Supporting Information

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SI Text

Carbon Nanotube Dispersion Preparation. All carbon materials, dispersed in ultrapure water, were sonicated from 5 min (functionalized carbon nanotubes, CNTs) to 45 min (pristine CNTs) in a Branson 3200 water bath sonicator and vortexed for few seconds before in vitro, ex vivo, and in vivo experiments. The long time of sonication does not affect the morphology of the pristine nanotubes. In the case of the functionalized multiwalled CNTs (MWCNTs) used in the experiments described in Fig. 1 *A*–*C*, the solid was just added to ultrapure water without any sonication or vortexing.

Animal Procedures, Histopathology, and Immunophenotypic Characterization of Inflammatory Cells. Three female healthy pigs (*Sus Scrofa* species) 8-wk-old and about 15 kg in weight were bought from Azienda Agricola Le cascine. The animals were housed in accordance to the guide of use of laboratory animals of the Italian Ministry of Health. For MWCNT administration, pigs were under anesthesia with azaperone (Strensnil; Janssen-Cilag) (1 mL/20 kg). Five milliliters of MWCNTs at a concentration of 1,000 μg/mL were injected into the bladder ($n = 2$) or intravenously ($n = 1$). Urine were collected immediately after ultrasonography and analyzed by transmission electron microscopy (TEM). Seven days after the injection of MWCNTs, pigs were killed with azaperone treatment and Tanax (Intervet International). For toxicity investigation ($n = 3$), blood samples were collected before the in vivo experiment and right before killing, and examined with Advia 2120 (Siemens) and Dimension RXL (Siemens).

For histopathology, samples from kidney, bladder, liver, lung, and heart were fixed in neutral buffered 4% (vol/vol) formalin and then embedded in paraffin. Paraffin-embedded tissue blocks were cut into 5-μm-thick sections and stained with H&E (Bio-optica), to be subsequently examined under a light microscope (Leica) coupled with a digital camera.

The in situ identification of macrophages and T and B lymphocytes was performed by detecting the cluster of differentiation antigen systems. CD163 was used to identify macrophages; CD79 and CD3 were used to recognize B cells and T lymphocytes, respectively. Briefly, 5- to 7-μm-thick sections were placed on poly-L-lysine-coated glass slides for paraffin wax removal with xylene and for rehydration by an ethanol series. For CD3 and CD79 antigen retrieval, sections were immersed in pH 9 Tris Buffer EDTA solution and treated two times in a microwave oven at 850 W for 10 min. For CD163 antigen retrieval we used a microwave oven at 850 W (three runs each of 5 min) with sections being immersed in a solution of 0.01 M citric acid (pH 6.1). Slides were then immersed in a solution of 0.3% H_2O_2 for 10 min at room temperature to quench endogenous peroxidase activity, rinsed with PBS; nonspecific sites were blocked in 5% BSA in PBS. Further steps included utilization of a biotin-streptavidin detection method (Vector Laboratories), using the following primary antibodies: MCA 2311 (AbD Serotec) anti-porcine CD163 MoAb, anti-human CD79 MoAb (DakoCytomation), and a rabbit polyclonal anti-human CD3 (DakoCytomation). Immune reactions were visualized by 3,3′-diaminobenzidine chromogen solution (Dako). Sections from the tonsil served as positive controls.

TEM Analysis. For TEM analysis, the collected urine was diluted five times and deposited on a carbon-coated copper TEM grid (Formvar-Carbon film on 300 square mesh copper grids from Electron Microscopy Sciences) and dried. Alternatively, the urine was dialyzed against deionized water for 1 d using Spectra/Por molecular weight cutoff 12,000–14,000 Da, diluted 2.5 times with deionized water, and deposited on TEM grids. TEM was performed on a Hitachi 600 microscope with an accelerating voltage of 75 kV.

Ultrasound Image Analysis. For ultrasonography analysis, we applied a method previously used (1). The ultrasound signal was calculated using Adobe Photoshop CS5 (Adobe Systems). This program had been previously used for ultrasound image analysis (2–4). Ultrasound signal is reported in 8-bit gray scale intensity from 0 to 255 shades of gray recordable. For all ultrasound images displayed, the areas of interest were selected; comparison between samples was performed on the same areas in terms of number of pixel. For the in vivo experiment, ultrasound signal calculation was not performed because of the normal small movement of living pigs, even under anesthesia.

