Electronic Supplementary Material (ESI) for Nanoscale Horizons.

Supporting Information

Nanoparticulates Reduce Tumor Cell Migration through Affinity Interactions with Extracellular Migrasomes and Retraction Fibers

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Experimental Section

Materials, Cell Culture, and Animals: Polystyrene nanoparticles (NPs) (70 nm, 200 nm, 500 nm, NBD-labeled) were purchased from Zhongke Leiming Technology Co., Ltd (Beijing, China). DMEM culture medium, Trypsin containing 0.25% EDTA, and Penicillin-Streptomycin were purchased from M&C Gene Technology (Beijing, China). Phosphate Buffer Solution (PBS), 4% Paraformaldehyde, Hoechst 33342, TRITC Phalloidin, Bouin's staining solution and Rat tail tendon collagen type I were purchased from Solarbio (Beijing, China). Fetal Bovine Serum (FBS) was purchased from Gemini Bio (USA). D-Luciferin potassium and Filipin was purchased from Meilunbio (Dalian, China). The Apoptosis and Necrosis Test Kit, Cell Counting Kit-8 (CCK-8 Kit), Trypan Blue Stained Cell Viability Assay Kit, RIPA Lysis Buffer, Primary Antibody Dilution, Secondary Antibody Dilution, Blocking Solution, Anti-fluorescence Quenching Sealing Solution, BCA Protein Assay Kit and DiD Cell Membrane Far-Infrared Fluorescent Probe were purchased from Beyotime Biotechnology (Shanghai, China). Sulfonhodamine B Sodium Salt was purchased from Adamas (Shanghai, China). Egg Phosphatidylcholine (EPC) was purchased from Yuanyebio (Shanghai, China). Trichloroacetic acid (TCA) was purchased from Coolaber (Beijing, China). Tris was purchased from Biodee (Beijing, China). Methyl-β-cyclodextrin (MβCD) was purchased from J&K (Beijing, China). Rabbit Anti-Human Fibronectin mAb, Rabbit Anti-Human TSG101 mAb, Rabbit Anti-Human TSPAN4 mAb, Goat Anti-Rabbit IgG H&L (Alexa Fluor® 647) and Goat Anti-Rabbit IgG H&L (HRP) were purchased from Abcam (Beijing, China). Rabbit Anti-Human TSPAN7 pAb were purchased from ABclonal (Wuhan, China). Glacial Acetic Acid (AR) and Xylene (AR) were purchased from Tongguang. (Beijing, China). Poly (d, l-lactic-co-glycolic acid) (PLGA) was purchased from Shandong Daigang Institute of Medical Instruments (Jinan, China). Ultrafiltration tubes were purchased from Millipore (USA). 8 μm Transwell polycarbonate membrane chambers were purchased from Corning (USA). Cholera Toxin Subunit B (CT-B) was purchased from Life Technologies Corporation (USA). Coumarin-6 (C6) and poloxamer 188 (Pluronic F68) was purchased from Sigma (USA).

Human breast cancer cells MDA-MB-231 were purchased from Chinese Academy of Medical Sciences (Beijing, China). MDA-MB-231 cells were cultured in DMEM culture medium supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin, in a humidified incubator containing 5% CO_2 at 37℃.

BALB/c mice (female, age of 6-8 weeks) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All animal experiments were conducted in Center of Experimental Animals of Peking University, China, and all these animals were approved by the Institutional Animal Care and Use Committee of Peking University, China.

Preparation of Nanoparticles: The required amount of polystyrene NPs or SiO_2-NPs mother liquor was taken out, diluted in blank medium at a concentration of 2 mg/mL, and then centrifuged at room temperature, 12000 rpm for 30 min. After centrifugation, the supernatant was discarded and NPs were resuspended in appropriate solvent (deionized water, PBS, or complete medium) at needed concentration. Finally, the suspension was treated by probe ultrasonic method at 50 w for 5 min, ice bathed.

PLGA-NPs were prepared according to the method described in our previous work.[1] Specifically, the original emulsion was gained by gradually adding 2 mL acetone dissolving 5 mg PLGA as well as 5 μg coumarin-6 in drops into 5 mL deionized water containing 0.05% Pluronic F68 followed by continuous stirring for 15 min. Subsequently, the organic solvent in the obtained emulsion was removed by a vacuum rotary evaporator at 60℃, solidifying the PLGA polymers into nano-scale. The PLGA-NPs were then washed for three times and the suspension was centrifuged at room temperature, 12 000 rpm for 30 min, to remove the remaining Pluronic F68. After being re-suspended with complete medium, the final PLGA-NPs suspension was obtained.

Characterization of NPs: NPs were dispersed at different concentrations (20, 100, 400 μg/mL) in deionized water. The hydrodynamic diameter of NPs was measured by dynamic light scattering (DLS) analysis using a Malvern Zetasizer Nano ZS (Malvern, United Kingdom) for three times after the probe ultrasound treatment. The morphology of NPs was characterized by transmission electron microscopy (TEM) imaging on a JEM 1400plus TEM (JEOL Japan), and scanning electron microscopy (SEM) imaging on a JSM-7900F FE-SEM (JEOL Japan). To prepare specimens for electron microscopes, NPs were dispersed in deionized water at 200 μg/mL. The suspension was then dropped onto a carbon-coated copper grid (for TEM imaging), non-carbon-coated copper grid (for Energy Dispersive X-ray analysis by SEM), or a silicon wafer (for SEM imaging), respectively, and was air dried before observation.[2] NPs were stained with

phosphotungstic acid (1%) for 1 min and then air dried before TEM imaging.[3] To determine the crystal structure of NPs, the freeze-dried powder of NPs was detected by X-ray diffraction (XRD) using a MiniFlex600 X-ray diffractometer (Rigaku, Japan).[4]

Cell Viability Test

SRB assay: MDA-MB-231 cells were seeded in a 96-well plate at a density of 5×10^3 cells per well and incubated for 12 h. NPs with different concentrations (50, 100, 200, 400, 800 μg/mL) were added to \ cells and incubated together for 48 h. Subsequently, the cells were washed with PBS for 3 times, and fixed with pre-cooled 10% trichloroacetic acid at 4℃ for 1h. After that, cells were washed with deionized water for 5 times and dried completely. Then, cells were stained with SRB working solution at room temperature for 30 min and washed with 1% acetic acid for 5 times. After the plate was dried completely again, 10 mmol/L Tris was added to solve the SRB with the plate shaken on a shaker at room temperature for 30 min. The OD value was evaluated using a 96-well microplate reader (Thermo Multiskan MK 3) at the wavelength of 540 nm. The cell viability ratio was calculated as follows and presented as mean±SD (n = 5):
(OD value－OD value of blank well)/(mean OD value of control group－OD value of blank well)×100 %

CCK-8 Assay: MDA-MB-231 cells were seeded in a 96-well plate at a density of 5×10^3 cells per well and incubated for 3 h. Polystyrene NPs with different concentrations (50, 100, 200 μg/mL) or PLGA-NPs with different concentrations (100, 200, 500, 1000, 1500, 2000 μg/mL) were added to cells and incubated together for 24 h. Subsequently, the cells were washed with PBS for 3 times and treated with 10% CCK-8 solution (solved in blank medium) at 37℃ for 2 h. The OD value was evaluated using the aforesaid 96-well microplate reader at the wavelength of 450 nm, and the cell viability ratio was calculated as mentioned above.

