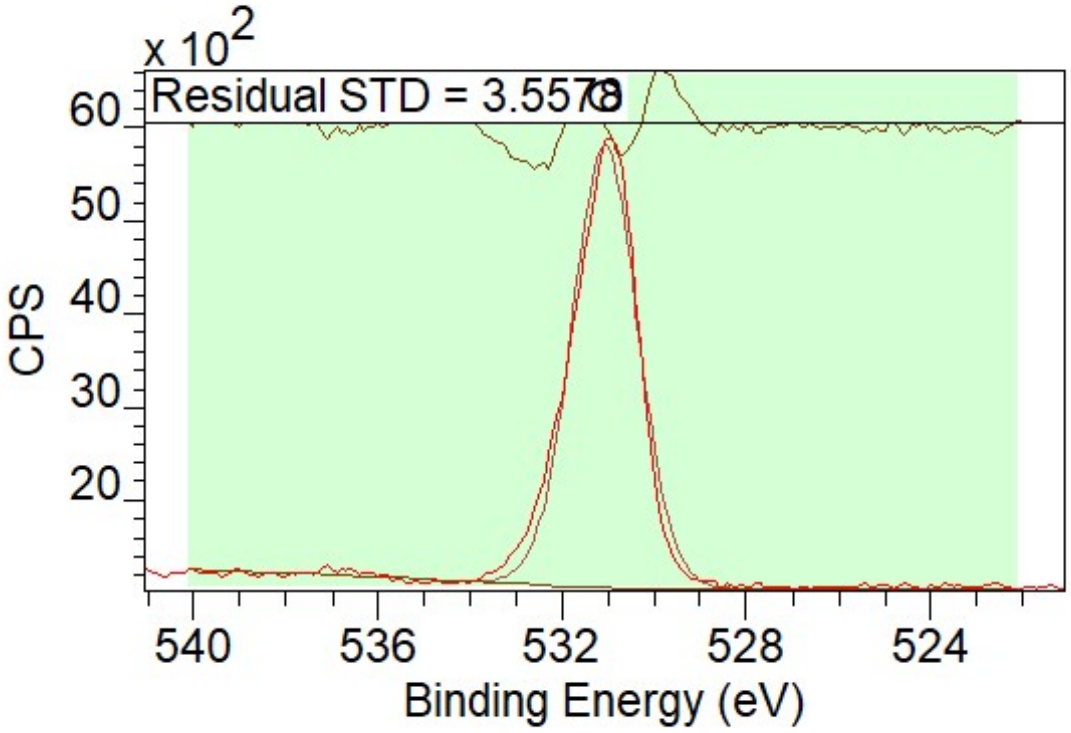

Figure S3. The XPS peak decomposition of oxygen region in PVP.

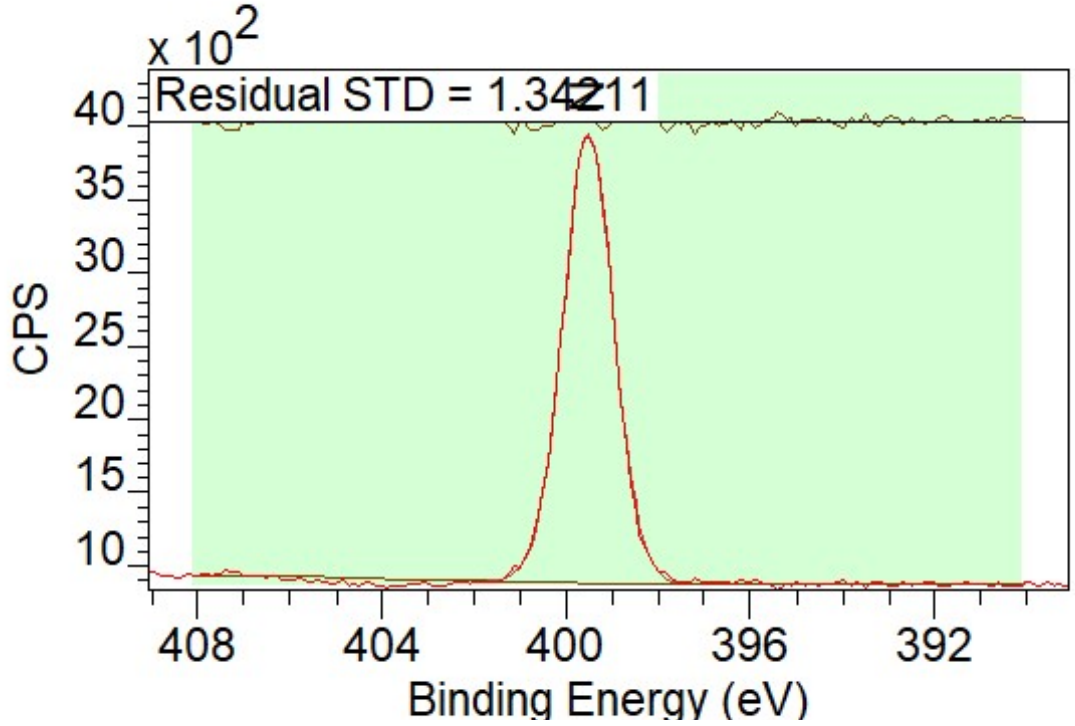

Figure S4. The XPS peak decomposition of nitrogen region in PVP.