Statistical Analysis. Statistical analyses on three different and independent incubations for each experiment were performed using Student *t* test for paired data. Data indicated with an asterisk were considered statistically significant (P value < 0.05). Data are presented as mean ± SD ($n = 3$). Ex vivo investigation ultrasound signal was calculated on measurements based on experiments performed in three different livers and three hearts from healthy pigs. For in vivo data, biocompatibility results were derived from three pigs treated with functionalized MWCNTs.

1. Martinez AW, Phillips ST, Whitesides GM (2008) Three-dimensional microfluidic devices fabricated in layered paper and tape. *Proc Natl Acad Sci USA* 105:19606–19611.
2. Liu P, Gao YH, Tan KB, Liu Z, Zuo S (2004) Grey scale enhancement of rabbit liver and kidney by intravenous injection of a new lipid-coated ultrasound contrast agent. *World J Gastroenterol* 10:2369–2372.
3. Lassau N, et al. (2006) Gastrointestinal stromal tumors treated with imatinib: Monitoring response with contrast-enhanced sonography. *AJR Am J Roentgenol* 187:1267–1273.
4. Iwamoto T, Shinozaki K, Kiuchi A, Umahara T, Takasaki M (2003) Evaluation of B-mode ultrasonographic images of carotid lesions by computer analysis as compared with visual assessment. *J Stroke Cerebrovasc Dis* 12:59–65.

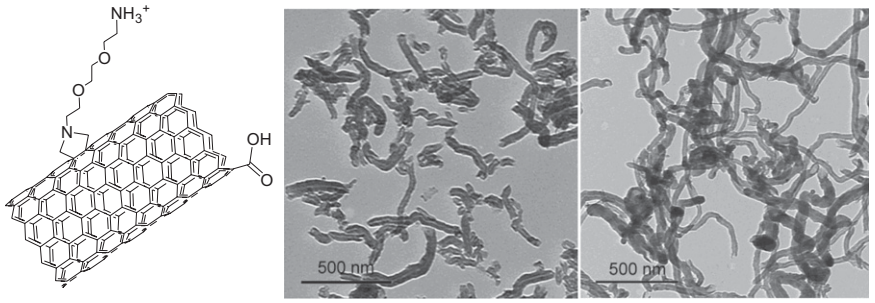


Fig. S1. Molecular structure (*Left*) and TEM of the ox-MWCNT-NH_3^+ (*Center*), and TEM of pristine MWCNTs (*Right*).

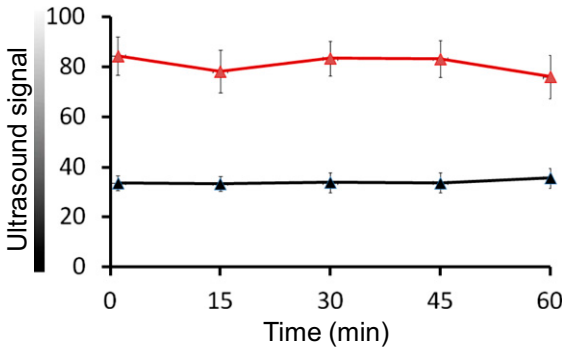


Fig. S2. Long-lasting ultrasound contrast of functionalized MWCNTs in vitro. The graph shows the ultrasound signal recorded for water, used as negative control (black line) and ox-MWCNT-NH_3^+ (red line) (1,000 μg/mL) aliquoted in a 96-well plate. Echoes were recorded after 1, 10, 15, 30, and 60 min of ultrasound irradiation. Ultrasound signal in the *y* axis dimension is expressed in gray shade value based on 8-bit scale intensity from 0 to 255. The error bars represent SD.