Trypan Blue Kit Assay: MDA-MB-231 cells were seeded in a 6-well plate at a density of 3×10^5 cells per well and incubated for 12 h. 200 μg/mL NPs or complete culture were added to cells and incubated together for 12 h. Later, the cells were digested, neutralized, centrifuged, resuspended in 20 μL of trypan blue staining solution (trypan blue solution and cell suspension at a ratio of 1:1), and counted by a Cellometer mini (Nexcelom, USA). The cell viability ratio was calculated as follows and presented as mean±SD (n = 3):
(1－the number of dead cells/the number of total cells)×100%

Apoptosis and Necrosis Assay: MDA-MB-231 cells were seeded in a 6-well plate at a density of 3×10^5 cells per well and incubated for 12 h. 200 μg/mL NPs or complete culture were added to cells and incubated together for 12 h. Then, the cells were digested, neutralized, centrifuged, resuspended in complete medium, counted, and seeded at 1×10^5 per well in a 24-well plate. 3 h later, 200 μg/mL NPs or complete culture were added to cells and incubated together for 24 h. Subsequently, the cells were washed with PBS for 3 times and stained with the mixed solution (cell staining buffer, Hoechst staining solution, and PI staining solution at a ratio of 200:1:1) at 4℃ for 30 min. The fluorescent images were shot by an XDS-1B inverted biological microscope (COIC, Chongqing, China). The number of cells in each field was counted using software ImageJ 1.52v. The cell viability ratio was calculated as follows and presented as mean±SD (n = 5):
(1－red dot/blue dot)×100%

Cellular Uptake: The mother liquor of NPs containing 25, 50, 100, 200, 800 μg/mL NPs was concentrated by a Savant SpeedVac vacuum concentrator (Thermo Scientific, USA) to remove the water phase. Then, 1 mL xylene was added into the NPs powders, and shaken overnight. The fluorescence intensity was measured by a RF-6000 Fluorescence Spectrophotometer (Shimadzu, Japan), and the NPs concentration－fluorescence intensity standard curves were calculated accordingly.

MDA-MB-231 cells were seeded in a 24-well plate at a density of 5×10^4 cells per well and incubated for 12 h and 21 h, respectively. 200 μg/mL NPs or complete culture were added to cells and incubated together for 12 h and 3 h, respectively. Then, the cells were washed with PBS for three times and lysed by RIPA lysis buffer. The mixture was concentrated by a Savant SpeedVac vacuum concentrator (Thermo Scientific, USA) to remove the water phase. Then, 1 mL xylene was added into the powder and shaken overnight. The mixture was centrifuged at 4℃, 12000 rpm for 15 min. The fluorescence intensity of supernatant was measured by an RF-6000 Fluorescence Spectrophotometer (Shimadzu, Japan), and cellular uptake was presented as intracellular mass of NPs and as mean±SD (n = 3).

The Effect of NPs on Cells' Migration

Transwell Assay: To exclude NPs' hindering effect on cell migration by blocking the Transwell membrane pores, 200 μg/mL NPs were added to the apical chamber of 8 μm Transwell polycarbonate membrane, and PBS was added to the basolateral chamber. Florescent intensity of NPs suspension in the basolateral chamber was measured at 0.5 h, 1 h, 1.5 h, 2 h, 3 h, 4 h, 6 h, 8 h and 10 h after the addition of NPs using a RF-6000 Fluorescence Spectrophotometer (Shimadzu, Japan).

MDA-MB-231 cells were seeded in a 6-well plate at a density of 3×10^5 cells per well and incubated for 12 h. 200 μg/mL NPs, PLGA-NPs or complete culture were added to cells and incubated together for 12 h. Then, the cells were digested, neutralized, centrifuged, resuspended in complete medium, counted, and seeded at 5×10^3 per well in the apical chamber of 8 μm Transwell polycarbonate membrane, and 500 μL complete medium was added to the basolateral chamber. 3 h later, 200 μg/mL NPs, PLGA-NPs or complete culture were added to the apical and basolateral chambers and incubated together for 24 h. Due to the high migratory feature, some of them could migrate through membrane pores to the basolateral side. Subsequently, cells were washed twice with PBS, fixed with 4% paraformaldehyde at room temperature for 15 min, and stained with Hoechst 33342 at room temperature for 20 min. The cells on the upper or lower side of the membrane were wiped with a cotton swab. Then, the membranes were cut down and placed on glass slides, sealed with the sealing solution and covered with coverslips. The central fields of the membranes were shot by an XDS-1B inverted biological microscope (COIC, Chongqing, China). The number of cells in each field was counted using software ImageJ 1.52v. The cell migration ratio was calculated as follows and presented as mean±SD (for polystyrene NPs, n = 4; for PLGA-NPs, n = 3):

lower side cells/upper side cells×100% (the data from control group was used as the benchmark for normalization calculation)

Wound Healing Assay: MDA-MB-231 cells were seeded in a 6-well plate at a density of 5×10^5 cells per well. The cell monolayer was scratched by a 200 μL pipette tip after cells reached 100% confluence. 200 μg/mL NPs or complete culture were added to cells and incubated together for 24 h. At 0 h, 12 h and 24 h, the images of the cell monolayer and the wounds were shot by an XDS-1B inverted biological microscope (COIC, Chongqing, China). The cell migration ratio was calculated as follows and presented as mean±SD (n = 9):

(area of the wounds at 0 h − area of the wounds at 12 h or at 24 h)/area of the wounds at 0 h×100% (the control group was used as the benchmark for normalization calculation)[5]

High Content Analysis: MDA-MB-231 cells were seeded in a 96-well plate at a density of 5×10^3 cells per well and incubated for 12h. 200 μg/mL NPs or complete culture were added to cells and incubated together for 3 h, 6 h, 9 h and 12 h, respectively. Subsequently, the cells were washed twice with PBS, fixed with 4% paraformaldehyde at room temperature for 15 min, stained with Hoechst 33342 at room temperature for 20 min, and stained with TRITC Phalloidin at room temperature for 30 min. The images were shot by an Operetta High-Content Analysis System (PerkinElmer, USA). The morphological parameters: (average per field) cell length, cell width, cell width to length ratio, cell area, and cell roundness were calculated by the "Calculate Morphology Properties" module in software Harmony 3.5.1, and presented as mean±SD (n = 51).

MDA-MB-231 cells were seeded in a 96-well plate at a density of 5×10^3 cells per well and incubated for 12h. 200 μg/mL polystyrene NPs or complete culture were added to cells. The images were shot by an Operetta High-Content Analysis System (PerkinElmer, USA) every 20 mins for consecutive 12 h. As for the experiment of other nanoparticles, 200 μg/mL PLGA-NPs, 200 μg/mL SiO_2-NPs and 2 mg/mL liposomes were added to cells, and images were shot every 30 mins for consecutive 10 h. The cell movement parameters: square displacement, displacement, distance and speed were calculated by Software Harmony 3.5.1, and presented as mean±SD.