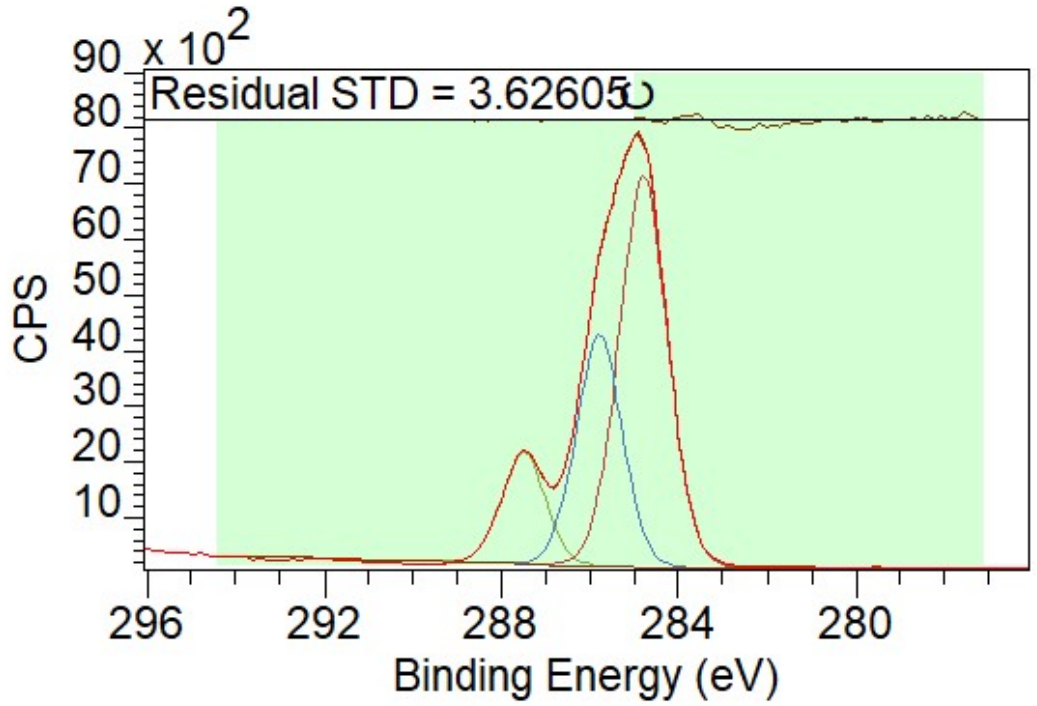

Figure S5. The XPS decomposition of carbon region in PVP.

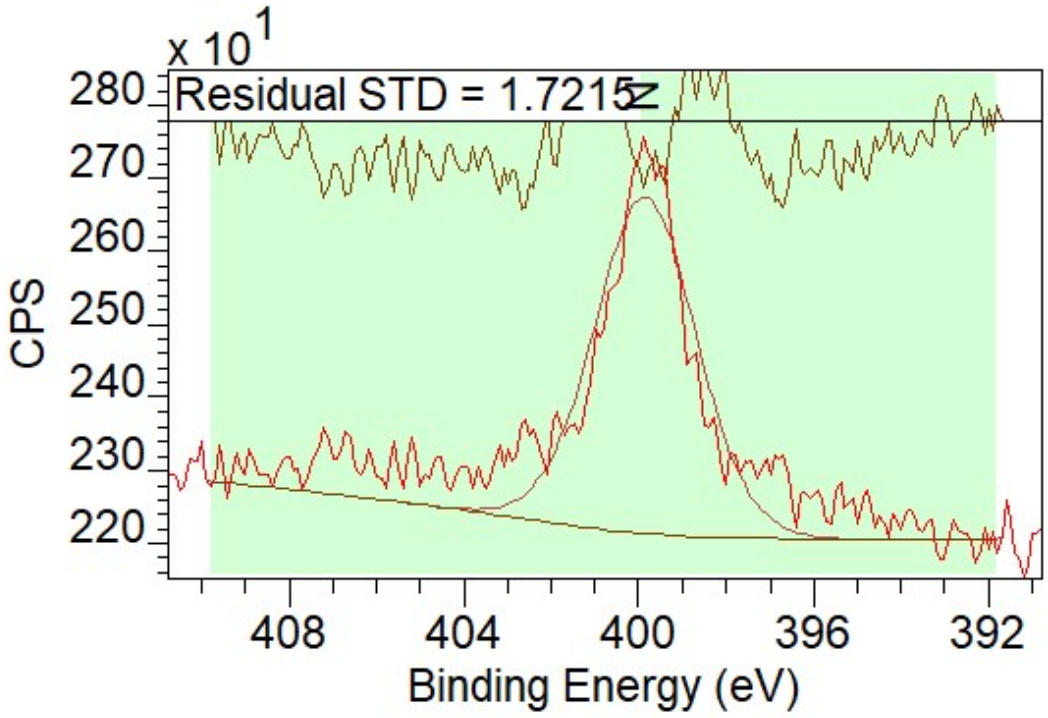

Figure S6. The XPS peak decomposition of P-HfO_2 NPs for N1s.

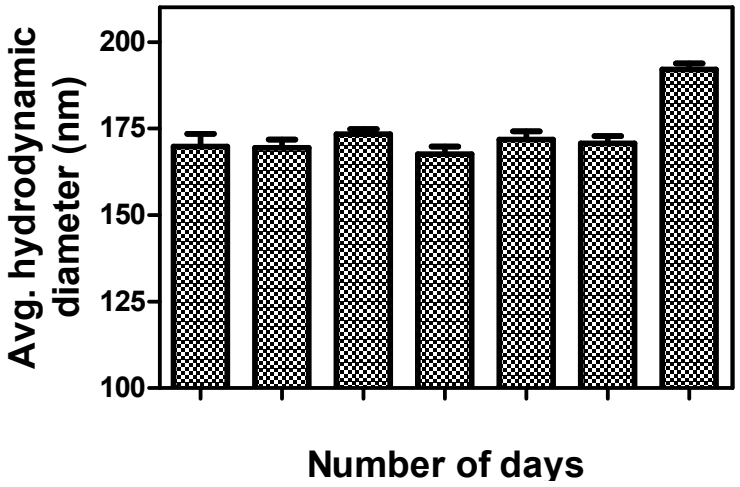

Figure S7. Change in hydrodynamic diameter of the hafnia nanoparticles over the period of seven days.