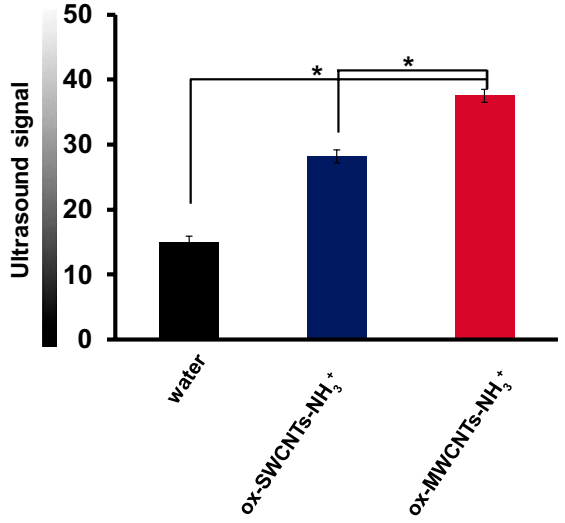


Fig. S3. Comparison between single-walled CNTs (SWCNTs) and MWCNTs. Ultrasound signal recorded from water, SWCNTs, and MWCNTs both functionalized by oxidation and further functionalized by 1,3-dipolar cycloaddition of azomethine ylides (ox-SWCNT-NH_3^+, ox-MWCNT-NH_3^+). Data were collected in a 96-well plate. CNTs were used at a concentration of 1,000 μg/mL. Signal is reported in 8-bit gray scale intensity from 0 to 255 shades of gray recordable. Intensity was calculated on a base of three experiments. The error bars represent SD ($n = 3$); *$P < 0.05$; analyzed pixel = 15,664.

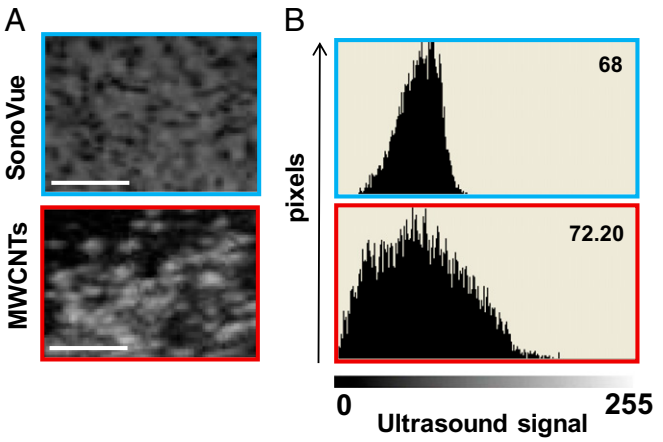


Fig. S4. Comparison between SonoVue and functionalized MWCNTs. (*A*) Ultrasonography images of sulfur hexafluoride (1 mL; 5,000 μg/mL) and MWCNTs (1 mL; 1,000 μg/mL) in 100 mL of water. (Scale bar, 2.5 mm.) (*B*) Ultrasound signal recorded for SonoVue (*Upper*) and functionalized MWCNTs (*Lower*) (analyzed pixel = 7,957). Ultrasound signal in the *y* axis dimension is expressed in gray scale value based on 8-bit scale intensity from 0 to 255; mean values for both samples are reported. Data are representative of one experiment of three.

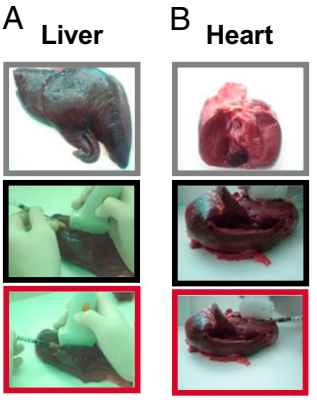


Fig. S5. Functionalized MWCNTs ex vivo in liver and heart from pigs. Images of liver (*A*) and heart (*B*) from a healthy pig before echography investigation. Insertion of a standard U-100 insulin syringe with 300 μL of water and 300 μL of functionalized MWCNTs at 1,000 μg/mL (black and red rectangles). Images are representative of three investigations.