The Effect of Extracellular Structures on Cells' Migration

Transwell Assay: MDA-MB-231 cells were seeded in the apical chamber of 8 μm Transwell polycarbonate membrane at a density of 2×10^4 per well, and 500 μL complete medium was added to the basolateral chamber. 12 h later, 200 μg/mL NPs or complete culture were added to the apical and basolateral chambers and incubated together for 3 h. Subsequently, the cells were burst due to unbalanced osmotic pressure by adding hypotonic solution to the apical and basolateral chambers for 30 min. Then, new MDA-MB-231 cells were seeded on the remaining extracellular structures in the apical chamber at a density of 1×10^4 per well for 24 h. Subsequently, the cells were washed twice with PBS, fixed

with 4% paraformaldehyde at room temperature for 15 min, and stained with Hoechst 33342 at room temperature for 20 min. The cells on the upper side of the membrane were wiped with a cotton swab. Then, the membranes were cut down, placed on glass slides, sealed with the sealing solution and covered with coverslips. The central fields of the membranes were shot by an XDS-1B inverted biological microscope (COIC, Chongqing, China). The number of cells in each field was counted using software ImageJ 1.52v. The cell migration ratio was calculated as follows and presented as mean±SD (n = 3):
the number of cells in NPs-treated group/the number of cells in control group×100%

High Content Analysis: MDA-MB-231 cells were seeded in a 24-well plate at a density of 8×10^4 cells per well and incubated for 12 h. 200 μg/mL NPs or complete culture were added to the cells and incubated together for 3 h. Subsequently, the cells were burst due to unbalanced osmotic pressure by adding hypotonic solution into the wells for 30 min. Then, new MDA-MB-231 cells were seeded on the remaining extracellular structures in the wells or directly in the blank wells at a density of 5×10^4 per well. The images were shot by an Operetta High-Content Analysis System (PerkinElmer, USA) every 30 mins for consecutive 10 h. The cell movement parameters: square displacement, displacement, distance, and speed were calculated by Software Harmony 3.5.1, and presented as mean±SD (n = 51).

Real Time Cellular Analysis: MDA-MB-231 cells were seeded in an RTCA electrode plate at a density of 5×10^3 cells per well and incubated for 12 h. 200 μg/mL NPs or complete culture were added to cells and incubated together for 3 h. Subsequently, the cells were burst due to unbalanced osmotic pressure by adding hypotonic solution into the wells for 30 min. Then, new MDA-MB-231 cells were seeded on the remaining extracellular structures in the wells or directly in the blank wells at a density of 5×10^3 per well. The number of cells among different groups was kept the same during the investigation. So, the higher index (i.e., higher resistance value) indicates greater adhesion of cells on substrate (the pre-coated ECM), and it also means a stronger restriction of cellular movement. The cell index was recorded by an xCELLigence RTCA S16 system (ACEA Biosciences, USA), and presented as mean±SD (n = 3).[6]

Real-time Affinity Interactions between NPs and Extracellular Structures

High Content Analysis: MDA-MB-231 cells were seeded in a 96-well plate at a density of 5×10^3 cells per well and incubated for 12h. 200 μg/mL NPs or complete culture were added to the cells. The images were shot by an Operetta High-Content Analysis System (PerkinElmer, USA) every 20 mins for consecutive 4 h.

Confocal Laser Scanning Microscopy: MDA-MB-231 cells were seeded in 8-well confocal chambers at a density of 5×10^3 cells per well for 12 h. The cells were washed twice with PBS and stained with DiD at 37℃ for 1 h. 200 μg/mL NPs or complete culture were added to the cells. The images were shot by Airyscan on an LSM880 confocal laser scanning microscope (Zeiss, Germany) every 10 mins for consecutive 2 h.

The Co-localization between PLGA-NPs, SiO2-NPs and Membrane Structures: MDA-MB-231 cells were seeded in 8-well confocal chambers at a density of 5×10^3 cells per well for 12 h. 200 μg/mL polystyrene NPs, C6-labeled PLGA-NPs or complete culture were added to the cells and incubated together for 3 h. Then, the cells were washed twice with PBS and stained with DiD at 37℃ for 1 h. The images were shot by an LSM880 confocal laser scanning microscope (Zeiss, Germany).

MDA-MB-231 cells were seeded in 8-well confocal chambers at a density of 5×10^3 cells per well for 12 h. 200 μg/mL ReF-labeled SiO_2-NPs or complete culture were added to the cells and incubated together for 3 h. Then, the cells were washed twice with PBS and stained with DiO at 37℃ for 1 h. The images were shot by an LSM880 confocal laser scanning microscope (Zeiss, Germany).

The Interaction between NPs and ECM Proteins

Confocal Laser Scanning Microscopy: MDA-MB-231 cells were seeded in 8-well confocal chambers at a density of 5×10^3 cells per well for 12 h. 200 μg/mL NPs or complete culture were added to cells and incubated together for 12 h. Subsequently, the cells were washed twice with PBS, fixed with 4% paraformaldehyde at room temperature for 15 min, blocked with immunostaining blocking solution at 37℃ for 30 min, incubated with Rabbit Anti-Human Fibronectin mAb at 4℃ for 12 h, incubated with Goat Anti-Rabbit IgG H&L (Alexa Fluor® 647) at 37℃ for 2 h, stained with Hoechst 33342 at room temperature for 20 min, and stained with TRITC Phalloidin at room temperature for 30 min. The images were shot by a A1R confocal laser scanning microscope (Nikon, Japan).

High Content Analysis: In order to detect whether the interaction of ECM proteins with NPs would affect cellular motion, a 24-well plate was pre-coated with rat tail tendon collagen type I. 200 μg/mL 200 nm NPs or complete culture were added to the coated wells and incubated together for 3 h. MDA-MB-231 cells were seeded in the coated wells at a density of 5×10^4 per well. The images were shot by an Operetta High-Content Analysis System (PerkinElmer, USA) every 30 mins for consecutive 8 h. The cell movement parameters: square displacement, displacement, distance, and speed were calculated by Software Harmony 3.5.1, and presented as mean±SD (n = 48).