Figure S8. The growth of bacteria in bio-HfO2 NPs after 24 h (right). Left vial is plain medium.

Figure S9. Phantoms prepared for materials discrimination experiment.

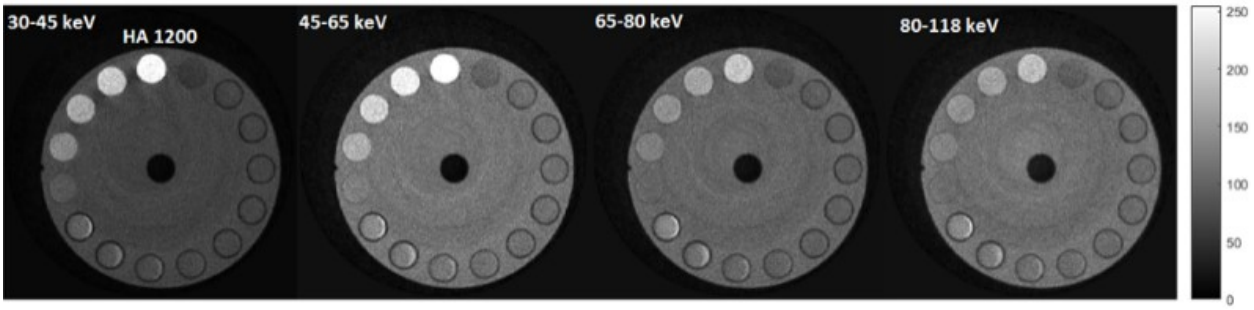

Figure S10. Energy images of the calibration phantom in four energy bins.

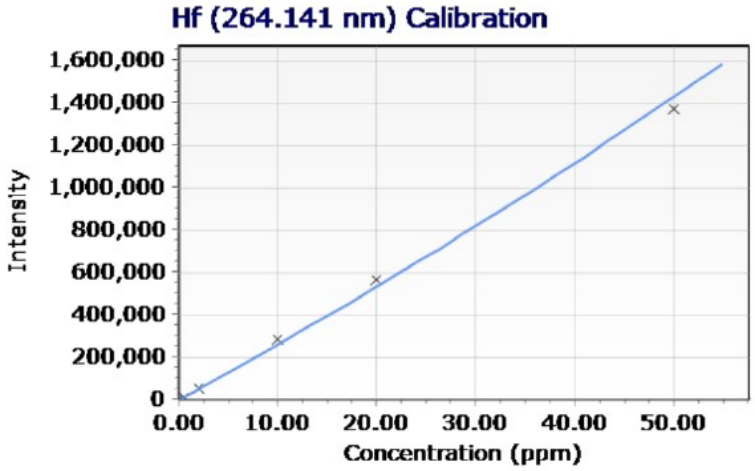

Figure S11. Standard curve of hafnium for ICP-OES analyses.

Intensity = (25786.20795819 * Concentration + 37.77476473) / (1 - 0.00197064 *

Concentration); Correlation coefficient: 0.99924; %RSE:6.22575458

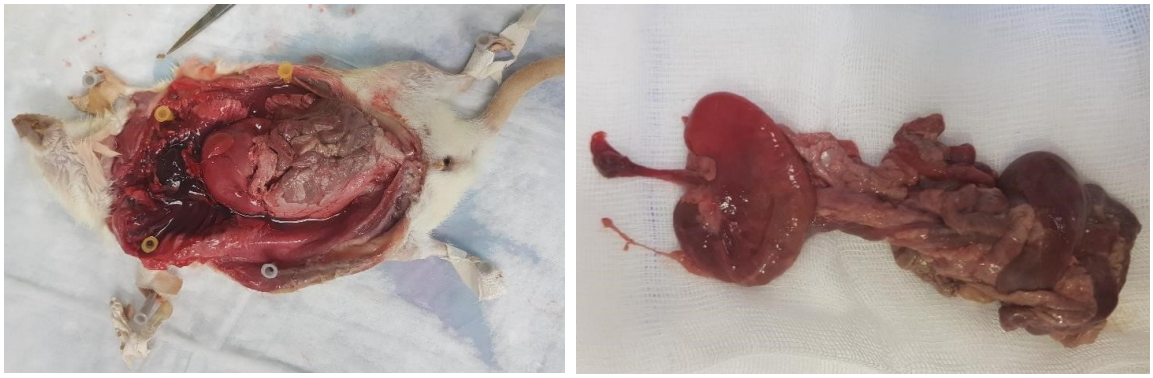

Figure S12. Images of dissected rat and GI tract.

