Supporting information

Size-dependent therapeutic efficiency of ^{223}Ra-labeled calcium carbonate carriers for internal radionuclide therapy of breast cancer

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1. Materials

1.1. For $CaCO_3$ MPs synthesis

Calcium chloride dihydrate ($CaCl_2 \times 2H_2O$, M_W = 147.01) and anhydrous sodium carbonate (Na_2CO_3, M_W = 105.99) were obtained from Sigma-Aldrich (Germany) and used without further purification. Purified water with a specific resistivity higher than 18.2 MΩ cm^{-1} was used from a three-stage Milli-Q Plus 185 purification system.

1.2. For $CaCO_3$ NPs synthesis

Calcium chloride dihydrate ($CaCl_2 \times 2H_2O$, M_W = 147.01), anhydrous sodium carbonate (Na_2CO_3, M_W = 105.99), and poly (acrylic acid) (PAA, № 32667) were obtained from Sigma-Aldrich (Germany) and used without further purification. Purified water with a specific resistivity higher than 18.2 MΩ cm^{-1} was used from a three-stage Milli-Q Plus 185 purification system.

1.3. For carrier modifications

Sulfo-cyanine 5 NHS ester (Cy5, M_W = 777.95) was obtained from Lumiprobe (Germany). Bovine serum albumin (BSA, M_W = 66 kDa) was obtained from Sigma-Aldrich (Germany) and used without further purification.

1.4. For $CaCO_3$ MPs radiolabeling

Bovine serum albumin (BSA, M_W = 66 kDa) was obtained from Sigma-Aldrich (Germany) and used without further purification.

1.5. For $CaCO_3$ NPs radiolabeling

Barium chloride dihydrate ($BaCl_2 \times 2H_2O$ M_W = 244.26) and sodium sulfate (Na_2SO_4, M_W = 142.04) were obtained from Sigma-Aldrich (Germany) and used without further purification.

1.6. For in vitro experiments

Roswell Park Memorial Institute medium (RPMI-1640), penicillin, and streptomycin (P/S) were obtained from Biolot (Russia). Fetal bovine serum (FBS) was obtained from HyClone (USA). Phosphate-buffered saline (PBS) was purchased from Lonza (Switzerland). Trypsin-EDTA solution was purchased from Capricorn Scientific (Germany). Rhodamine B (RhB, M_w = 479.01), AlexaFluor 488 (AF488, M_w = 1252.44), and Propidium Iodide (PI, M_w = 668.39) were purchased from Sigma-Aldrich (Germany). A kit for detecting dead cells with 7-AAD was purchased from BioLegend (USA). Ficoll solution was purchased from Biolot (Russia).

1.7. For in vivo experiments

BALB/c mice 3-4 weeks old were used. A sterile syringe with a removable 29 G needle was purchased from Vogt Medical (Germany). Isoflurane was obtained from Baxter (USA). Zolazepam hydrochloride was obtained from Zoletil (France). Xylazine hydrochloride was obtained from Bioveta (Czech Republic). Phosphate buffered saline was purchased from Sigma-Aldrich (Germany).

1.8. For histological analysis

Formaldehyde, paraffin, 1% hydrochloric acid solution, methanol, potassium hexacyanoferrate (II) trihydrate, and PBS were obtained from Thermo Fisher Scientific (USA). Ehrlich's hematoxylin was purchased from Labiko (Russia). Eosin Y (M_W = 647.89) was obtained from Sigma-Aldrich (Germany). Glycergel mounting medium was obtained from Agilent Dako (USA). PI (M_W = 668.39) was obtained from Sigma-Aldrich (Germany).

2. Synthesis of $CaCO_3$ carriers

2.1. $CaCO_3$ MPs

A co-precipitation approach was used to obtain $CaCO_3$ MPs. For this, 2.5 mL of Milli-Q water, 615 μL of $CaCl_2$ aqueous solution (1 M), and 615 μL of Na_2CO_3 aqueous solution (1 M) were stirred together at room temperature for 30 sec. The resulting suspension was washed three times with Milli-Q water and purified by

centrifugation for 1 min at 4000 rpm after the reaction. Finally, the pellets were obtained and stored for further investigations.

2.2. $CaCO_3$ NPs

A co-precipitation approach was used to obtain $CaCO_3$ NPs. For this, 1 mL of $CaCl_2$ aqueous solution (0.1 M) was first added separately to 1 mL of PAA aqueous solution (3.0 mg/mL) and stirred at room temperature for 1 h. Following that, 1 mL of Na_2CO_3 aqueous solution (0.1 M) was added to the resulting mixture and left for 1 h under the same conditions. The resulting suspension was washed multiple times with ethanol and purified by centrifugation for 3 min at 13000 rpm after the reaction. Finally, the pellets were obtained and stored for further investigations.

3. $CaCO_3$ carriers characterization

3.1. Scanning electron microscopy (SEM)

The developed carriers were characterized by scanning electron microscopy (SEM), performed on a Quanta 200, FEI (Netherlands) with an accelerating voltage of 10 kV and ZEISS Merlin® FE-SEM (Germany) with an accelerating voltage of 30 kV. The day before measurements, 5 μL of samples dispersed in water were placed on a coverslip and left to dry overnight. The next day, the dry samples were imaged with SEM.

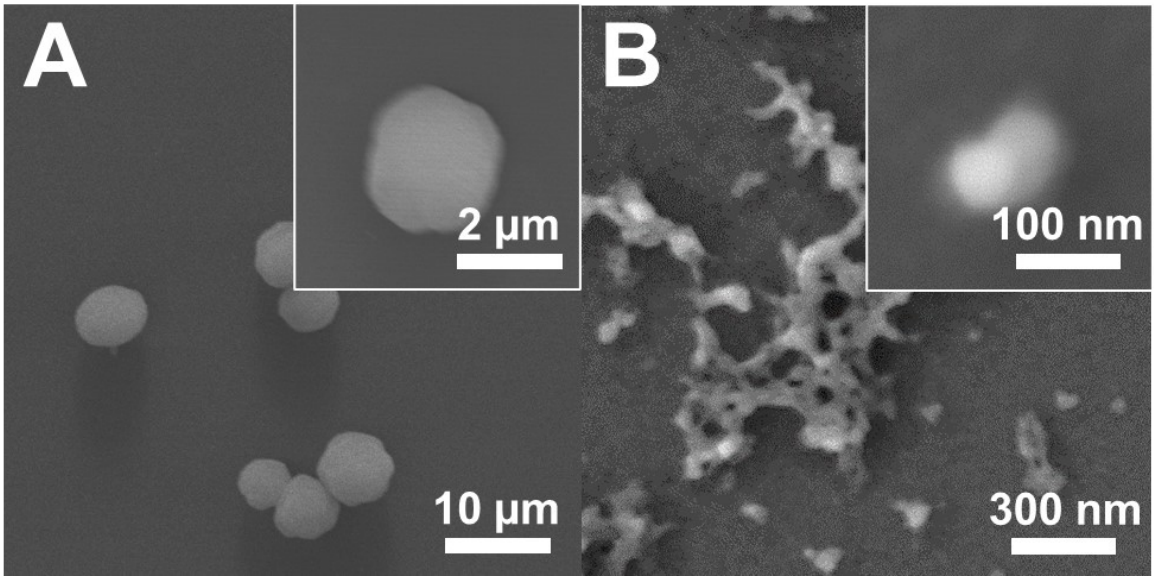

Figure S1. SEM images of (A) $CaCO_3$ MPs and (B) $CaCO_3$ NPs.

3.2. Transmission electron microscopy (TEM)

$CaCO_3$ carriers were visualized using transmission electron microscopy (TEM). To obtain TEM images, 5 μL of diluted samples were dropped on the top of a copper grid coated with a carbon layer, and the samples were imaged with a TEM JEOL JEM-1011 (Japan) and TEM Zeiss Libra 200 FE (Germany).