The Co-localization between NPs and Lipid Rafts

Field Emission Scanning Electron Microscopy: MDA-MB-231 cells were seeded in glass bottom dishes at a density of 8×10^4 cells and incubated for 12 h. 200 μg/mL NPs or complete culture were added to cells and incubated together for 12 h. Then, the cells were washed twice with PBS, fixed with 2.5% glutaraldehyde at room temperature for 2 h, stained with 1% osmic acid at room temperature for 60 min, dehydrated with a series of ethanol (50%, 70%, 90%, 95%, and 100%) at room temperature for 10 min each, frozen at −20℃, and then dried with a freeze dryer. Subsequently, dried samples were mounted onto an aluminum stub through a conductive carbon tape and coated with Platinum in a JEC-3000FC Auto Fine Coater (JEOL Japan). The images were obtained by a JSM-7900F FE-SEM (JEOL Japan).[7, 8]

Confocal Laser Scanning Microscopy: MDA-MB-231 cells were seeded in 8-well confocal chambers at a density of 5×10^3 cells per well for 12 h. 200 μg/mL NPs or complete culture were added to cells and incubated together for 3 h. Then, the cells were washed twice with PBS and stained with CT-B at 0℃ for 30 min. The images were shot by an LSM880 confocal laser scanning microscope (Zeiss, Germany). The surface plot was processed and obtained by software Image-Pro Plus 6.0.0.260.

MDA-MB-231 cells were seeded in 8-well confocal chambers at a density of 5×10^3 cells per well for 12 h. 200 μg/mL NPs or complete culture were added to cells and incubated together for 3 h. Then, the cells were washed twice with PBS, fixed with 4% paraformaldehyde at room temperature for 15 min and stained with Filipin at room temperature for 2 h. The images were shot by an LSM880 confocal laser scanning microscope (Zeiss, Germany).

The Effect of NPs on ECM Proteins

Western Blot: MDA-MB-231 cells were seeded in 100 mm cell culture dishes at a density of 3×10^6 cells for 12 h. 200 μg/mL NPs or complete culture were added to cells and incubated together for 3 h. Subsequently, the cells and extracellular matrix were washed twice with PBS and collected by a cell scraper. Then, the mixture was centrifuged at 4℃, 1000 rpm for 10 min and 2000 rpm for 10 min, successively. The supernatant was retained and concentrated by a 3 k, 4 mL ultrafiltration tube and then lysed by RIPA lysis buffer at 4℃ for 30 min. 15 μL collected proteins was loaded on a 4%-20% gradient precast gel for SDS-PAGE at 100 mV, 90 min. Then proteins were transferred to a polyvinylidene difluoride membrane at 4℃, 110 mV, 70 min. Subsequently, the membrane was blocked with western blot blocking solution at 37℃ for 30 min, incubated with Rabbit Anti-Human TSG101 mAb, Rabbit Anti-Human TSPAN4 mAb, or Rabbit Anti-Human TSPAN7 pAb, respectively, at 4℃ for 12 h and incubated with Goat Anti-Rabbit IgG H&L (HRP) at 37℃ for 2 h. The images were shot by a Tanon 5200 multi-imaging system (Tanon, Shanghai, China).[9]

Proteomics Analysis: The protein samples were prepared by the same method as that of western blot assay. 15 μL collected proteins was loaded on a 10% precast gel for SDS-PAGE at 100 mV, 15 min.

Next, the tube-gel method was applied to digest proteins (2 μL sample, 14.25 μL ddH2O, 35 μL 30% acrylamide solution, 45 μL Tris-HCl buffer (pH 8.8), 2.5 μL 10% ammonium persulfate, and 1 μL 10% SDS). Then, the gel was fixed with 50% methanol and 12% acetic acid at room temperature for 30 min and was cut into small pieces. Subsequently, the gel was dehydrated with acetonitrile (ACN), reduced with Tris(2-carboxyethyl)phosphine(TCEP), alkylated with iodoacetamide, washed with 50% ACN/50 mM NH_4HCO_3 buffer, rehydrated with Trypsin (10 ng/μL) in 25 mM NH_4HCO_3 buffer at 4℃ for 2 h, and incubated at 37℃ for 12 h to obtain peptides. Peptides mixture got from the last step was extracted twice with ACN/5% trifluoroacetic acid (TFA) (v/v, 2/1) and lyophilized for follow-up steps.

The LC-MS/MS (Mass Spectrometric) analysis was conducted depending on Label-Free Quantification method. 10 μL peptides were loaded on a C18 pre-column (Easy-column C18-A1, 100 μm I.D.×20 mm, 5 μm, Thermo Fisher Scientific, USA) and separated by nano-LC-MS/MS based on an Easy-LC nano-HPLC (Thermo Fisher Scientific, USA). Through this

process, H_2O/TFA was used as mobile phase A and ACN/TFA was mobile phase B. With a 300 nL/min flow rate, the gradient was set as: from 5% to 30% for 90 min, from 30% to 50% for 10 min, from 50% to 100% for 10 min, and then remained at 100% for 10 min. MS investigation was conducted by an LTQ Orbitrap Velos pro or Q-Exactive HF (Thermo Fisher Scientific, USA). MS/MS spectra were collected by a data-dependent collision-induced dissociation (CID) model, and the full MS was obtained from m/z 350 to 2000 with a resolution at 60,000. The ions which were top 15 most intense were selected for MS/MS.

The primary data was analyzed by MaxQuant (version 1.5.6.0), and the identification of different proteins was conducted using the UniProt database. Up- and down-regulated proteins were picked out according to a 'significantly altered ratio', i.e., P value < 0.01, calculated by Perseus (version 1.6.0, http://www.perseus-framework.org). Cluster analysis of DAVID function was conducted on the Database for Annotation, Visualization and Integrated Discovery (https://david.ncifcrf.gov/). Venn diagrams were obtained by TBtools 1.064. Heatmaps were gained from Graphpad Prism 8.0.2. [10-12]

The Effect of NPs on the Cellular Uptake of Large Multivesicular Liposomes (LMVs)

The Preparation and Characterization of LMVs: 10 mg EPC and 10 μg DiD were dissolved in trichloromethane in a pear-shaped flask, and the organic solvent was dried under reduced-pressure conditions. The dry lipid film was then hydrated by 1 mL PBS to obtain LMVs. [13]

The Cellular Uptake of LMVs

Flow Cytometry: MDA-MB-231 cells were seeded in glass bottom dishes at a density of 8×10^4 cells for 12 h. Subsequently, the cells were burst due to unbalanced osmotic pressure by adding hypotonic solution to the dishes for 30 min. Then, the mixture of 200 μg/mL NPs and 1 mg/mL DiD-labeled LMVs was added onto the remaining extracellular structures in the dishes and incubated together for 6 h. New MDA-MB-231 cells were seeded on the mixture remaining in the central area of the glass bottom dishes at a density of 3×10^4 cells. 12 h later, the cells were digested, neutralized, washed twice with ice-bathed PBS, and resuspended in 100 μL ice-bathed PBS. The fluorescent signals of the cells were recorded on an FACS Calibur dual laser flow cytometer (BD, USA). The cellular uptake value was demonstrated as the intracellular fluorescent intensity of APC-A and presented as mean±SD (n = 3).

Confocal Laser Scanning Microscopy: MDA-MB-231 cells were seeded in glass bottom dishes at a density of 8×10^4 cells for 12 h. Subsequently, the cells were burst due to unbalanced osmotic pressure by adding hypotonic solution to the dishes for 30 min. Then, the mixture of 200 μg/mL NPs and 1 mg/mL DiD-labeled LMVs was added onto the remaining extracellular structures in the dishes and incubated together for 6 h. New MDA-MB-231 cells were seeded on the mixture remaining in the central area of the glass bottom dishes at a density of 3×10^4 cells. 12 h later, the cells were washed once with complete culture. The images were shot by an LSM880 confocal laser scanning microscope (Zeiss, Germany).