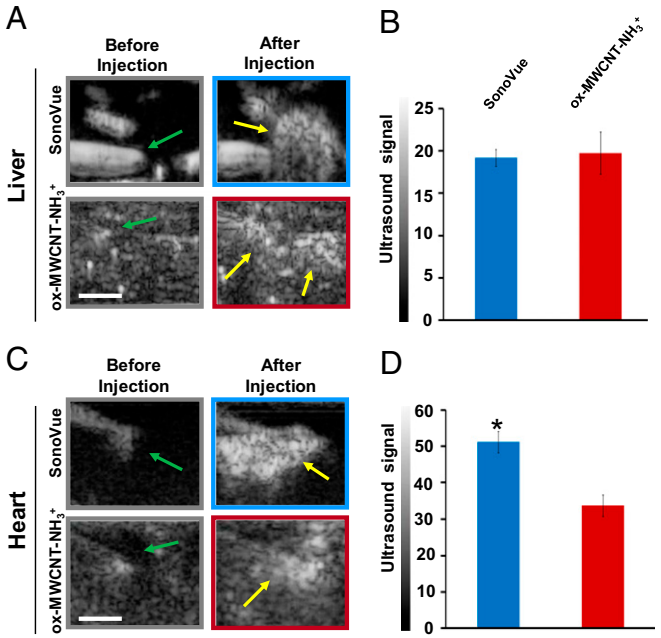


Fig. S6. Comparison between SonoVue and functionalized MWCNTs ex vivo in liver and heart from pigs. (*A* and *C*) Ultrasound images of liver and heart. In light gray and blue rectangles (*Upper*) are images before and after injection of 500 μL SonoVue; in light gray and red rectangles (*Lower*) are images before and after injection of 500 μL of functionalized MWCNTs at 1,000 μg/mL concentration. Green arrows indicate the tip of syringe before injection. Yellow arrows indicate the ultrasound signal after injections. (Scale bars, 5 mm.) (*B* and *D*) Signal analysis in liver and heart after the echogenicity subtraction of images before injection, for SonoVue and MWCNTs (*$P < 0.05$, $n = 3$) (analyzed pixel: liver = 34,578, heart = 26,026). Ultrasound images are representative data of three investigations.

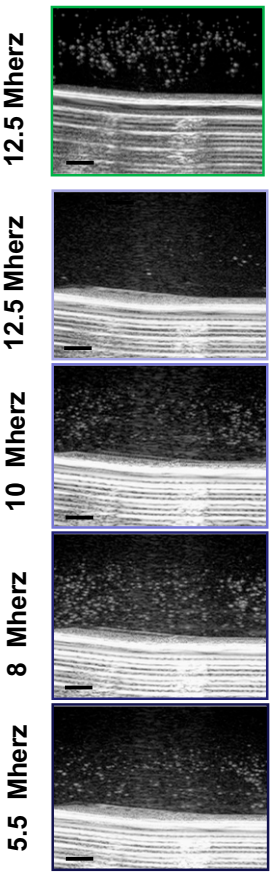


Fig. S7. Bladder phantom detection of functionalized MWCNTs at different frequencies. A bladder phantom was built specifically to assess the echogenic property of MWCNTs at different wave frequencies in megahertz. The phantom was constituted of a Plexiglas reservoir with a diameter of 10 cm and a vertical height of 12 cm, able to hold ~1,000 mL. We used water as a solution because it gives the same ultrasound signal of urine. The bladder phantom was filled with 250 mL, the amount of two glasses of water, at 37 °C. MWCNTs (2 mL at 1,000 μg/mL) were injected and an ultrasound image recorded at 12.5 MHz in tissue harmonic imaging (THI) modality (top green rectangle); all of the other images from top to bottom were captured without THI at 12.5, 10, 8, 5.5 MHz, respectively, depth 41 mm, gain 130. (Scale bars, 5 mm.)