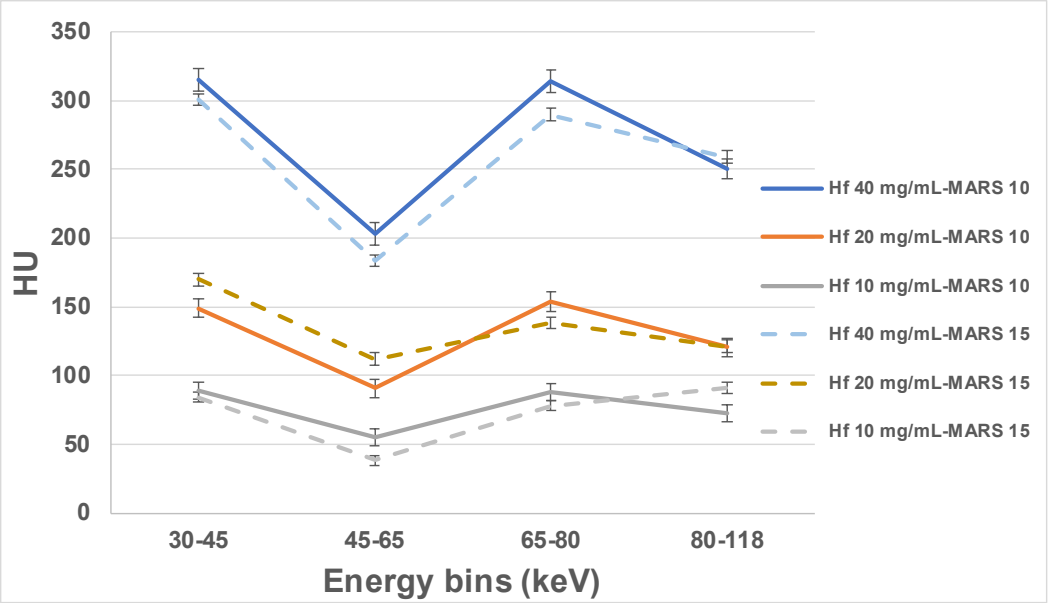

Figure S13. Graph comparing the attenuation profiles for two scans obtained from MARS-10 and MARS-15.

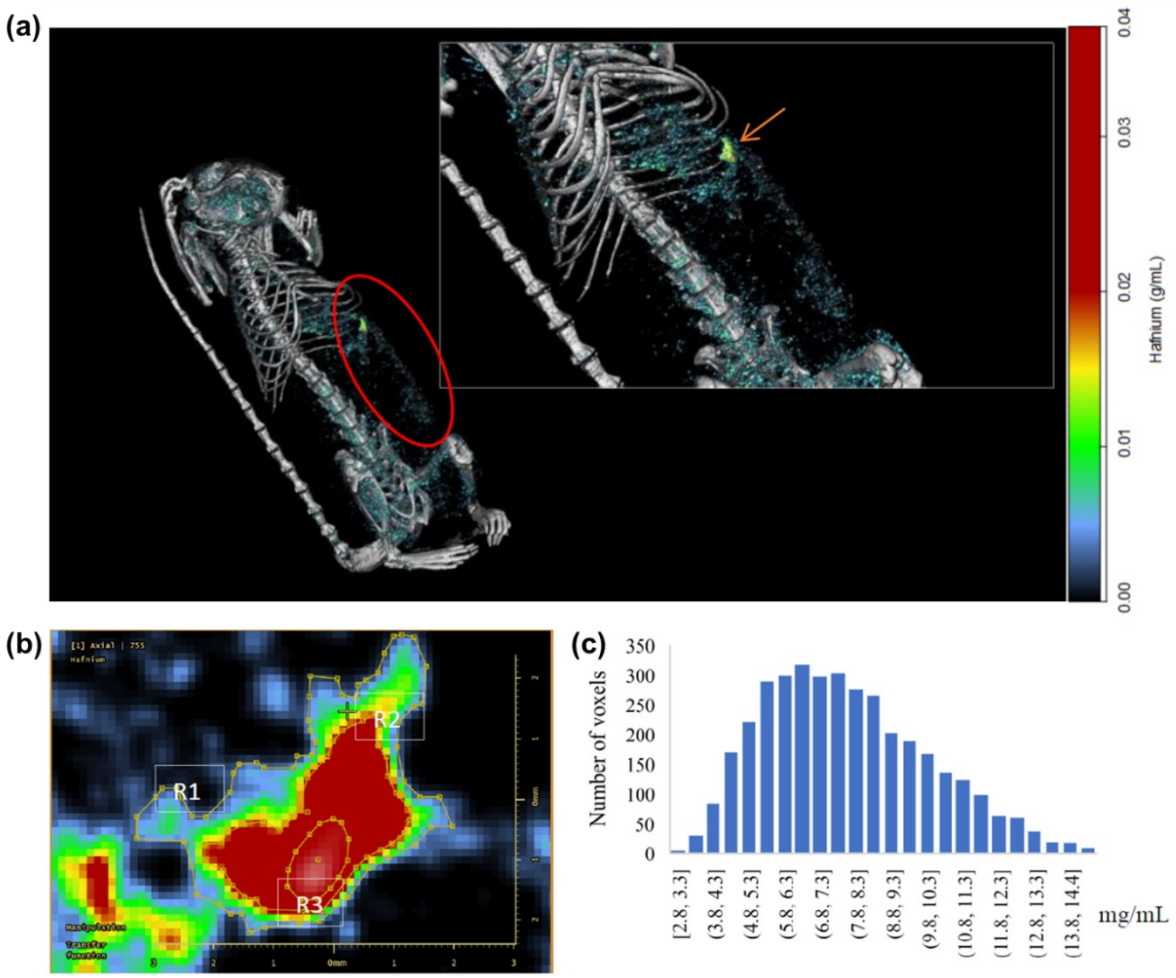

Figure S14. (a) MARS scan of the animal following oral gavage of Hf NPs. 3D spectral image: Hf (indicated by the colour bar) in the sample is differentiated from the bone (white/grey), and soft tissue (removed from view). (b) 2D axial view of the region of interest indicated by the red arrow in Figure 8a. Region 1 (R1), region 2 (R2), and region 3 (R3) correspond with low, mid, and high concentration, respectively. (c) Example distribution (R1; histogram) with concentrations in 24 bins along the x-axis. R1 measured a 3D volume excluding R2 and R3 and R2 excludes R3.