The Effect of Disturbed Extracellular Structures on Cells' Migration

Confocal Laser Scanning Microscopy: MDA-MB-231 cells were seeded in 8-well confocal chambers at a density of 5×10^3 cells per well for 12 h. 200 μg/mL NPs or complete culture were added to cells and incubated together for 3 h. Then, the cells were washed twice with PBS, incubated with 10 mM Methyl-β-cyclodextrin at 37℃ for 30 min and stained with DiD at 37℃ for 1 h. The images were shot by an LSM880 confocal laser scanning microscope (Zeiss, Germany). The length of retraction fibers was measured by ImageJ 1.52v, and the number of retraction fibers and migrasomes were counted field by field. The data were presented as mean±SD.[14]

High Content Analysis: MDA-MB-231 cells were seeded in a 24-well plate at a density of 8×10^4 cells per well and incubated for 12 h. Subsequently, the cells were burst due to unbalanced osmotic pressure by adding hypotonic solution into the wells for 30 min. 10 mM MβCD was added to cells for 30 min to remove extracellular lipid components. In contrast, PBS was added to cells of control group. Then, new MDA-MB-231 cells were seeded on the remaining extracellular structures in the wells at a density of 5×10^4 per well. The images were shot by an Operetta High-Content Analysis System (PerkinElmer, USA) every 30 mins for consecutive 8 h. The cell movement parameters: square displacement, displacement, distance, and speed were calculated by Software Harmony 3.5.1, and presented as mean±SD (n = 48). [14]

MDA-MB-231 cells were seeded in a 24-well plate at a density of 8×10^4 cells per well and incubated for 12 h. Subsequently, the cells were burst due to unbalanced osmotic pressure by adding hypotonic solution into the wells for 30 min. RIPA lysis buffer was added to cells for 30 min to totally destroy extracellular membrane structures. In contrast, PBS was added to cells of control group. Then, new MDA-MB-231 cells were seeded on the remaining extracellular structures in the wells at a density of 5×10^4 per well. The images were shot by an Operetta High-Content Analysis System (PerkinElmer, USA) every 30 mins for consecutive 8 h. The cell movement parameters: square displacement, displacement, distance, and speed were calculated by Software Harmony 3.5.1, and presented as mean±SD (n = 48).

The Effect of Nano-ECM on Cellular Proteins

Proteomics Analysis: MDA-MB-231 cells were seeded in a 6-well plate at a density of 4×10^5 cells for 12 h. 200 μg/mL 200 nm NPs or complete culture were added to cells and incubated together for 3 h. Subsequently, the cells were burst due to unbalanced osmotic pressure by adding hypotonic solution into the wells for 30 min. Then, new MDA-MB-231 cells were seeded on the remaining extracellular structures in the wells at a density of 4×10^5 per well. 4 h or 12 h later, the cells were digested by Trypsin containing 0.25% EDTA, washed twice with PBS, lysed by RIPA lysis buffer at 4℃ for 30 min and collected. Then, the mixture was centrifuged at 4℃, 10000 rpm for 15 min, and total protein in supernatant was quantified by BCA Protein Assay Kit. 10.5 μg collected proteins was loaded on a 10% precast gel for SDS-PAGE at 100 mV, 15 min.

The subsequent steps of protein samples treatment, proteomics analysis, and data processing were the same as mentioned above.

In Vivo **Tumor Cells Metastasis Analysis:** The 4T1-luc breast cancer model was established by injecting 2×10^5 4T1-luc cells *s.c.* in the mammary fat pads of female BALB/c mice. When the tumor volume reached approximately 150 mm^3, the mice were sacrificed and 8 mm^3 tumor blocks were implanted to the right mammary fat pads of a new batch of female BALB/c mice. Then 1 mg/mL NPs or blank medium was injected to the peripheral area of the tumor blocks. At 14, 16, 18, 20, 22, 24, 26 days after the implantation and injection, 150 mg/kg D-Luciferin potassium was injected *i.p.* to the mice, and the bioluminescent images were shot by an IVIS Lumina II Imaging System (PerkinElmer, USA). At 26 days after the implantation and injection, the mice were sacrificed, and the lungs, kidneys, livers and spleens were harvested and observed for the metastatic nodes via Bouin's solution staining. Metastatic nodes on the lungs were counted and presented as mean±SD (n = 8).[3, 15-17]

Statistical Analysis: All data in this study were analyzed by GraphPad Prism 8.0.2 and presented as mean±SD. The comparison between two groups was conducted as unpaired Student's t-test (two-tailed), and P value was considered to be statistically significant if it was lower than 0.05. Specifically, * represented P value<0.05, ** represented P value<0.01, *** represented P value<0.001, and **** represented P value<0.0001.[10]