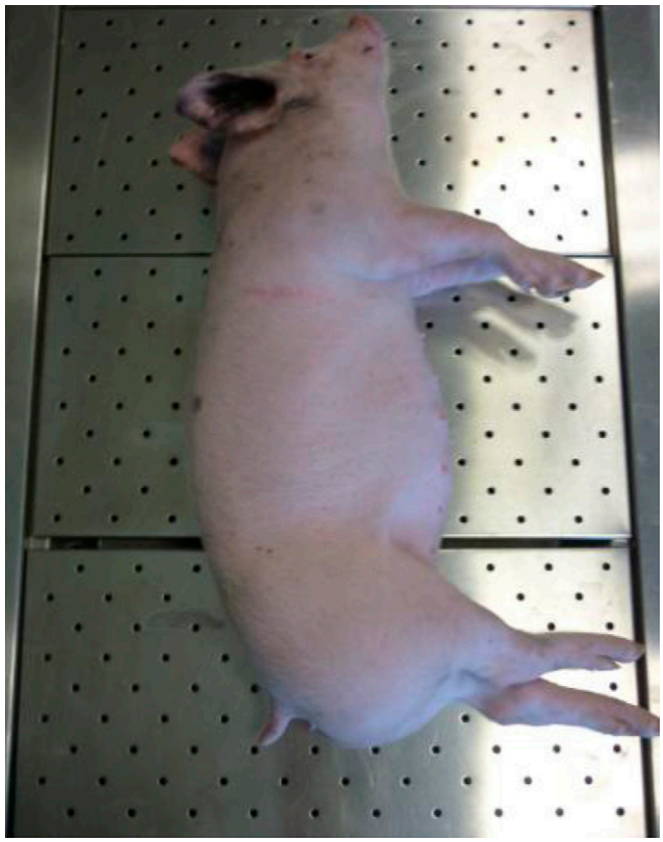


Fig. S8. Anesthetized pig. A representative 8-wk-old female healthy pig, 15 kg, under azaperone treatment (1 mL/20 kg) (n = 3).

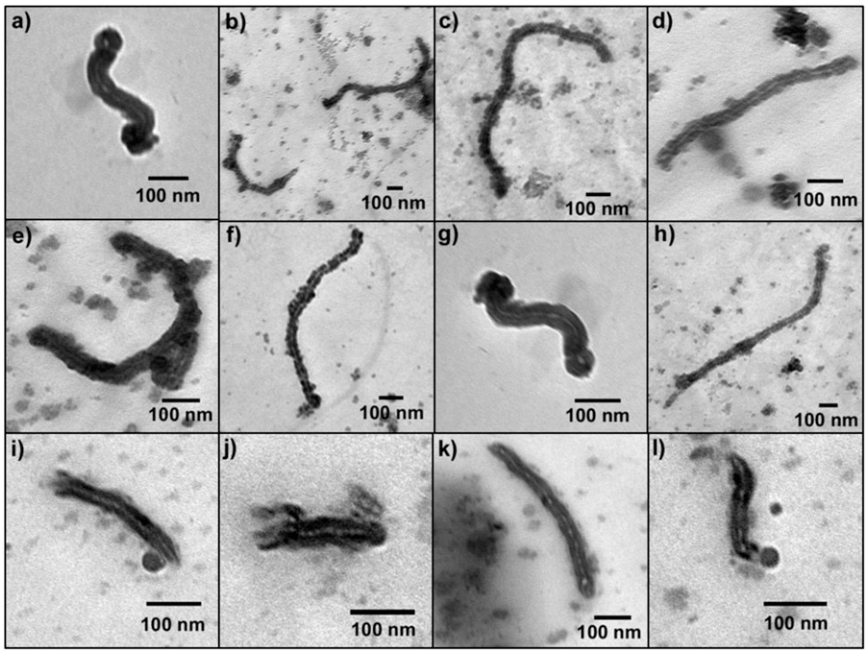


Fig. S9. TEM images of functionalized MWCNTs in pig urine. (*A–H*) Images were taken from grids prepared by deposition of diluted (2.5 times) and dialyzed urine against deionized water. (*I–L*) Images were taken from grids prepared by direct deposition of diluted urine (five times).