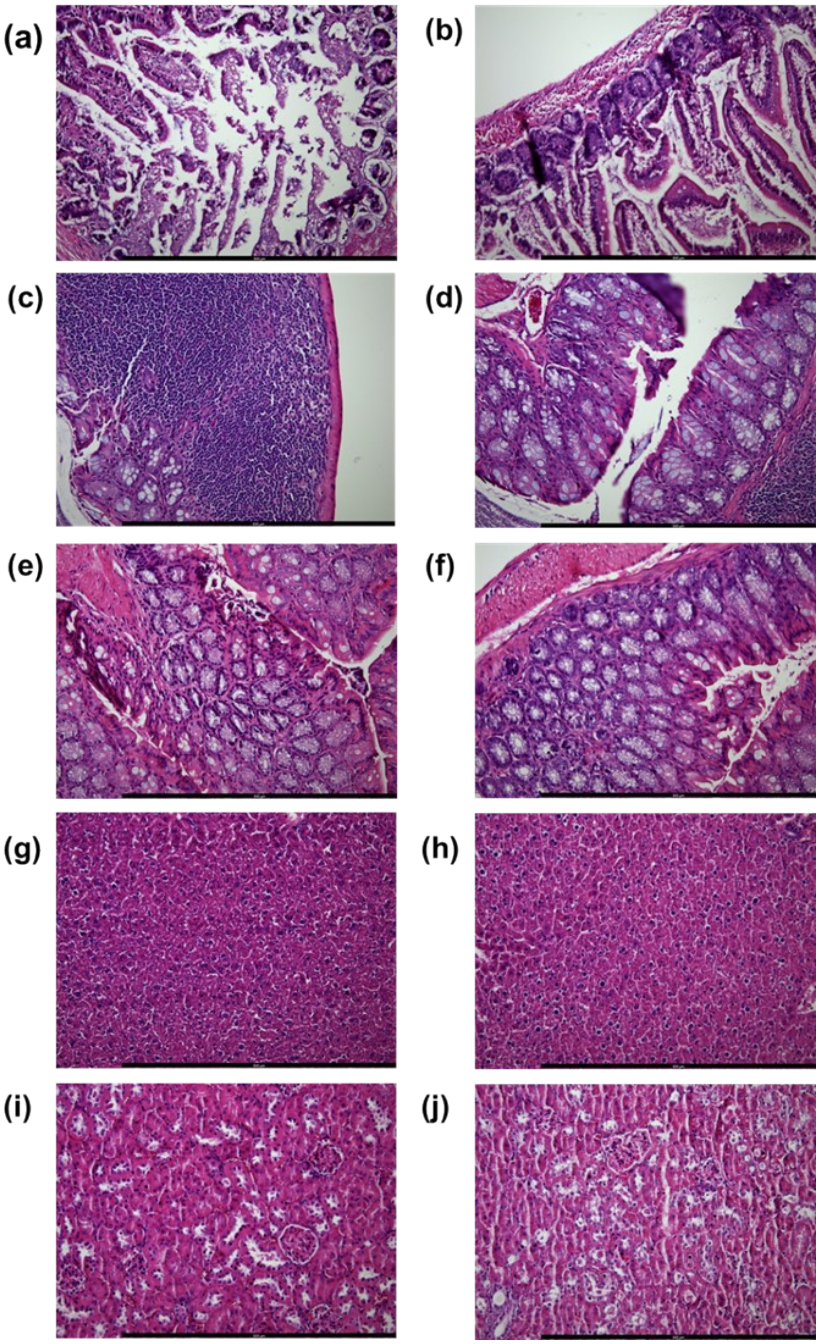

Figure S15. Hematoxylin and eosin assay on different organs (b, d, f, h, j) treated with HfO_2 NPs compared to the (a, c, e, g, i) non-treated ones. The organs were: (a, b) small intestine; (c, d) colon; (e, f) rectum; (g, h) liver and (i, j) kidney.

Table S1. Hafnium concentration calculated from ICP-OES analyses.

Suspension	**Treated HfO_2-NP concentration**	**Hf Concentration found from ICP-OES**
As-synthesized HfO_2 NPs	54 mg in 10 ml, i.e., 5400 ppm	5.4025 ppm
Non-treated probiotics	--	Not detected
Treated probiotics	54 mg in 10 ml, i.e., 5400 ppm	4.582 ppm

Table S2: Protocol's specifications of MARS-10 and MARS-15.

Protocol parameters	**MARS-15**	**MARS-10**
Scan type	Continuous motion	Continuous motion
Tube voltage	118 kVp	118 kVp
Tube current	25 µA	21 µA
Exposure time	150 ms	150 ms
SDD, SOD	286.1 mm, 215 mm	271.95 mm, 211.95mm
Field of view	68 mm	51 mm
Circular projections over 360°, Flat	720, 720	720, 720

field		
Voxel size (isotropic)	0.18 mm	0.1 mm
X-ray filtration	3.8 mm (2mm Al + 1.8 mm Al intrinsic)	3.8 mm (2mm Al + 1.8 mm Al intrinsic)
Energy thresholds	30, 45, 65, 80 keV	30, 45, 65, 80 keV
Detector model	CZT-Medipix3RX Five-chip	CZT-Medipix3RX Seven-chip

Materials and Methods

Hafnium nitrate $Hf(NO_3)_4$ was purchased from Onyxmet. Sodium borohydride ($NaBH_4$), polyvinyl pyrrolidine (PVP), and anhydrous ethanol (EtOH) were obtained from Sigma Aldrich (St. Louis, MO, US) while Glycerol was procured from Alfa Aesar (Haverhill, Massachusetts, United States). Nano pure water (0.2×10^{-6} m, 18 MΩ cm) was used throughout the experiments. The culture tube of *lactococcus lactis* was bought from VWR International (Chicago, IL, US) and *Escherichia coli* (*E. Coli*) Nissle 1917 was collected from capsules (Mutaflor®). The bacteria were sub-cultured in BHI broth which was obtained from Becton Dickinson (Franklin Lakes, NJ, US).