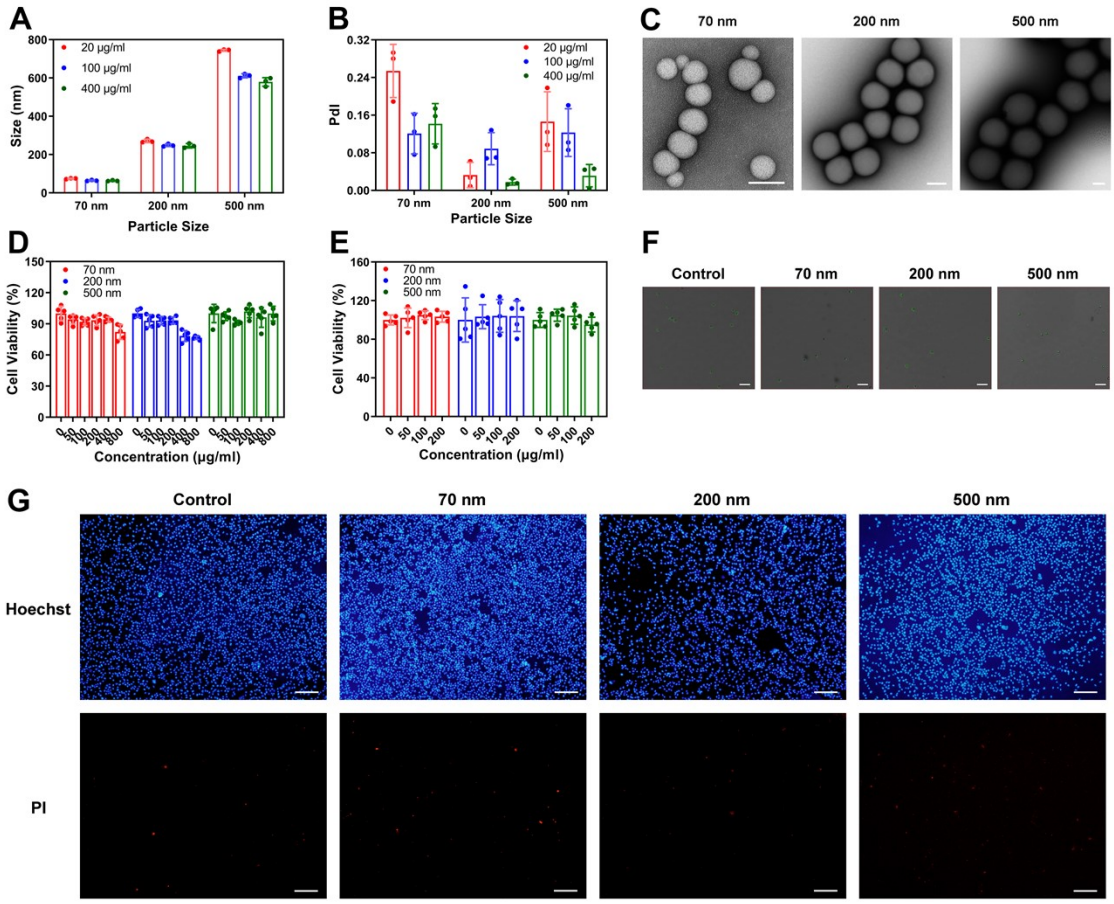

Figure S1. (A) The intensity size of polystyrene nanoparticles (NPs) at different concentrations (n = 3). (B) The PDI of NPs at different concentrations (n = 3). (C) Representative TEM images of NPs with different particle sizes. Scale bar, 100 nm. (D) Cell viability of MDA-MB-231 cells incubated with different concentrations of NPs tested by SRB assay (n = 5). (E) Cell viability of MDA-MB-231 cells incubated with different concentrations of NPs tested by CCK-8 assay (n = 5). (F) Representative images of MDA-MB-231 cells stained by Trypan Blue Kit. Scale bar, 100 μm. (G) Representative florescent images of MDA-MB-231 cells stained by Apoptosis and Necrosis Kit. Scale bar, 200 μm.

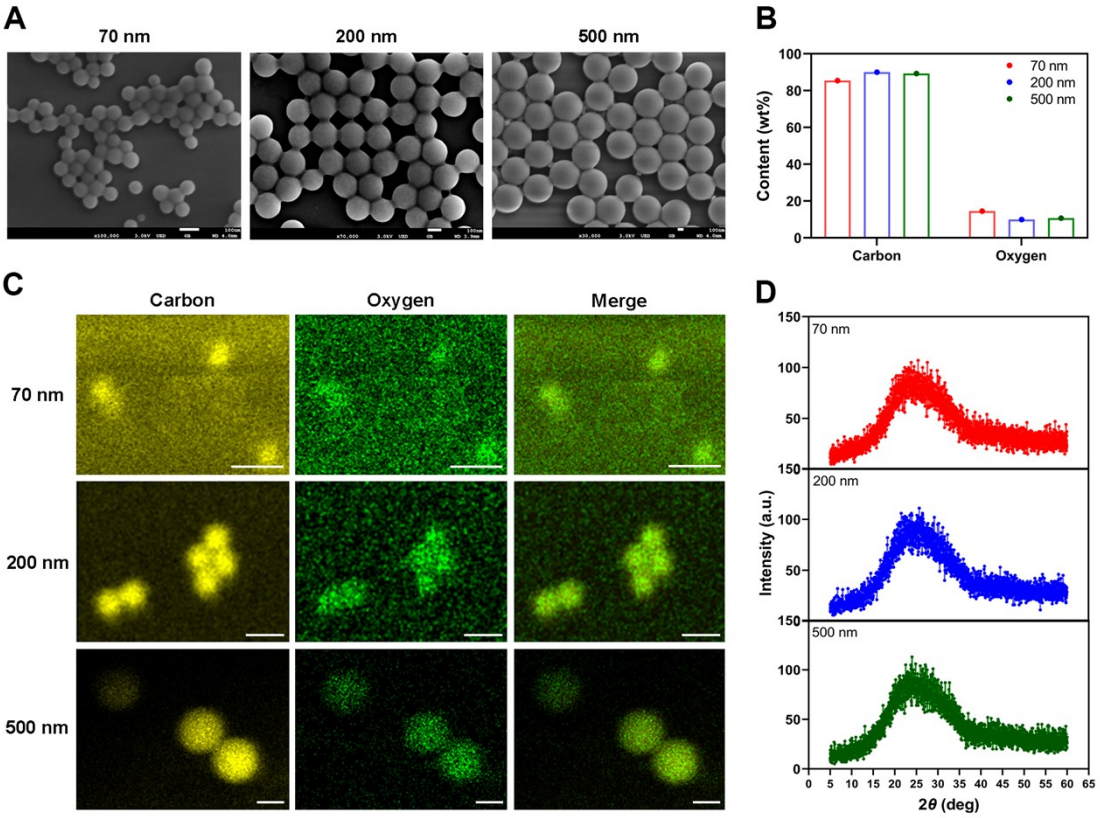

Figure S2. Surface chemical structure investigation of NPs. (A) Representative SEM images showing the surface morphology of NPs with different particle sizes. Scale bar, 100 nm. (B) Chemical composition (EDX) of the surface of NPs with different particle sizes. (C) EDX elemental mapping of NPs with different particle sizes (C and O). Scale bar, 250 nm. (D) XRD pattern of NPs with different particle sizes.