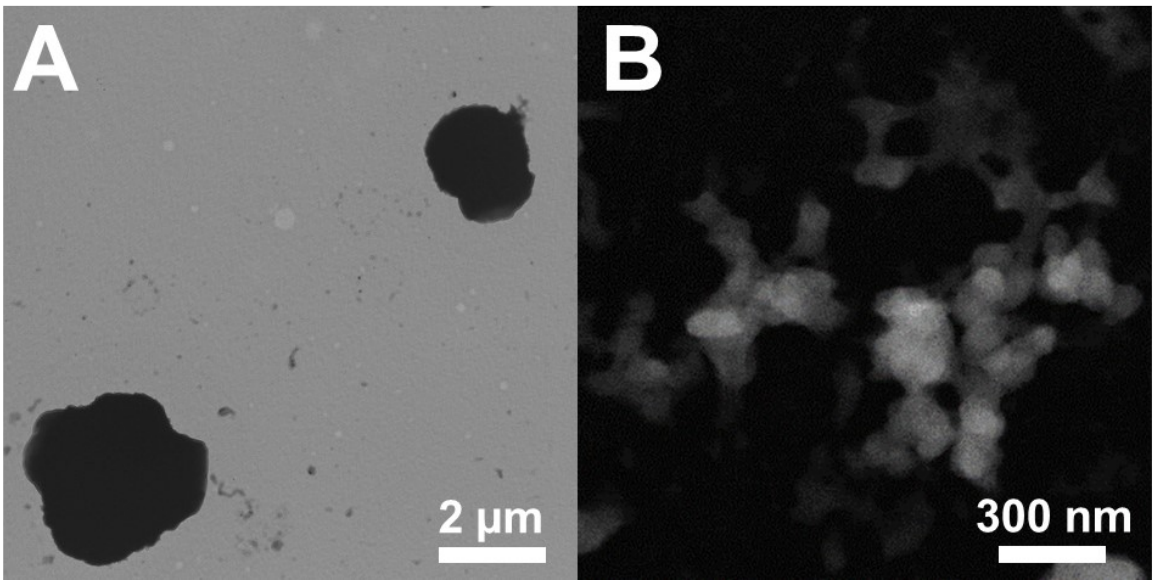

Figure S2. TEM images of (A) $CaCO_3$ MPs and (B) $CaCO_3$ NPs.

3.3. Dynamic laser scattering (DLS)

The hydrodynamic diameters (D_h) of the $CaCO_3$ carriers were measured using Malvern Instruments, model Zetasizer NanoZS.

3.4. Powder X-Ray Diffraction (PXRD)

The crystalline phase of the $CaCO_3$ MPs and NPs were studied using PXRD analysis. Diffraction patterns of the $CaCO_3$ carriers were recorded on a Shimadzu 7000-maxima X-ray diffractometer with a 2 kW characteristic CuKα (Kα1 $\lambda = 1.54059$ Å, angular range $2\theta = 5°–60°$) X-ray radiation source and a Bragg-Brentano goniometer geometry. The angular resolution during the analysis was 0.01 degree at a scanning speed of 1 degrees/min.

3.5. Fourier-transform infrared (FTIR) spectroscopy

Fourier transform infrared spectroscopy peaks were obtained with a Shimadzu IRTracer-100 combined with a Specac The Quest Single Reflection ATR Accessory, scans were made in the range from 400 to 4000 cm^{-1}, with a resolution of 1 cm^{-1}.

3.6. Energy Dispersive X-Ray Analysis (EDX)

Energy-dispersive elemental analysis was performed using a ZEISS Supra 55-VP (Germany) at an acceleration voltage of 25 kV to analyze the elemental composition of the particles.

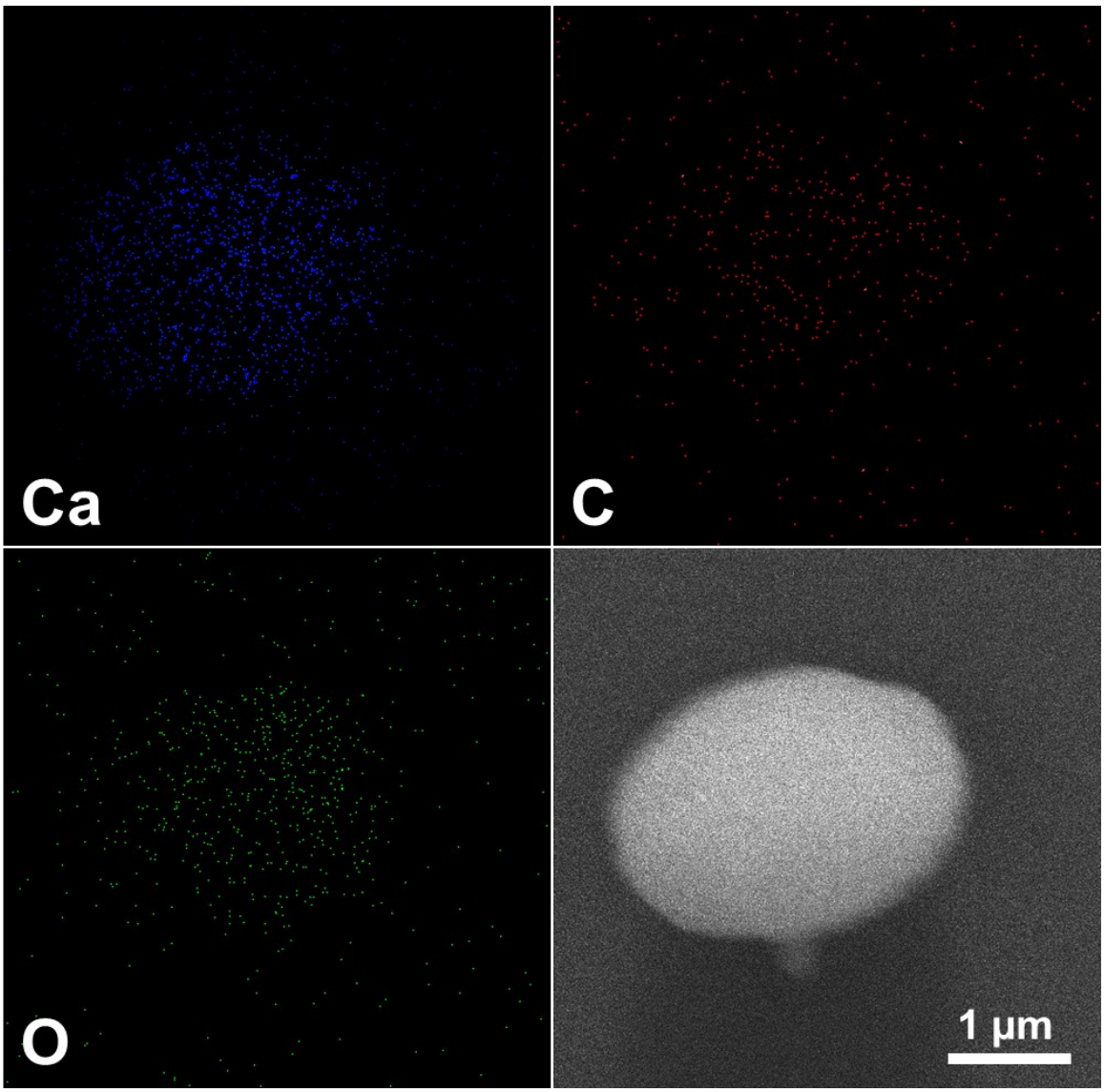

Figure S3. Energy-dispersive X-ray spectrometry (EDS) elemental mapping of $CaCO_3$ MPs (Ca, C, O).

4. *In vitro* stability of $CaCO_3$ MPs and NPs

We analyzed the hydrodynamic diameters (D_h) of the carriers as a part of the *in vitro* stability assay. For this, the $CaCO_3$ carriers were incubated either in 0.9% NaCl (pH 6.5), PBS (pH 7.4), or 0.1% serum (pH 7.4) for 24 h. The change in the hydrodynamic diameters was analyzed at several moments of time (0, 1, 5, 7 and 24 h). The samples were then measured using a Zetasizer Nano ZS90 (Malvern, USA).

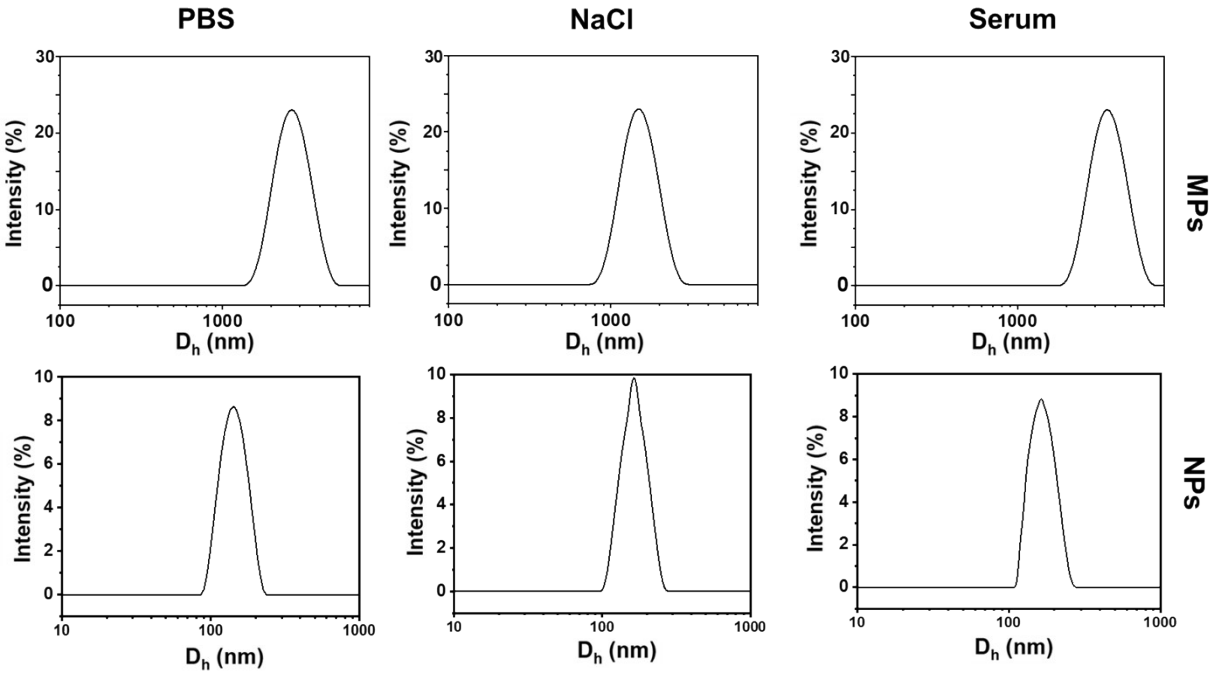

Figure S4. Hydrodynamic diameters (D_h) determined for $CaCO_3$ MPs and NPs in different media (PBS, 0.9% NaCl, and 0.1% human serum).