Nanoparticle synthesis and characterizations

Three grams of PVP and 1 g of $Hf(NO_3)_4$ were admixed in 100 ml of glycerol and 50 ml of anhydrous EtOH at 35 °C under ambient condition and with constant stirring. To this solution, 500 mg of $NaBH_4$ was added rapidly and stirred vigorously for 2 min to yield ultrasmall P-HfO_2 NPs (supporting video 1). The NPs were washed excessively by anhydrous EtOH for three times and then water (three times) and the obtained white powder was dried over night by vacuum oven and/ or freeze drying. For every experiment, the NPs were facilely dispersed in water by a probe sonication instrument for 5 min (Q700, Qsonica Sonicators, Newtown, CT) at Pulsed Amp, 1; 2 s on, and 1 s off.

The sample for Transmission electron microscopy (TEM) was prepared by drop casting the dilute suspension of NPs on a carbon-coated copper grid (TED PELLA Inc., Redding, CA, US). The excess of liquid was wicked away by filter paper. The imaging was conducted on a JEOL 2100 Cryo TEM (Peabody, MA, US) equipped with Gatan UltraScan 2kx2k CCD camera operating at 200 kV. The selected area diffraction pattern was obtained on the same instrument. X-ray powder diffraction (XRD) pattern was collected on a Brucker D8 advance XRD system (Billerica, MA, US) in a $\theta - 2\theta$ configuration with Cu K_α radiation of 1.5418 Å with an operation voltage and current maintained at 40 kV and 40 mA. Atomic force microscopy (AFM) was carried out on a dried diluted sample on a mica attached to steel disk. The images were acquired on a Bruker MultiMode Nanoscope IIIA (Billerica, MA, US) and the images were processed with Gwyddion software. Thermogravimetric analysis (TGA) was done at the heating rate of 20 °C.min^{-1} under N_2 (40 ml.min^{-1}) and air flow (60 ml.min^{-1}) in a Pt-pan using Q50 TGA system (Q50, TA instruments, Pittsburgh, PA, USA).

To determine the dispersity and stability of the HfO_2 nanoparticles, the hydrodynamic diameter of the aqueous suspension was measured over the period of 7 days. The nanoparticles were suspended at a concentration of 1.8 mg/mL with brief probe sonication as is indicated in the synthesis methods and diluted 50 times to a concentration of 36 μg/mL before being monitored using a particle tracking analyzer (Zetaview Particle Metrix). 1 mL of diluted samples were injected into the machine for measurements. The chamber of the machine was properly cleaned prior to each measurement.

The absorbance of the dispersed NPs was performed on GENESYS 10 UV−vis spectrometer (Thermo Scientific, MA, US). For Fourier transform infrared (FTIR) spectroscopy, the samples were deposited on MirrIR IR-reflective glass slides (Kevley Technologies, Chesterland, OH, USA). The transmission of dried samples was subsequently recorded in attenuated total reflectance (ATR) mode on a Nicolet Nexus 670 FT-IR instrument (ThermoFisher scientific, Waltham, MA, US). For further chemical characterizations, x-ray photoelectron spectroscopy (XPS) was conducted on small piece of sample dried overnight on glass slide. A Physical Electronics PHI 5400 spectrometer using Al Kα (1486.6 eV) radiation was utilized for acquiring the spectra. CasaXPS software was used for peak decomposition and referencing to the adventitious C-C bond (284.8 eV) [1]. Finally, to quantify the amount of Hf in the elemental form inductively coupled plasma (ICP)-optical emission spectroscopy (OES) was done by dissolving the samples in 511FAR (5 ml of HNO3, 1 ml of HCl, and 1 ml of HF) acidic mixture. After digestion by Discover SPD 80 (CEM Corporation, NC, USA) digestion system, the sample was run in ICP-OES system and the amount of Hf was determined by calibration and background correction with 511FAR.

In vitro biocompatibility of P-HfO_2

MTT cell viability assay: Hs 1.Int cells (ATCC® CRL-7820™) were cultured in DMEM high glucose media with 10% FBS supplement. Standard cell culture techniques were followed to maintain the culture of the intestinal cells. Cytotoxicity of the nanoparticles at different concentrations was determined using 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. In brief, 15,000 cells/well were plated in 96 well plates. After 24 h, all the samples at pre-defined concentration were added to the cells in presence of 10% FBS. The compounds were removed after 6 h of desired incubation and 200 μl of fresh media with 10% FBS was added. After 42 h, % viability was calculated as,

[{A_{590}(treated cells)-background]/ [A_{590}(untreated cells)-background}] x 100.

Blood-smear experiment: The protocol has been followed by a previous literature report.[2] A single smear was made per slide by putting a drop of fresh rabbit blood on the slide (near the end). The drop was spread by using another slide ("spreader"), placing the spreader at a 45° angle and backing into the drop of blood. The spreader catches the drop, and it spreads by capillary action along its edge. The smear was allowed to air-dry for 1 min and a coverslip was applied before placement directly on the microscope, and samples were observed under 100× magnification by Nikon Ti2 inverted microscope (Figure 4D). Initially blood was incubated with different concentration of ultrasmall HfO_2 nanoparticle. The samples were diluted 5 times before being imaged under the microscope.