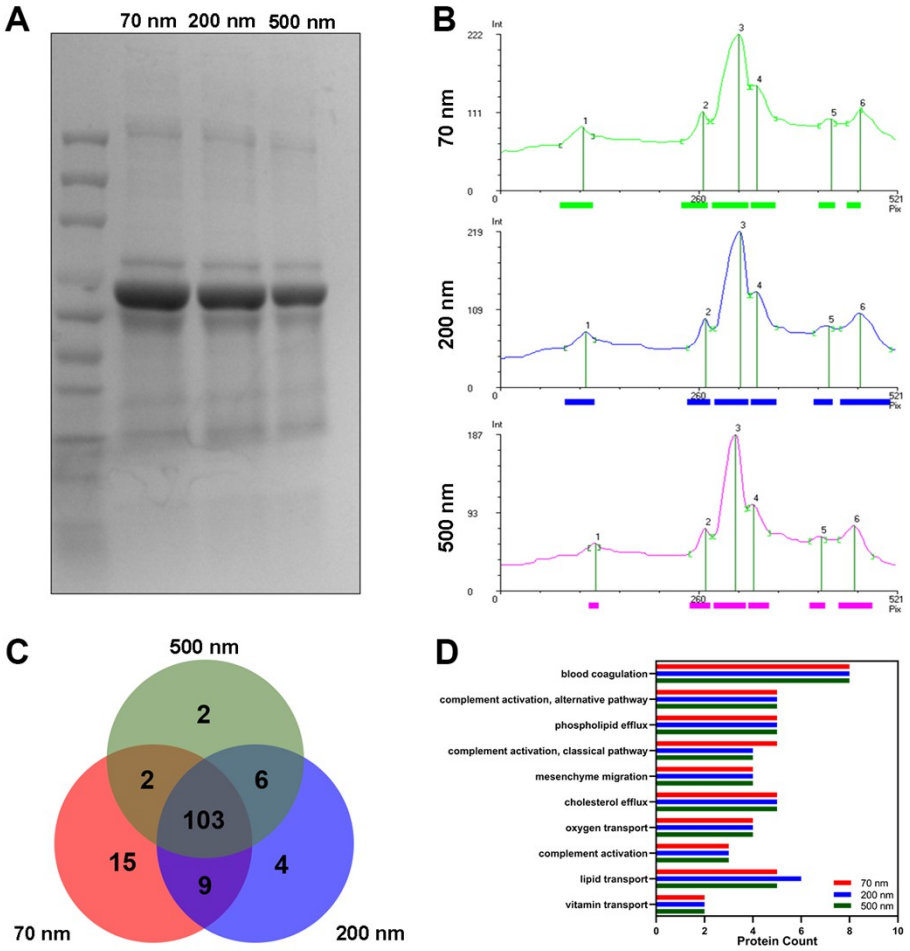

Figure S3. Influence of the surface states of the NPs on their interaction with ECM/migrasomes. (A) SDS-PAGE image showing the composition of the protein corona of NPs with different particle sizes. (B) Grayscale analysis of the protein bands in SDS-PAGE image. (C) Venn diagram illustrating the proteins identified in both biological replicates in the protein corona of three kinds of NPs. (D) DAVID cluster analysis of surface proteins of NPs with different particle sizes.

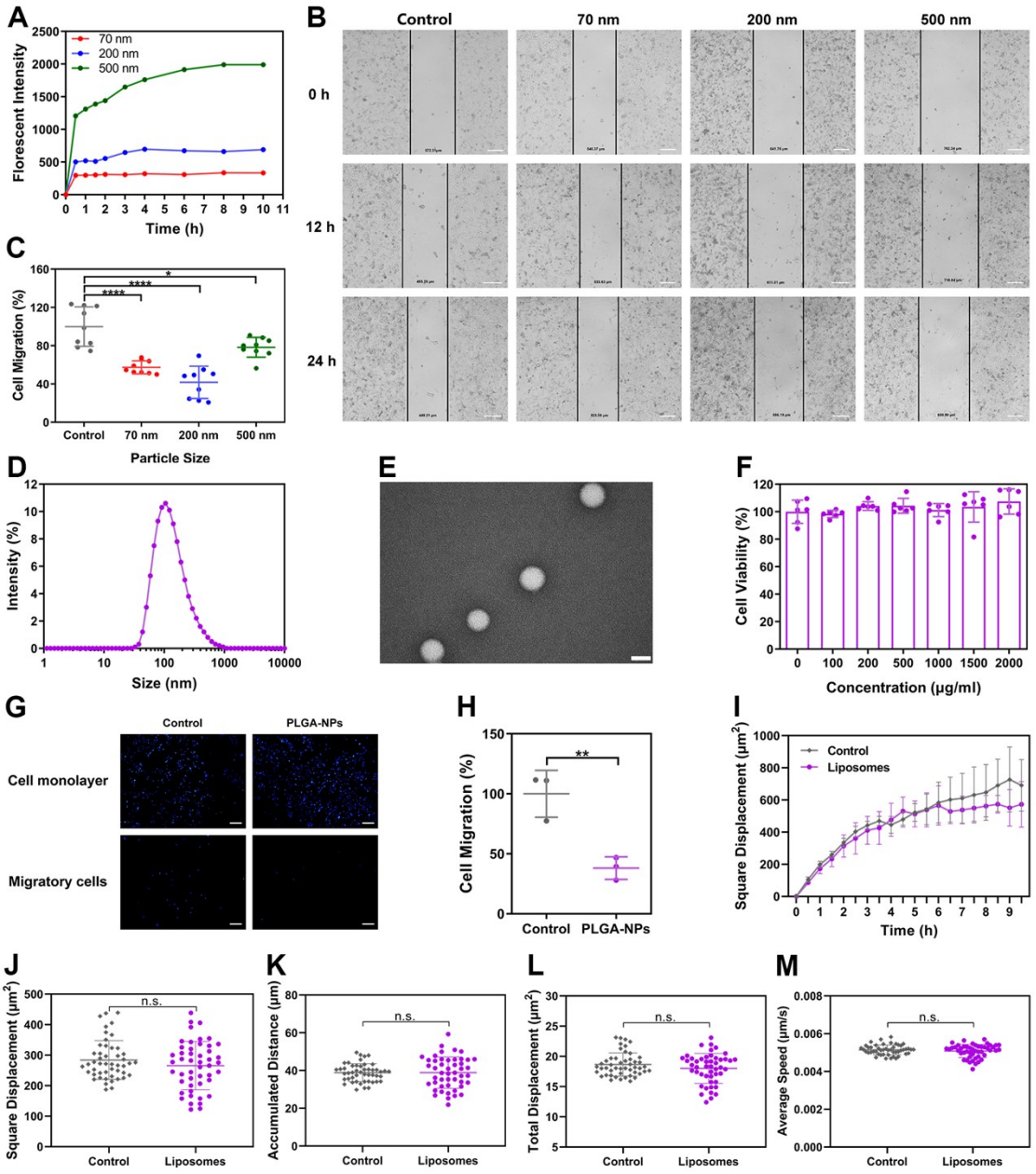

Figure S4. (A) Florescent intensity of polystyrene nanoparticles (NPs) in basolateral chambers of Transwell insert at different time points. (B) Representative images of the wound healing assay on MDA-MB-231 cells at 0 h, 12 h, and 24 h. Scale bar, 200 μm. (C) MDA-MB-231 cells migration ratio incubated with NPs for 24 h tested by wound healing assay (n = 9). (D) The intensity size distribution of PLGA-NPs. (E) Representative TEM images of PLGA-NPs. Scale bar, 50 nm. (F) Cell viability of MDA-MB-231 cells incubated with PLGA-NPs tested by CCK-8 assay (n = 6). (G) Transwell membrane apical and basolateral images. Scale bar, 200 μm. (H) MDA-MB-231 cells migration ratio incubated with PLGA-NPs tested by Transwell assay (n = 3). (I) Square displacement of MDA-MB-231 cells incubated with liposomes at each time point calculated by high content analysis system (n = 3). Mean square displacement over 10 h (J), accumulated distance (K), total displacement (L) and average speed (M) of MDA-MB-231 cells incubated with liposomes calculated by high content analysis system (n = 48). (Data were presented as mean±SD. Statistical significances were calculated by Student's t-test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.)