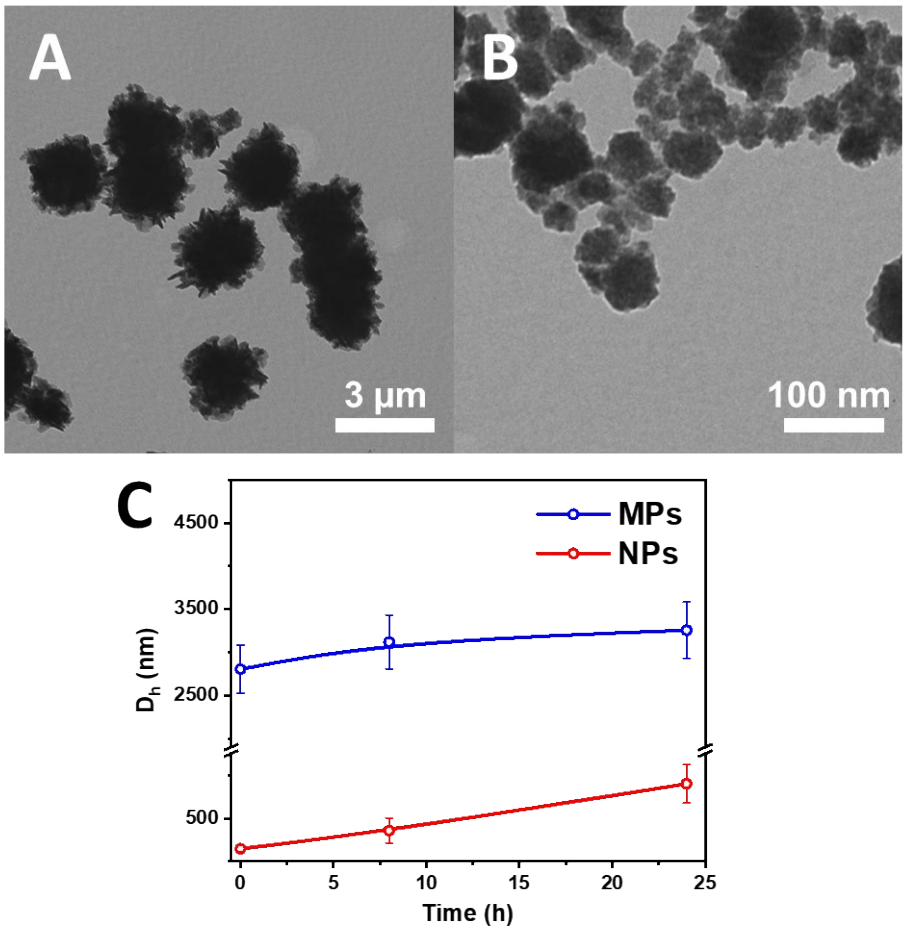

Figure S5. TEM images of $CaCO_3$ (A) MPs+BSA and (B) NPs after 24 h incubation in acidic condition (pH 1.5) with corresponding (C) colloidal stability. TEM samples were prepared without wash from buffer salts.

5. Hemolysis assay

The impact of the $CaCO_3$ carriers on the destruction of human erythrocytes was studied using the hemolysis assay[1]. For this, 1 mL of 3.8% sodium citrate solution was added to 10 mL of donor blood to prevent blood clotting. Further, 5 mL of blood was added dropwise to a tube with 5 mL of Ficoll and then centrifuged for 20 min at 312 g. The sedimented erythrocytes were collected from the bottom of the tube and washed in a PBS (pH 7.4) three times. Then, the human erythrocytes were diluted to a final concentration (1%, v/v) in PBS. At the same time, the $CaCO_3$ carriers were resuspended in 1×PBS solution and further added to human erythrocytes at different concentrations (50, 100, 200, 400, 600, 800, and 1000 μg/mL) to a final volume of 1 mL. After the $CaCO_3$ carriers were added, the reaction

mixture was incubated for 2 h, and then centrifuged for 5 min at 312 g. The hemoglobin concentration in the supernatant was quantitatively assessed using a spectrophotometer at an absorption wavelength of 540 nm. As a positive control, an erythrocyte lysis buffer was added to the human erythrocytes, and PBS was added as a negative control. The percentage of hemolysis was calculated using the formula:

$$Hemolysis\ rate\ (\%) = \frac{optical\ density\ (sample)\ -\ optical\ density\ (negative\ control)}{optical\ density\ (positive\ control)\ -\ optical\ density\ (negative\ control)} \times 100\%$$

6. Cy5-fluorescent labeling of $CaCO_3$ carriers

6.1. Fluorescence labeling of bovine serum albumin (BSA)

HSA was fluorescently labeled with Cy5 dye using the method described previously[2]. Briefly, 144 mg of BSA was added to 40 mL of PBS (pH 7.4) to a final concentration of 3.85 mg/mL. Further, 10 μg of Cy5-dye was resuspended in 4.4 mL of DMSO. Afterwards, the dye mixture was mixed with BSA (the final BSA concentration was 7 mg/mL) and incubated for 24 h at 4 °C. Then, the unbound dye was removed with a dialysis procedure[3]. For this, the resulting solution was poured into a dialysis bag, which was then placed in a beaker and stirred for 3 d (500 rpm, 4 °C). The water with unreacted dye was changed every 24 h.

6.2. $CaCO_3$ MPs

To obtain Cy5-fluorescent labeled MPs, 2 mL of Milli-Q water, 615 μL of $CaCl_2$ aqueous solution (1 M), 615 μL of Na_2CO_3 aqueous solution (1 M), and 500 μL of Cy5-BSA complex were stirred together at room temperature for 30 sec. The resulting MPs fluorescently labeled with Cy5 were washed multiple times with Milli-Q water and purified by centrifugation for 1 min at 4000 rpm after the reaction. Finally, pellets were obtained and stored for further investigations.

6.3. $CaCO_3$ NPs

To obtain Cy5-fluorescent labeled NPs, 1 mL of $CaCl_2$ aqueous solution (0.1 M) was first added separately to 500 μL of Cy5-BSA complex and stirred at room temperature for 1 h. Afterwards, 1 mL of PAA aqueous solution (3.0 mg/mL) was

added and stirring was continued for 1 h. Following that, 1 mL Na_2CO_3 aqueous solution (0.1 M) was added to the resulting solution and left for 1 h under the same conditions. The resulting suspension was washed multiple times with ethanol and purified by centrifugation for 3 min at 13000 rpm after the reaction. Finally, pellets were obtained and stored for further investigations.

7. *In vitro* studies

7.1. Cell culture

Murine breast cancer line (4T1 cells) was obtained from the American Type Culture Collection. The cells were cultured in RPMI supplemented with 10 vol% FBS. The cell culture was maintained in a sterile humidified atmosphere containing 95% air and 5% CO_2 at 37 °C.

7.2. Cellular uptake

7.2.1. Confocal laser scanning microscopy (CLSM).

To visualize cellular uptake of the $CaCO_3$ carriers (MPs and NPs) by 4T1 breast cancer cells, they were seeded into 48-well plates (1.0×10^4 per well). The next day, the Cy5-fluorescent labeled $CaCO_3$ carriers were added to the cells at different concentrations (200 μg/mL, 400 μg/mL, 800 μg/mL), and the cells were left overnight. Next day, the cell cytoskeletons were stained with phalloidin conjugated with AF488, and the cell nuclei were stained with PI. For this, cells were washed 2 times with PBS and permeabilized with PBS supplemented with 10 vol% formalin for 40 min. Afterwards, the cells were washed again with PBS, and 1 μM of AF 488 was added. The 4T1 cells were left for another 1 h in an incubator at 37 °C and 5% CO_2. After that, 20 μM of PI was added and cells were left incubating for another hour. Afterwards, the cells were washed again with PBS. Cellular uptake of the $CaCO_3$ carriers was visualized using a confocal laser scanning microscope (CLSM) Leica TCS SP8 (Germany). To visualize the cell cytoskeletons, an argon laser emitting at 488 nm was used. To visualize the cell nucleus a helium neon laser emitting at 543 nm was used. To visualize the Cy5-labeled $CaCO_3$ carriers, a helium

neon laser emitting at 633 nm was used. The confocal pinhole was set to 1 Airy unit, and the images were taken with an HC PL FLUOTAR 10x/0.30 PH2 Objective.