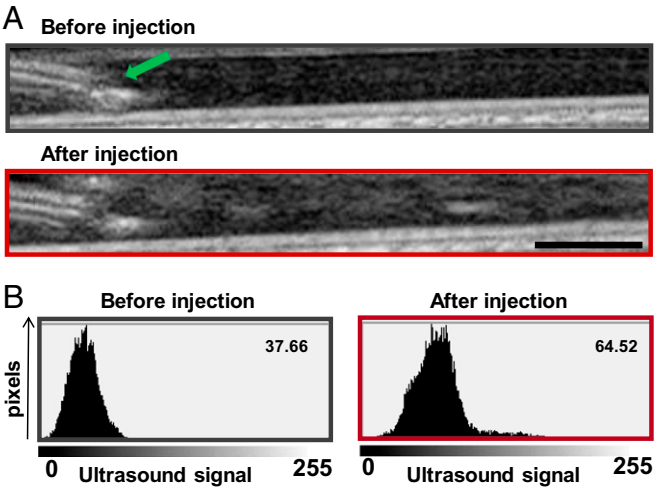


Fig. S10. Ultrasonography of MWCNTs in total blood ex vivo. (*A*) Ultrasonography images of whole blood (9 mL in a tank of 2.5 × 6 cm) before and after injection of MWCNTs (0.5 mL, 1,000 μg/mL). Green arrow indicates the syringe tip. Ultrasound images are representative data of three investigations. (Scale bar, 2.5 mm.) (*B*) Signal analysis before injection (gray rectangle) and after injection (red rectangle) (n = 3) (analyzed pixel = 15,762). Mean values is reported up right in the rectangles (37.66 ± 4.03; 64.52 ± 3.72).

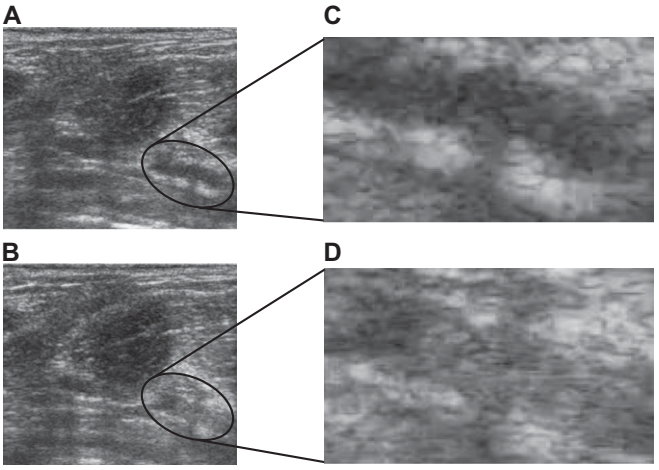


Fig. S11. Snapshots from Movie S2. Images are recorded before (*A*) and during (*B*) MWCNT injection (1,000 μ/mL, 5 mL). Black circles indicate the area of injection in the anterior vena cava. (*C* and *D*) Magnifications of *A* and *B* circles, respectively. (Magnification: 4×.)

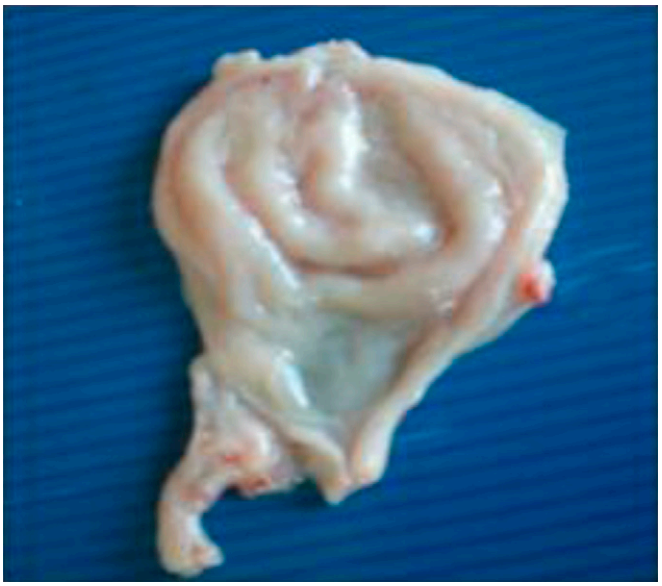


Fig. S12. Autopsy of the experimental pigs. A representative image of a pig bladder after 7 d from MWCNT ultrasonography.