CYP3A4 metabolic assay: The metabolic assay for inhibition of human CYP3A4 enzyme activity was performed in multi-well plates using the CYP inhibition assay kit (Promega; CYP3A4 Screening System with Luciferin-IPA; cat. no. V9920) as described in the manufacturer's protocol.

***In vitro* studies of bio-HfO_2**

At first, to understand the cytocompatibility of P-HfO_2 NPs, we incubated an overnight culture of *E.Coli* Nissle with various concentrations of NPs (0.3-10 $mg.ml^{-1}$) for 4 h and then LIVE/DEAD® BacLight™ Bacterial Viability Kit (L7012, ThermoFisher Scientific, Grand Island, NY, USA) as performed. The bacterial OD_{600} was adjusted to 0.3 and after incubation, the bacteria were spun down and washed with 0.85% NaCl solution twice. The bacteria were stained with the kit's dyes with equal volumes: PI and SYTO™9 at 2X the working concentration. The incubation was done for 15 min in the dark and then the fluorescence was recorded with a multi-well plate reader at $\lambda_{excitation}/\lambda_{emission}$ = 480/500 and 490/635 nm. The confocal images were collected on the samples entrapped between two cover slips with a Leica SP8 UV/Visible laser confocal microscope (Leica Micosystems, Germany) in immersion oil medium with a refractive index of 0.6.

For investigating the uptake of NPs in bacteria, we incubated *E.Coli* Nissle and *L. Lactis* (OD_{600}=0.7) with NPs (2.5 $mg.ml^{-1}$) for 4 h and then the samples were centrifuged for 4000 × *g* for 2 min and the medium was discarder. The cells were then washed with Tris HCl buffer for three times and were fixed in an EM-grade fixative reagent (2.0% paraformaldehyde and 2.5% glutaraldehyde) overnight under mild vacuum at 4 °C. The samples were spun into a tight pellet, embedded in epoxy resin, and allowed to harden overnight in an 80 °C oven. They were then

sectioned using an ultramicrotome (Ultracut UCT, Leica Micosystems, Germany). The images were obtained using a Hitachi H600 TEM (Hitachi Instrument Co., Tokyo, Japan) at 75 kV.

As a proof-of-concept study, we carried out SEM imaging on the freeze-dried sample. To do this, we incubated *E. Coli* with OD_{600}=0.7 was incubated with 2.5 $mg.ml^{-1}$ for 4 h and then the samples were washed excessively with Tris-HCl to remove any unbound NPs. The bacteria were then lyophilized with Labconco Freezone 2.5L freeze dry system by flash freezing the samples in the liquid N_2 and then lyophilizing under 0.018 Torr at -50 °C. Scanning electron microscopy (SEM) was done on these samples by XL30 ESEM-FEG Field-Emission Environmental SEM instrument (Philips/FEI, Hillsboro, OR) equipped with energy dispersive spectroscopy (EDS). Prior to imaging the samples were sputter coated with Au and Pd for 40 s.

To ensure the viability of bacteria after the above process, 5 mg of bio-HfO_2 was dispersed in BHI broth and then optical density at 600 nm was measured.

X-ray and CT imaging

A small-bore MARS spectral CT scanner was used to scan specimens. The scanner works in the clinical diagnostic energy range (20-120 keV), is equipped with a micro-focus polychromatic X-ray source, and the Medipix3RX photon counting detector (Medipix3 Collaboration, CERN). The Medipix3RX detector has eight energy counters (1 arbitration counter, 4 charge summing mode (CSM) counters, and 3 single pixel mode (SPM) counters) that extract information over the wide X-ray spectrum and enables the identification and quantification of multiple materials simultaneously.

To cover the K-edge of Hf, energy thresholds in charge summing mode (CSM) were set at 30, 45, 65, and 80 keV, and an aluminum filter was employed (2mm Al + 1.8 mm Al intrinsic). CSM mode enables inter-pixel communication to mitigate the charge sharing effect and improves the overall energy resolution of the system.

For the current study, eight Eppendorf PCR tubes (200µl) of P-HfO_2 (100, 50, 25, 12.5, 6.25, 3.75, 1.87, 0.98 $mg.ml^{-1}$ which translate to 282.4, 141.2, 70.6, 35.3, 17.6, 8.8, 4.4, 2.2 mM of Hf as calculated by ICP), five Hydroxyapatite (HA) rods (HA50, 100, 200, 400 600 $g.cm^{-3}$ which translate to 49.8, 99.6, 199.2, 398.4, 598.6 mM), water, and lipid were placed within a custom made 12-hole Polymethyl methacrylate (PMMA) phantom (Figure S7). MARS CT scanning protocol used 118 kVp, 13 µA tube current, and 300 ms exposure time. The sample-to-detector (SDD) distance and sample-to-object (SOD) distance were set to 250 and 200 mm, respectively. 720 projections over 360^o rotation were acquired, as well as 720 flat-field (open-beam) projections. A field of view (FOV) of 32 mm, with 2 camera translations, was of sufficient size to cover the phantom and bone sample.

Image reconstruction of both the calibration phantom and biological sample was performed using MARS Algebraic Reconstruction Technique (mART) [3]. To assess the detection of the K-edge effect, the linear attenuations (LA) of each material in all four CSM energies were measured and converted into Hounsfield Units (HU). MARS Material Decomposition (MD) images were obtained [3, 4]. Several slices from the MD datasets of the calibration phantom were used to quantify Hf and analyze the accuracy of identification for each calibration vial.