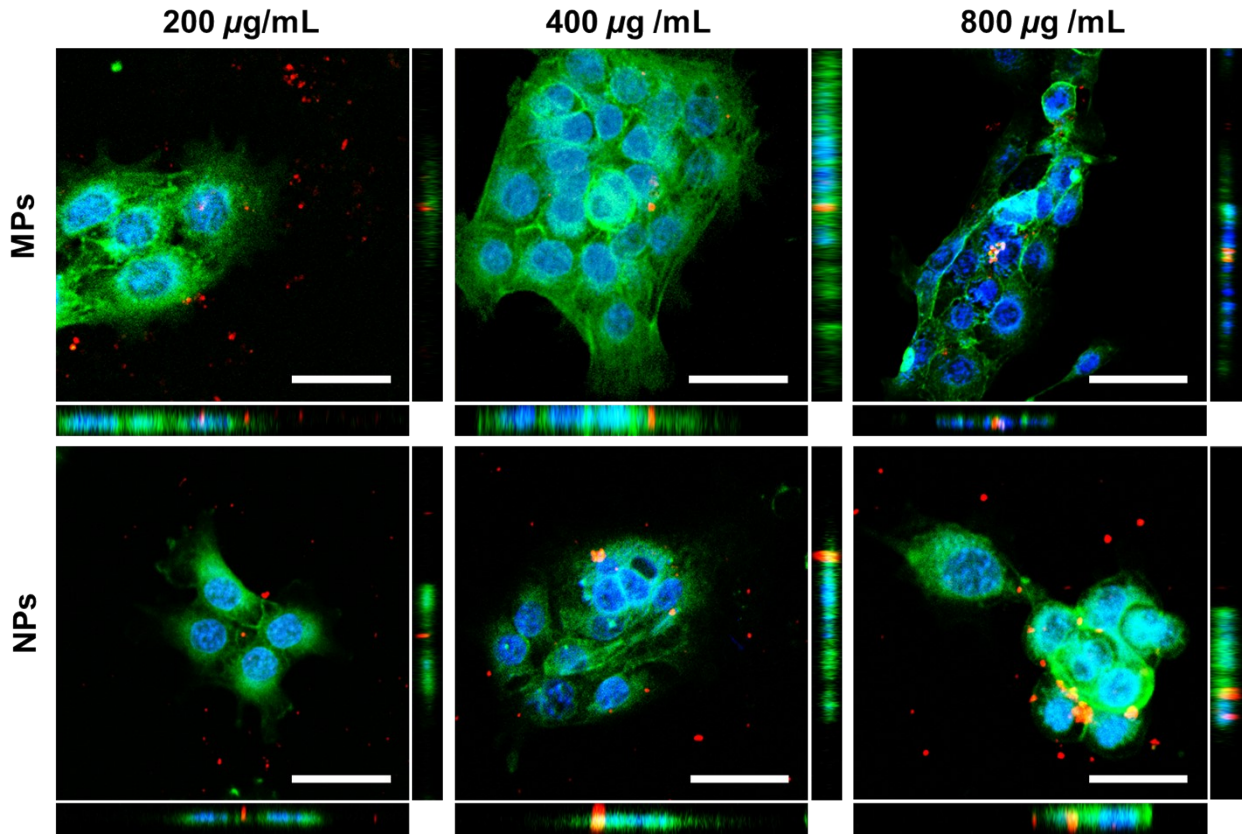

Figure S6. CLSM z-stack images of the internalization of $CaCO_3$ MPs and NPs in 4T1 cells at different concentrations of MPs and NPs (200, 400, 800 μg/mL). Scale bars correspond to 60 μm.

7.2.2. Flow cytometry

To further confirm the association of 4T1 cells with Cy5-labeled $CaCO_3$ carriers, flow cytometry analysis was performed. For this, the cells were seeded into 6-well plates (1.0×10^5 per well). The next day, Cy5-labeled $CaCO_3$ carriers (MPs and NPs) were added to the cells (to 2 mL of cell medium) at different concentrations (200, 400, 800 μg/mL), and the cells were left overnight. The next day, the cells were detached from the well, centrifuged (300 g for 5 min) and washed twice with PBS to remove non-internalized capsules. After that, the cells were diluted in 100 μL of PBS. The measurements were performed at a wavelength of 650 nm with a long-pass filter for Cy5 and analyzed using flow cytometry (FACS Aria, BD, USA).

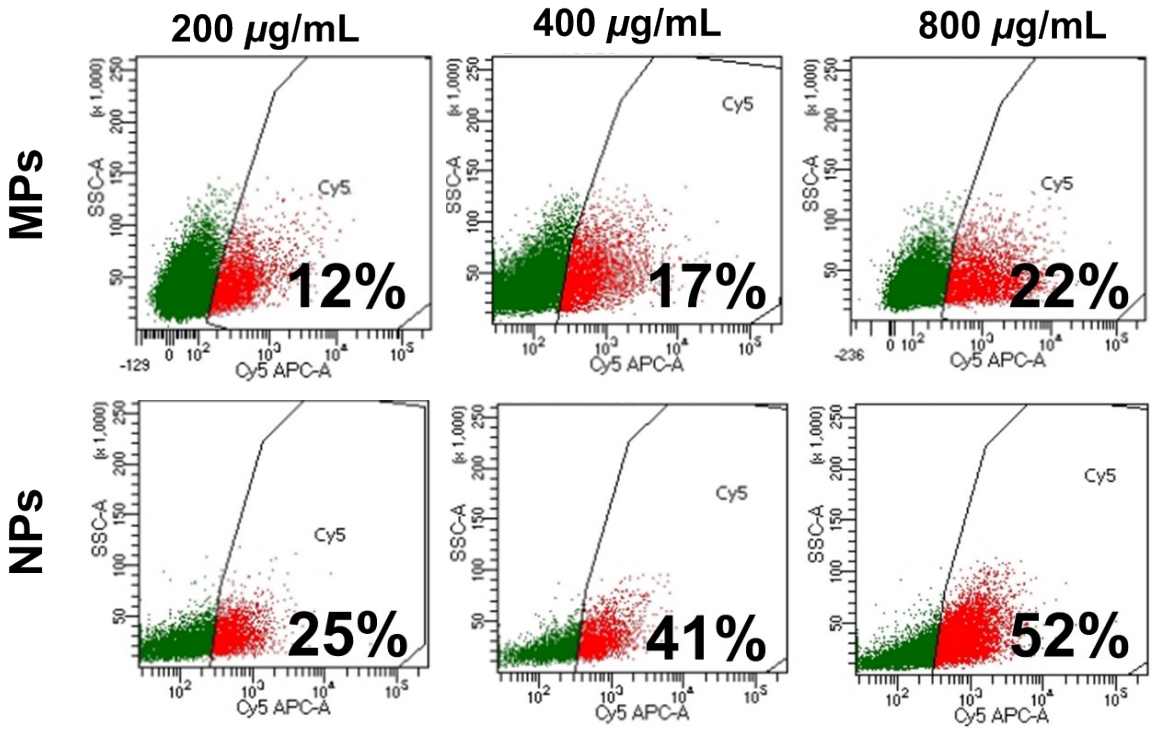

Figure S7. Flow cytometry assay of 4T1 cells treated with different concentrations of Cy5-MPs and NPs (200, 400, 800 μg/mL).

7.3. Toxicity studies

7.3.1. CLSM

To study the toxicity of the $CaCO_3$ carriers (MPs and NPs) at different concentrations (200, 400, 800 μg/mL), LIVE/DEAD assay was used. For this, 4T1 cells were seeded into a 24-well plate at 25 000 cells/well. On the same day, the samples were added to the cells. The next day, the 4T1 cells were stained with 0.2 μM Calcein AM (live staining) and 3 μM of PI (dead staining) for 30 min at 37 °C and 5% CO_2. Afterwards, the cells were observed under a confocal microscope (Leica TCS SP8, Germany). To visualize live cells (cells stained with Calcein AM), an argon laser emitting at 488 nm was used. To visualize dead cells (cell nuclei stained with PI), a helium neon laser emitting at 543 nm was used. The confocal pinhole was set to 1 Airy unit, and the images were taken with an HC PL FLUOTAR 10x/0.30 PH2 Objective.