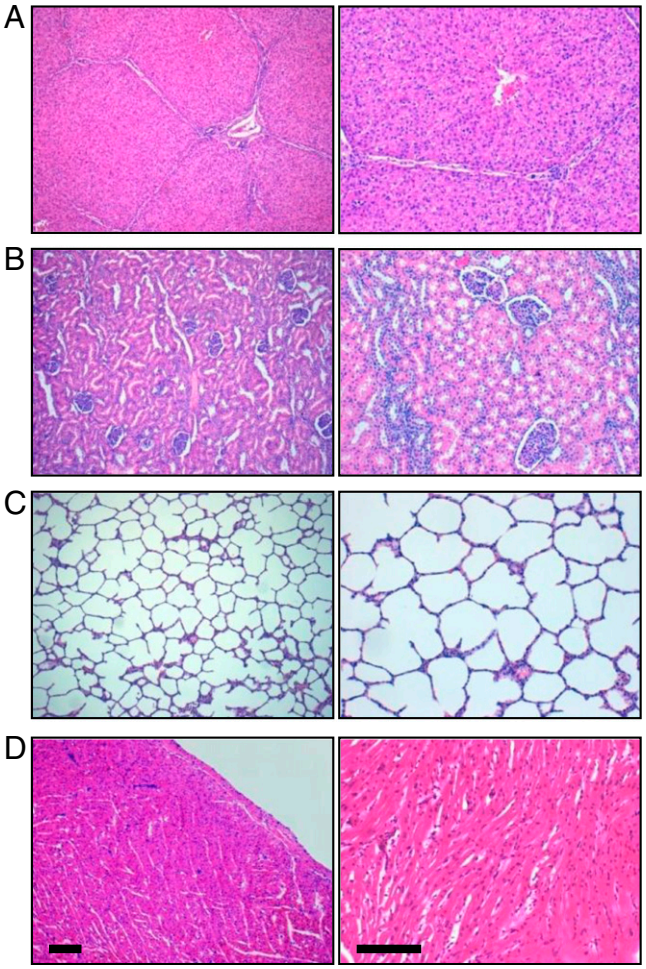
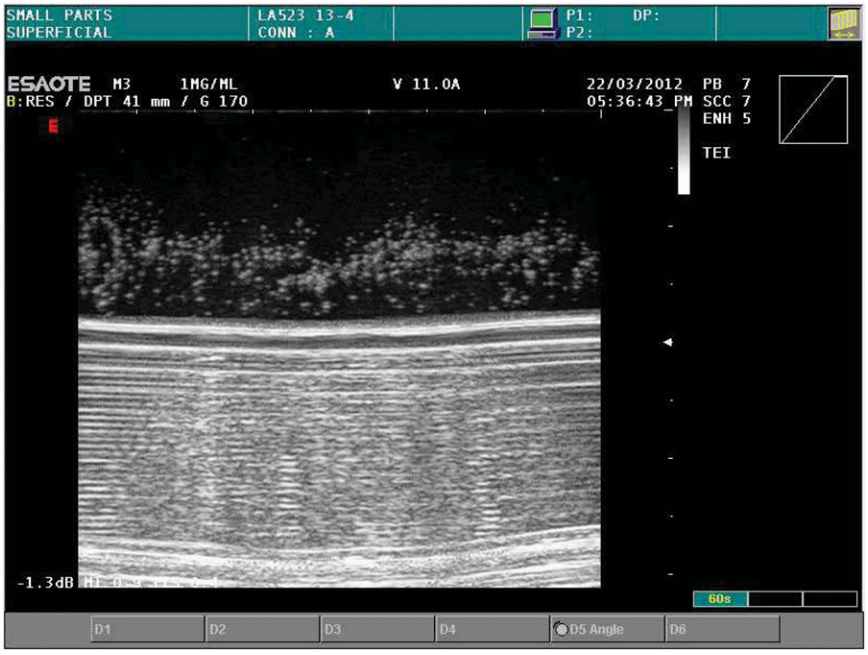


Fig. S13. Histology of liver, kidney (cortex), lung, and heart. Paraffin-embedded sections stained with H&E of liver (*A*), kidney (cortex) (*B*), lung (*C*), and heart (*D*) after 7 d from the MWCNT injection. The images are representative results of three investigations. (Scale bars, 100 μm.)