Quantification of internalized Hf by Inductively coupled plasma - optical emission spectrometry (ICP-OES) analyses

54 mg of P-HfO_2 NPs was incubated with 27 ml of *E. coli* Nissle, sub-cultured in BHI broth, for 2, 4 and 6 h. It was then centrifuged for 4000 x g for 2 mins. The supernatant was discarded, and the cell pellet was washed at least three times with Tris HCl buffer to remove any traces of unbound NPs. The sample was then freeze-dried overnight. In a similar process, non-treated 27 ml of probiotic culture was also centrifuged and freeze-dried for ICP-OES analyses. The freeze-dried samples were then added with 10 ml of aqua regia overnight for digestion. Similarly, 54 mg of as-synthesized HfO_2 NPs was also added with 10 ml of aqua regia overnight for digestion. 800 µl of the aqua regia solutions were then diluted to 40 ml with milli-Q water and used directly for ICP-OES analyses. The standard curve for Hf was generated with the hafnium standard for ICP purchased from Sigma (Cat. no. 04617).

Optimization of in vivo treatment conditions

The choice of weight of HfO_2 nanoparticles, incubating volume of probiotics and incubation time was made from suitable design of experiments. Here, we have shown the contrast of P-HfO_2 NPs even in the fed state of Sprague Dawley rats. Average body weight of the studied male rats was found to be 540 gm. It was observed that suitable Hf contrast was found when the rats were gavage with a nanoparticle concentration of ~100 mg/kg. Hence, 54 mg of P-HfO_2 NPs, which was the required nanoparticle weight maintaining 100 mg/kg of nanoparticle concentration per rat, was first incubated with 27 ml of *E.Coli* Nissle for 4 h. The suspension was then washed several times to get rid of unbound NPs. The sample was freeze-dried overnight.

The sample was resuspended in 1 ml of PBS. Sprague Dawley rats (n=3) were subsequently gavage orally with flexible tube attached to a syringe.

Again, while testing the cell compatibility of P-HfO_2 NPs with the bacteria, we found that there is no significant toxicity of the NPs till the concentration of 10 mg/mL (Fig. 4A and 4B). Hence, we thought of treating the probiotics at a much safer concentration, *i.e.*, 2 mg/mL concentration. Therefore, we treated 54 mg of P-HfO_2 NPs to 27 ml of *E.Coli* Nissle culture.

On the other hand, incubation time of 4 h is necessary for optimum loading of HfO_2 NPs inside the probiotic cells. We have carried out ICP-OES analysis of probiotics incubated with HfO_2 NPs (54 mg in 27 mL of probiotic culture) for different time points (2 h, 4h and 6h). The content of Hf loading post 2 h was found to be 2.563 ppm, whereas for post 4 h and 6 h of incubation, the Hf loading was observed to be 4.582 ppm and 4.598 ppm respectively. Thus, we have optimized the Hf incubation period as 4 h.

***In vivo* study of bio-HfO_2**

Approval for this study was granted by the Illinois Institutional Animal Care and Use Committee at University of Illinois Urbana-Champaign (protocol # 17125). All animal experiments were conducted according to the ethical guidelines outlined by the aforementioned institution. 54 mg of P-HfO_2 NPs was incubated with 27 ml of *E.Coli* Nissle for 4 h and then was washed several times to get rid of unbound NPs. Then the sample was freeze-dried overnight. The sample was resuspended in 1 ml of PBS. Sprague Dawley rats (n=3) were gavage orally with flexible tube attached to a syringe. The animals were then scanned after 30 min with conventional multislice CT (General Electric Light speed 16-slice CT scanner (GE, Milwaukee, WI, USA)) using the following

parameters: slice thickness: 0.625 mm; tube voltage: 80 kV; tube current: 100 mA; gantry rotation time: 0.8 s; and pitch: 9.38 mm. The images were then processed with 3DSlicer 4.8.1 software.

For animal scan studies in multispectral CT, the following parameters were used:

Scan type	**Continuous Motion**
Tube voltage	120 kVp
Tube current	25 µA
Exposure time	150 ms
SDD, SOD	281.6 mm, 215 mm
Field of view	68 mm
Circular projections, Flat-fields	360 over 360^0, 360
Voxel size	0.180 mm^3
Sample diameter (FOV)	68 mm
Filtration	3.8 mm (2mm Al + 1.8 mm Al intrinsic)
Energy bins (CSM)	30-45, 45-65, 65-80, 80-120 keV.

A dense region of Hf material within the GI tract was selected for quantification. By selecting the region of interest within several slides, Hf was quantified. Especially, to visualize NPs to the lining of the GI tract, the rat was dissected, and the GI tract was excised. This included removal of the trachea, stomach, large intestine, and small intestine. **Figure S12** shows the rat dissection and excised GI tract. In this case, we used MARS 10 to scan the GI tract, resulting in a slightly different protocol setting than was used for previous scans those were taken on MARS 15. However, we kept the count rate equal to the count rate in our previous scans. This means we do not expect to have a significant difference in attenuation profiles. In **table S2**, the main difference in protocols is highlighted for tube current, SDD, and SOD. For MARS 10, the SDD is a bit smaller than MARS 15. Therefore, we reduced the flux to have the same attenuation profile for both

scans. **Figure S13** shows the attenuation profile for both scans. It can be seen, the attenuations (HU) calculated from MARS 10 are close to the attenuations (HU) obtained from MARS 15.

Hematoxylin and Eosin assay.

After the *in vivo* experiment, the mice were sacrificed, and different organs were collected from either the control rats or the rats treated with nanoparticles. The organs were then dissected to prepare slides and evaluated by hematoxylin and eosin assay using manufacturers' protocol. We have used the H&E staining kit available from abcam (catalogue no. ab245880).

References.

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