7.3.2. Flow cytometry

To study the toxicity of $CaCO_3$ carriers (MPs and NPs) at different concentrations (200, 400, 800 μg/mL), we used flow cytometry (FACS Aria, BD, USA). For this, 4T1 cells were seeded into a 6-well plate at an amount of 1.0×10^5 cells/well. Next day, the $CaCO_3$ carriers were added to the cells (200, 400, 800 μg/mL). The next day, the cells were trypsinized with trypsin-EDTA solution and centrifuged for 4 min at 4000 rpm, then resuspended in PBS. Before the study, 1 μL of 7-AAD was added and incubated for 15 min in the dark at room temperature.

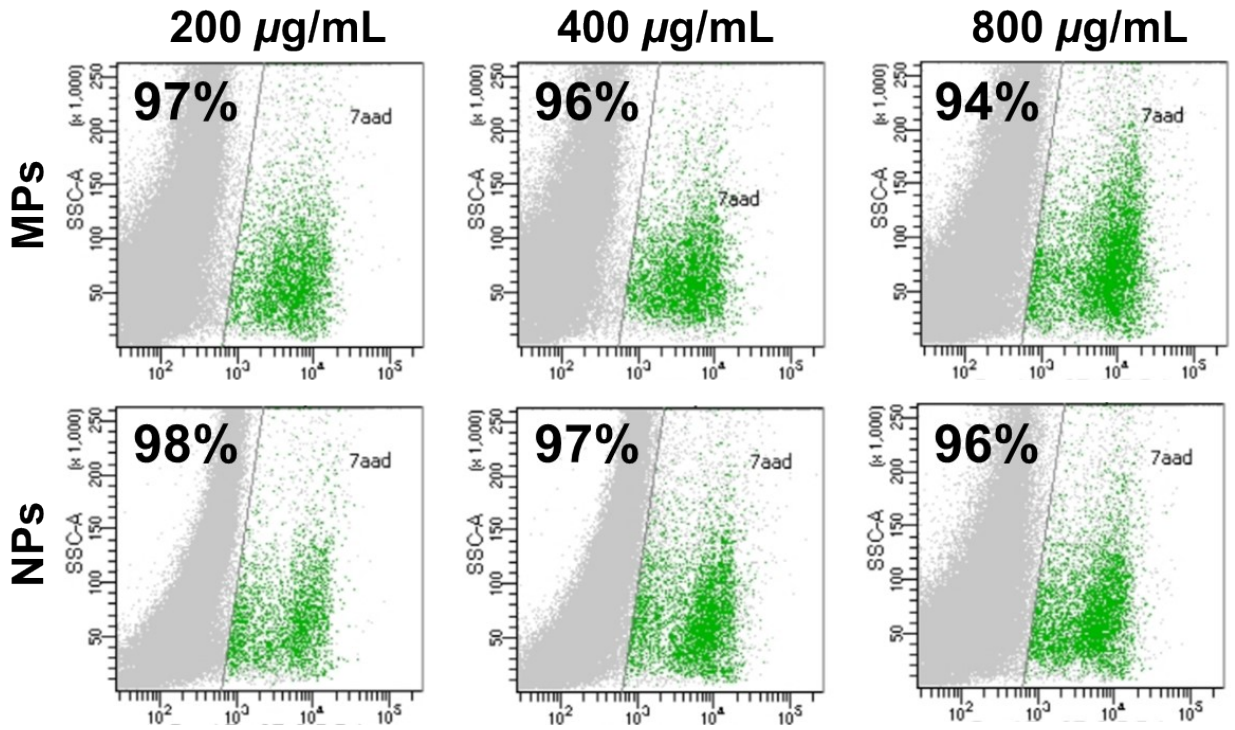

Figure S8. Flow cytometry assay of 4T1 cells treated with different concentrations of MPs and NPs (with % of alive cells).

7.4. Spheroid penetration efficiency

Spheroids (4T1, 3×10^3 cells per drop) were obtained using the method of hanging droplets cultured on cups. About 20×30 μL of cell droplets were distributed over the inverted lids of 3 cm Petri dishes, and the lids were carefully placed on cups that were pre-filled with 2 mL of PBS to ensure high humidity, and then incubated for 120 h at 37 °C, 5% CO_2. Every 48 h, the drops were washed with 30 mL of medium and further incubated in a fresh cell culture medium. After 120 h, the formed

spheroids were washed with PBS, 10 μL of RhB was added, and the spheroids were left to incubate for another 24 h. Afterwards, the spheroids were washed with PBS, and Cy5-labeled $CaCO_3$ carriers (MPs and NPs) were added at a concentration of 50 μg per spheroid. At different moments of time (1 h and 24 h), 20 μL of formaldehyde (10%) was added, and the spheroids were fixed for an hour at 4 °C. After the fixation, the spheroids were transferred to a PBS solution for subsequent CLSM examination.

7.3.1. CLSM

To study the penetration ability of $CaCO_3$ carriers, CLSM was used. To visualize RhB-spheroids, an argon laser emitting at 514 nm was used. To visualize the Cy5-labeled $CaCO_3$ carriers, a helium-neon laser emitting at 633 nm was used. The confocal pinhole was set to 1 Airy unit, and the images were taken with an HC PL FLUOTAR 10x/0.30 PH2 Objective.

8. Animals

For *in vivo* studies, healthy, immunocompetent mice of the BALB/c line (females, eight weeks old, 18-22 g, Rappolovo, Saint-Petersburg, Russia) were used. Mice were kept at room temperature 22-24 °C and humidity 40-60% in standard cages with unlimited access to food and water for at least 10 days before the experiments. To evaluate the results of all the experiments, mice were sacrificed by cervical dislocation, since this method does not cause chemical contamination of samples and organs for further research. All the animal studies have been approved by the local authorities and conducted in accordance with the Guidelines for the Placement and Care of Animals (European Convention for the Protection of Vertebrates Used for Experimental and Other Scientific Purposes).

9. Mice tumor model (4T1 breast cancer)

Breast cancer cell line was used as a cancer model. For this purpose, 4T1 cells were trypsinized in the exponential growth phase. Then, they were washed with PBS and resuspended in RPMI medium at a concentration of 1.0×10^7 cells/mL. After that,

BALB/c line mice (females, age eight weeks, 18-22 g, Rappolovo, St. Petersburg, Russia) were injected subcutaneously with a suspension of cells in the volume of 100 μL (0 d) into the hind limbs. After the infusion of tumor cells (7 d), the mice were examined, and animals with a sufficient tumor size (approximately 0.05 ± 0.01 cm^3) were used for further experiments.

10. Biodistribution studies

10.1. SPECT

Single-photon emission computed tomography/computed tomography (SPECT) imaging of mice injected with either ^{99m}Tc-MPs (10 MBq, 50 μL) and ^{99m}Tc-NPs (10 MBq, 50 μL) was performed to observe the $CaCO_3$ carriers' biodistribution.

The *in vivo* biodistribution experiments were performed under combined anesthesia with an intramuscular injection of zoletil solution (Virbac, France) at the rate of 6 mg of zolazepam hydrochloride per kilogram of animal body mass and 0.5 mL/kg of xylazine hydrochloride solution (Bioveta, a.s., Czech Republic). The ^{99m}Tc-MPs and ^{99m}Tc-NPs were redispersed in 200 μL of sterile PBS to obtain the required concentration of radioactivity (100 MBq per mL), and 50 μL of this solution was administered via 30-gauge needle intratumorally. Planar SPECT imaging was performed with a Discovery NM 630 (GE Healthcare) using a low-energy general-purpose high-resolution collimator (GE Healthcare). The data was collected at the 140 keV photopeak (^{99m}Tc), and the scan acquisition was started immediately after the dose administration (100 kilocounts for each animal on a 256×256 matrix, 1.28 zoom). SPECT image reconstruction was performed with a pixel-ordered subset expectation-maximization algorithm using GE Workstation (GE Healthcare). To visualize the anatomy of mice and localization of SPECT signals, digital outlines of mice were used for further data formation. The SPECT images were superimposed on the outlines and analyzed using an image editor with a merge function.

10.2. IVIS bioluminigraph

To confirm the biodistribution and retention of $CaCO_3$ carriers inside tumors, the Cy5-labeled $CaCO_3$ carriers were intratumorally injected to BALB/c mice carrying 4T1 tumor. At certain points in time, the mice were sacrificed, and then the main organs (the liver, spleen, lungs, kidneys, and heart) and tumors were removed. Then, the fluorescent signals were monitored at different moments of time after the injection (the 1st and 3rd d) in all organs using IVIS Lumina II (IVIS Lumina II, PerkinElmer Inc, USA) in epifluorescence mode with excitation at 675 nm and radiation at 720 nm. During further processing by the IVIS device, the received data were corrected by removing background noise. The region of interest (ROI) of each animal was detected by IVIS with the selected settings in a specific area.