Table S1. Complete blood count and chemical profile of experimental pigs

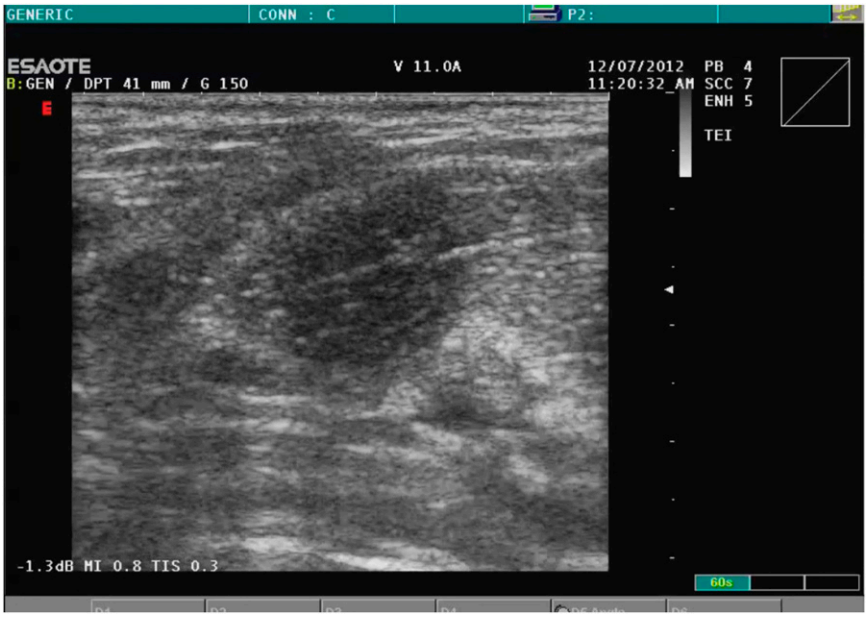
	Preultrasonography			Postultrasonography		
Blood count and chemical profile	N1	N2	N3	N1	N2	N3
Blood count (%)						
Neutrophils	33.7	28.8	25.9	43.4	26.0	24.2
Lymphocytes	55.7	61.9	66.8	51.4	67.3	66.1
Monocytes	5.4	7.0	4.8	2.2	4.5	7.4
Eosinophils	4.5	0.6	0.7	1.5	0.7	0.8
Basophils	0.3	1.3	1.2	0.2	0.8	0.9
Leukocytes	0.5	0.9	0.6	1.2	0.7	0.5
Chemical profile						
Total bilirubin(mg/dL)	0.27	0.3	0.3	0.15	0.2	0.2
Total protein(g/dL)	5.1	5.4	5.1	4.9	4.8	5.0
Albumin (g/dL)	2.1	2.2	2.1	2.0	1.8	1.9
Urea nitrose (mg/dL)	18	25	31	18	26	23
Creatinine (mg/dL)	0.8	1.3	0.9	1.1	1.0	1.0
Alkaline phosphatase (U/dL)	200	234	287	199	224	300
Alanine aminotransferase (U/L)	63	60	57	64	67	57
Aspartate (U/L)	91	58	36	64	77	37

Values are reported for preultrasound investigation and after 7 d from MWCNT administration (n = 3, N1 and 2 indicate the pigs treated intravesically and N3 indicates the pig injected with MWCNTs intravenously).



Movie S1. Functionalized MWCNTs injected in the bladder phantom. ox-MWCNT-NH_3^+ were injected in the bladder sample at a concentration of 1 mg/mL in water and detected at 12.5 MHz in THI modality.

Movie S1



Movie S2. Functionalized MWCNTs injected in the anterior vena cava of the pig. ox-MWCNT-NH_3^+ were administered in vivo by intravenous injection into the anterior vena cava of the pig. The movie was recorded using a 7.5–12 MHz linear probe in THI modality.

Movie S2