11. Histological analysis

11.1. Preparation of the histological slices

Mice were euthanized for histological examinations. Next, biopsy samples were isolated from the studied tissues of organs (the heart, lungs, liver, kidneys, spleen, and lungs) and tumors. The tissue samples were soaked in PBS and transferred to a buffered formalin solution for no longer than 3 d. The samples were then dehydrated with an isopropanol solution on a Leica TP1020 Tissue Processor. After the dehydration, the tissue samples were embedded in paraffin blocks using a Microm EC350-1 unit (Thermo Scientific). The paraffin-embedded samples were cut into 2-4 μm sections using a HM 340E rotary microtome (Thermo Scientific) and placed on glass slides for further staining.

11.2. Fluorescent staining

Histological tissue sections on slides were washed in a solution of 1x PBS 3 times, transferred to a flask with a solution of PI (10 mg/mL), and then stained for 1 h. After staining, the sections were washed in a PBS solution 2 times. Then, the tissue sections were fixed with a Glycergel Mounting Medium and placed under a

coverslip. Further, the samples were visualized on CLSM (objective C Plan-Neofluar 40x/1.30 Oil DIC).

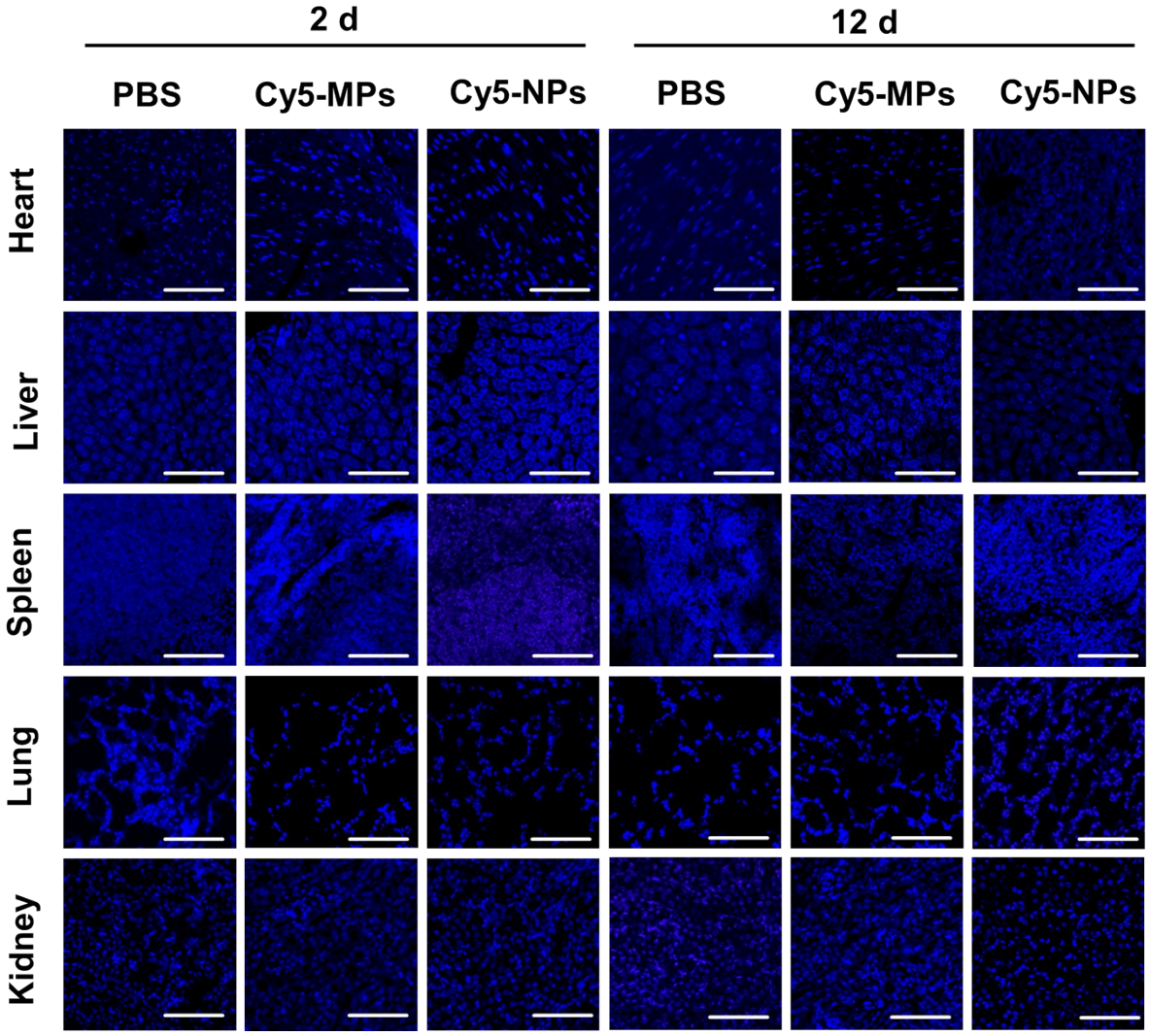

Figure S9. PI staining of different organs (the lungs, spleen, kidneys, liver, and heart) of 4T1 breast cancer-bearing mice on the 2nd and 12th d of treatment. Scale bars correspond to 100 μm.

12. Radiolabeling of MPs and NPs with ^{223}Ra

12.1. MPs radiolabeling with ^{223}Ra

$CaCO_3$ MPs were labeled using two methods: (i) co-precipitation and (ii) adsorption. For co-precipitation approach, 2 mL of Milli-Q water, 615 μL of $CaCl_2$ aqueous solution (1 M), 615 μL of Na_2CO_3 aqueous solution (1 M), and 500 μL of $^{223}RaCl_2$ solution (37 kBq) were mixed together and stirred for 30 sec (**Protocol 1)**. For adsorption, MPs were obtained as described in **§2.1**. Then, 500 μL of $^{223}RaCl_2$

solution was added to the MPs. The resulting suspension was mixed for 1 h (1000 rpm, 25 °C) (**Protocol 3)**. ^{223}Ra-labeled MPs were resuspended in 1 mL of BSA water solution (1 mg/mL) and mixed for 10 min (1000 rpm, 25 °C) to obtain a BSA layer on the surface of ^{223}Ra-labeled MPs (**Protocol 2-4**). The resulting ^{223}Ra-labeled MPs were washed twice with Milli-Q water (4000 rpm, 3 min) and resuspended in 1 mL of 0.9% NaCl solution.

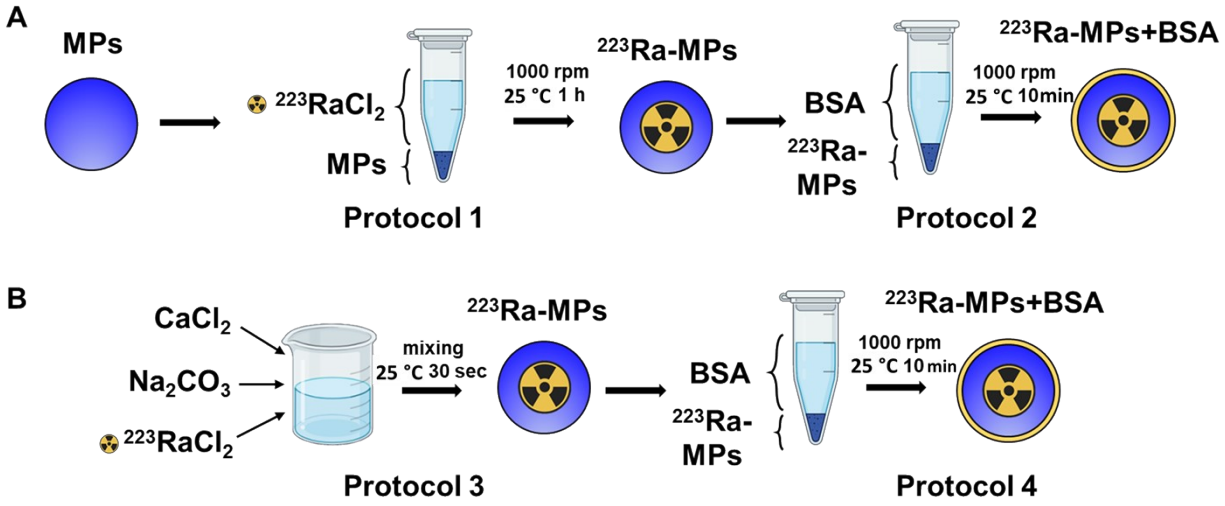

Figure S10. Schematic illustration of ^{223}Ra incorporation into the MPs (A) using co-precipitation (**Protocol 1-2**) and (B) adsorption (**Protocol 3-4**) approaches.

Table S1. Parameter variations for the improvement of MPs' radiolabeling efficiency and isotope retention.

	Radiolabeling method	**organic additive**	**inorganic additive**	**incubation time**	**Average RE (%)**
Protocol 1	co-precipitation	–	–	–	67%
Protocol 2	**co-precipitation**	**BSA**	–	–	**99%**
Protocol 3	adsorption	–	–	1 h	75%
Protocol 4	adsorption	BSA	–	1 h	83%

12.2. NPs radiolabeling with ^{223}Ra

Radiolabeling of $CaCO_3$ NPs was performed using two methods: (i) co-precipitation and (ii) adsorption. For co-precipitation approach, two solutions were prepared: (1) 500 μL of Na_2CO_3 aqueous solution (0.1 M) was added to 80 μL of $^{223}RaCl_2$ solution (37 kBq) and (2) 500 μL of $CaCl_2$ aqueous solution (0.1 M) was added to 500 μL of PAA water solution (3 mg/mL) and stirred separately at room temperature for 1 h. After that, solution (1) and solution (2) were mixed together and stirred at room temperature for another 1 h. The resulting suspension was washed multiple times with ethanol and resuspended in 1 mL of 0.9% NaCl solution (**Protocol 1**). For the adsorption approach, NPs were obtained as described in **§2.2**. Then, 500 μL of $^{223}RaCl_2$ solution (37 kBq) was added to NPs. The resulting suspension was mixed for 1 h (1000 rpm, 25 °C) (**Protocol 2**). To obtain additional organic layer on the surface of NPs, ^{223}Ra-labeled NPs were resuspended in 1 mL of BSA water solution (1 mg/mL) (**Protocol 3**) or PSS water solution (2 mg/mL) (**Protocol 4**) and mixed for 10 min (1000 rpm, 25 °C). Then, ^{223}Ra-labeled NPs+PSS were washed with Milli-Q water, resuspended in 1 mL of PAH water solution (2 mg/mL) and mixed for 10 min (1000 rpm, 25 °C) to obtain a PAH layer (**Protocol 4**). Surface modification was performed one more time for PSS to be the outer layer. As for inorganic additive, $BaCl_2$ water solution (0.07 M) and Na_2SO_4 water solution (0.1 M) were added before incubation, Na_2SO_4 in the range from 5 to 100 μL for and $BaCl_2$ from 43 μL to 860 μL (1x, 2x, 5x, 10x, 20x). The resulting suspension was mixed for 1.5 h (1000 rpm, 25 °C) (**Protocol 5**). Finally, ^{223}Ra-NPs were washed 3 times and resuspended in 1 mL of 0.9% NaCl solution.

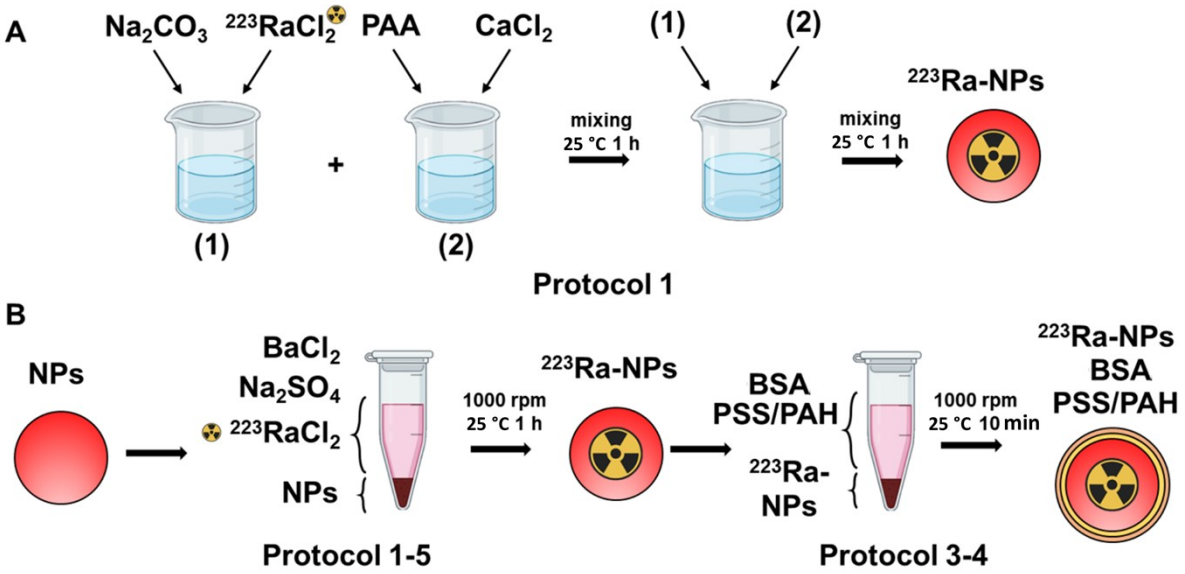

Figure S11. Schematic illustration of ^{223}Ra incorporation into the NPs (A) using coprecipitation (**Protocol 1**) and (B) adsorption (**Protocol 2-5**) approaches.

Table S2. Parameter variations for improvement of NPs' radiolabeling efficiency and isotope retention.

	Radiolabeling method	**organic additive**	**inorganic additive**	**incubation time**	**Average RE (%)**
Protocol 1	co-precipitation	PAA	-	-	99%
Protocol 2	adsorption	PAA	-	1 h	70%
Protocol 3	adsorption	PAA, BSA	-	1 h	86%
Protocol 4	adsorption	PAA, PSS, PAH	-	1 h	82%
Protocol 5	**adsorption**	**PAA**	**Ba^{2+}, SO_4^{2-}**	**1.5 h**	**90%**

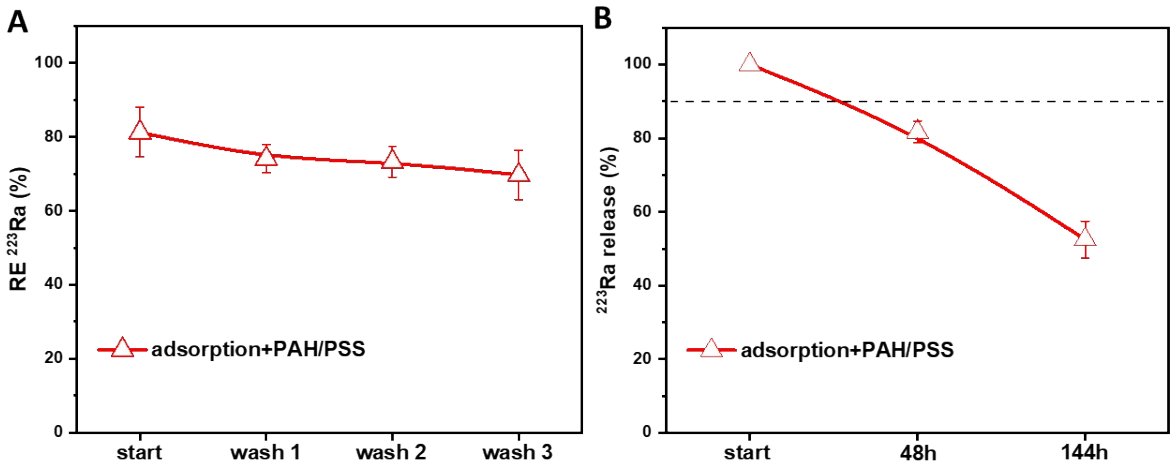

Figure S12. (A) RE (%) of ^{223}Ra-labeled NPs and (B) retention of ^{223}Ra within the ^{223}Ra-labeled NPs using **Protocol 4**. The results are shown as an average value ± standard deviation (n = 4).

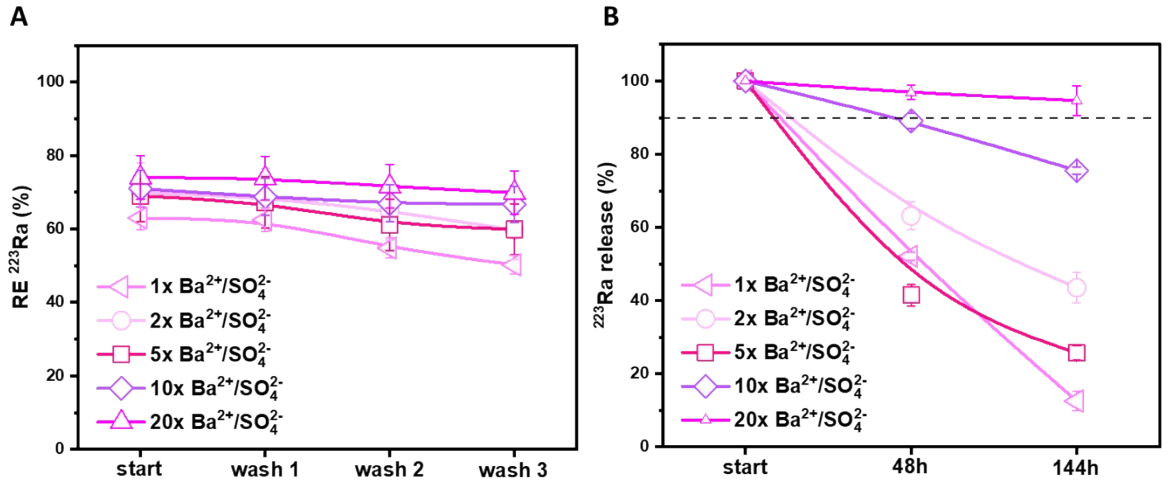

Figure S13. (A) RE (%) of ^{223}Ra-labeled NPs and (B) retention of ^{223}Ra within the ^{223}Ra-labeled NPs using **Protocol 5**. The results are shown as an average value ± standard deviation (n = 4).

12.3. In vitro radiochemical stability (isotope retention)

The studies on the retention of isotopes within the $CaCO_3$ carriers were carried out as follows. The obtained MPs and NPs labeled with ^{223}Ra (37 kBq) were washed 3 times with 0.9% NaCl solution by centrifugation at 12000 rpm for 3 min. The radioactivity of the precipitate was measured after each wash and correlated with the initial activity. Then, the pellets were resuspended in 1 mL of 0.9% NaCl solution and incubated in a thermoshaker (1000 rpm, 25 °C). We measured the radioisotope

retention level for 6 d. For this, the samples were centrifuged at each time point. The retention level was obtained by dividing the radioactivity of the pellet by the sum of the activities of the pellet and the supernatant. The activity of ^{223}Ra was corrected for decay to obtain the actual values of retention. The radioactivity was measured with a calibrated gamma counter (Triathler with Nal detector, Hidex Oy, Finland).

$$Retention\ (\%) = \frac{A_{pellet}}{A_{pellet} + A_{supernatant}} \times 100\%$$

where A_{pellet} is the radioactivity from solid MPs and NPs and $A_{supernatant}$ is the radioactivity from the supernatant after the centrifugation of MPs and NPs.

13. Radiometry of organs

Direct radiometry analysis of the $CaCO_3$ carriers' distribution in the tumor-bearing BALB/c mice was performed using a TRIATHLER portable spectrometric radiometer (Hidex Oy, Finland). Each animal was injected intratumorally with the ^{223}Ra-labeled $CaCO_3$ carriers (^{223}Ra-MPs, ^{223}Ra-NPs) at the same dose (37 kBq, 50 μL). The animals were sacrificed on the 2nd, 5th, 8th and 12th d after the administration of $CaCO_3$ carriers, and the organs (the heart, liver, lungs, spleen, and kidneys) and tumors were removed from the body, placed in plastic tubes and weighed. Total radioactivity for each tested sample was calculated as the percentage of the injected activity adjusted by the weight of tissues (% ID/g).

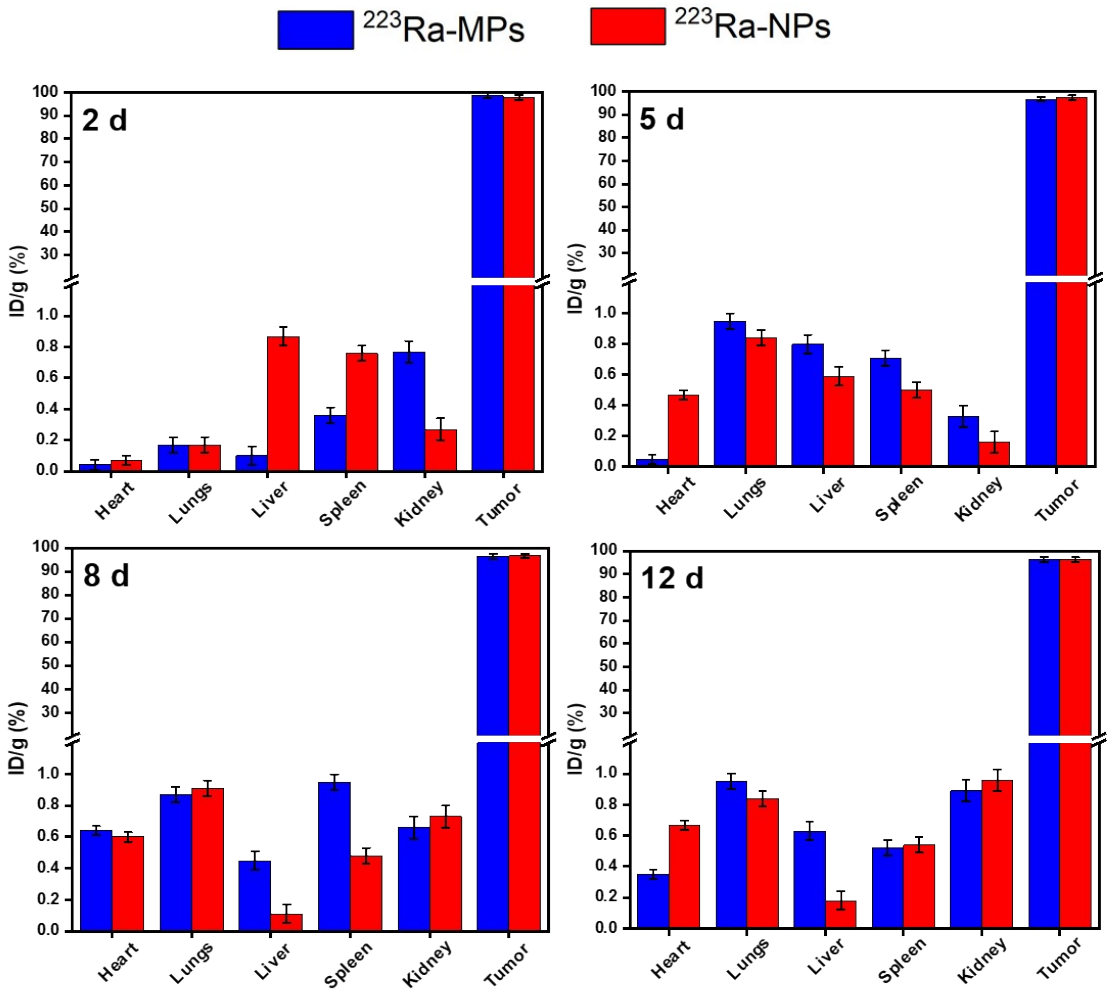

Figure S14. Direct radiometry analysis for each of the tested organs (the heart, liver, lungs, spleen, and kidneys) and tumors after the intratumoral injection of the ^{223}Ra-labeled $CaCO_3$ MPs and NPs in the 4T1 tumor-bearing mice on the 2nd, 5th, 8th and 12th d post-injection, performed using γ-spectrometry. The results are shown as average value ± standard deviation (n = 3).

14. Therapy studies

For the therapy study, BALB/c mice were injected with 4T1 cells and a model of breast cancer was developed as described in **§9**. The animals were treated with: PBS (50 μL) as control, ^{223}Ra-MPs, and ^{223}Ra-NPs at the same dose (37 kBq, 50 μL). Each experimental group consisted of at least 10 animals. The therapeutic potential of ^{223}Ra-MPs and ^{223}Ra-NPs was examined by measuring the tumor size during the time of the experiment and comparing it with the control group (without treatment). On the 2nd, 5th, 8th, and 12th d after the therapy, the mice were sacrificed, collected

on the 2nd and 12th d tissue samples (the tumor, lungs, kidneys, heart, liver, and spleen) were prepared for histological analysis (hematoxylin and eosin (H&E) staining).

14.1. Histological analysis with H&E staining

Histological samples were stained with (H&E) to evaluate the tumor progression and to analyze the toxicity of the compounds to healthy organs. For this, the sections were transferred to distilled water. Then, Ehrlich's hematoxylin solution was added, and the samples were stained for 5 min. Next, the sections were transferred to distilled water and washed for 3 min. Then, the samples were transferred to a 1% solution of hydrochloric acid diluted in 70% ethanol for 3 sec. Then, the samples were again washed in pure distilled water for 20 min. Afterwards, the samples were transferred to a 1% aqueous solution of eosin for 1-2 min. The stained sections were washed with distilled water and dehydrated with 96% ethanol. Clarification was carried out with a mixture of carbol-xylene for 1 min. Finally, the samples were fixed in glycergel.

References

1 T. E. Karpov, A. R. Muslimov, D. O. Antuganov, A. S. Postovalova, D. A. Pavlov, Y. V. Usov, S. V. Shatik, M. V. Zyuzin and A. S. Timin, *J Colloid Interface Sci*, 2022, **608**, 2571–2583.

2 M. V. Zyuzin, D. Antuganov, Y. V. Tarakanchikova, T. E. Karpov, T. V. Mashel, E. N. Gerasimova, O. O. Peltek, N. Alexandre, S. Bruyere, Y. A. Kondratenko, A. R. Muslimov and A. S. Timin, *ACS Appl Mater Interfaces*, 2020, **12**, 31137–31147.

3 A. I. Catarino, A. Frutos and T. B. Henry, *Science of The Total Environment*, 2019, **670**, 915–920.