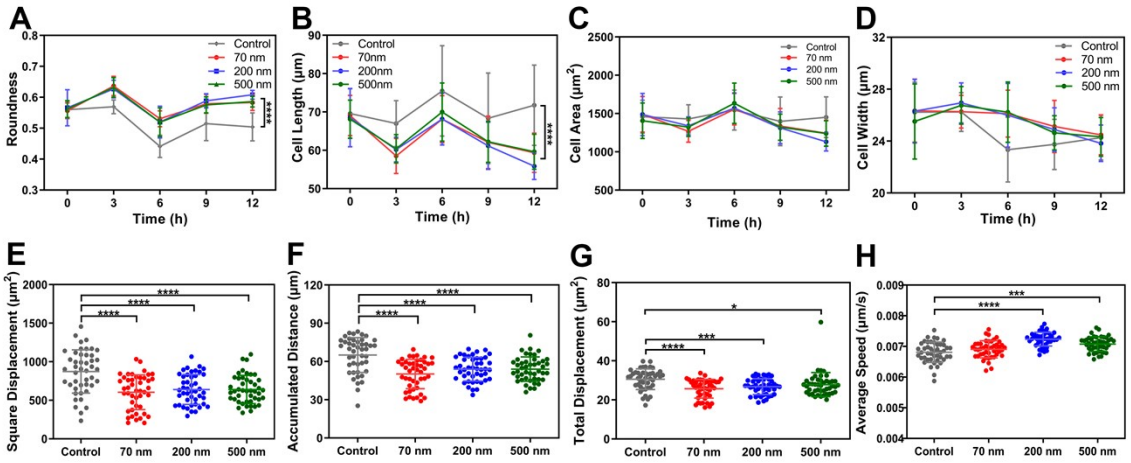

Figure S5. Roundness (A), length (B), area (C) and width (D) of MDA-MB-231 cells incubated with polystyrene nanoparticles (NPs) calculated by high content analysis system (n = 51). Mean square displacement over 12 h (E), accumulated distance (F), total displacement (G) and average speed (H) of MDA-MB-231 cells incubated with NPs calculated by high content analysis system (n = 51). (Data were presented as mean±SD. Statistical significances were calculated by Student's t-test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.)

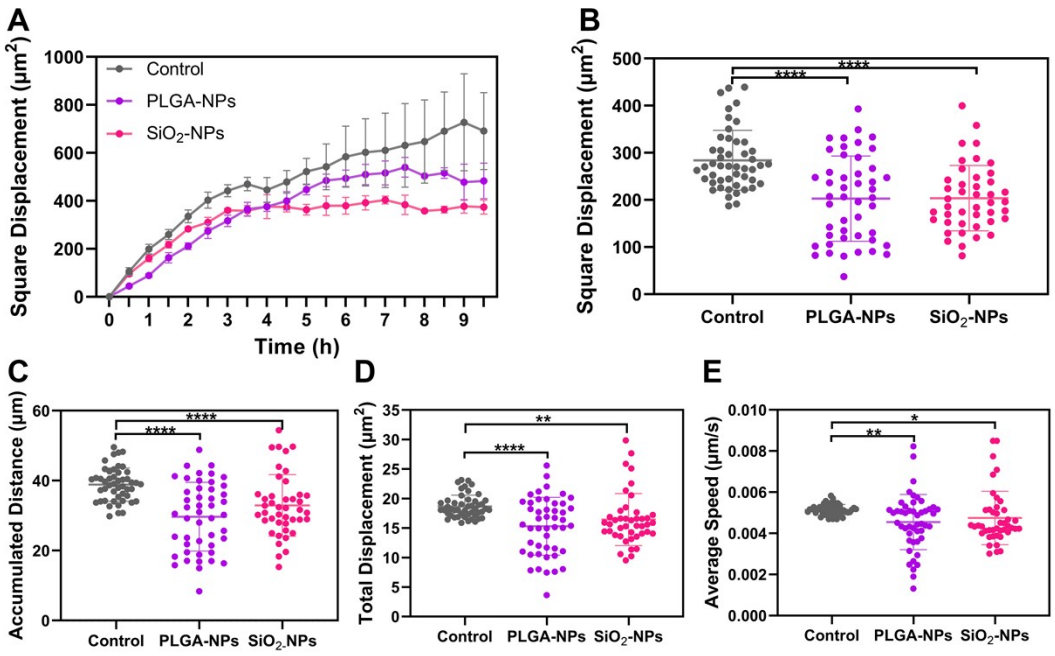

Figure S6. (A) Square displacement of MDA-MB-231 cells incubated with PLGA-NPs and SiO_2-NPs at each time point calculated by high content analysis system (n = 3). Mean square displacement over 10 h (B), accumulated distance (C), total displacement (D) and average speed (E) of MDA-MB-231 cells incubated with PLGA-NPs and SiO_2-NPs calculated by high content analysis system (n = 48). (Data were presented as mean±SD. Statistical significances were calculated by Student's t-test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.)

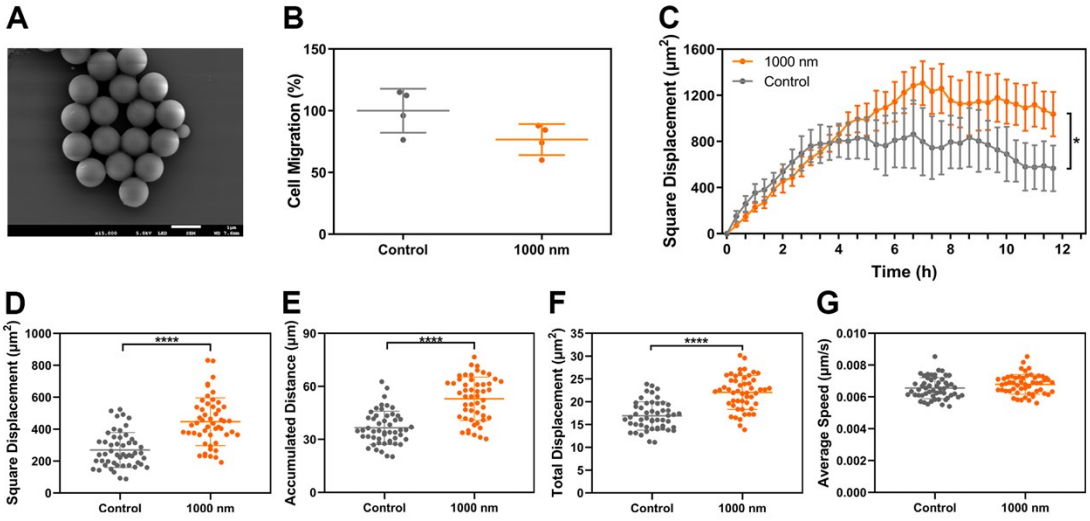

Figure S7. Cellular migration and movement with the incubation of 1000 nm polystyrene nanoparticles. (A) Representative SEM image of 1000 nm NPs. Scale bar, 1 μm. (B) MDA-MB-231 cells migration ratio after being incubated with 1000 nm NPs tested by Transwell assay (n = 4). (C) Square displacement of MDA-MB-231 cells incubated with 1000 nm NPs at each time point calculated by high content analysis system (n = 5). Mean square displacement over 12 h (D), accumulated distance (E), total displacement (F) and average speed (G) of MDA-MB-231 cells incubated with 1000 nm NPs calculated by high content analysis system (n = 51). (Data were presented as mean±SD. Statistical significances were calculated by Student's t-test: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.)

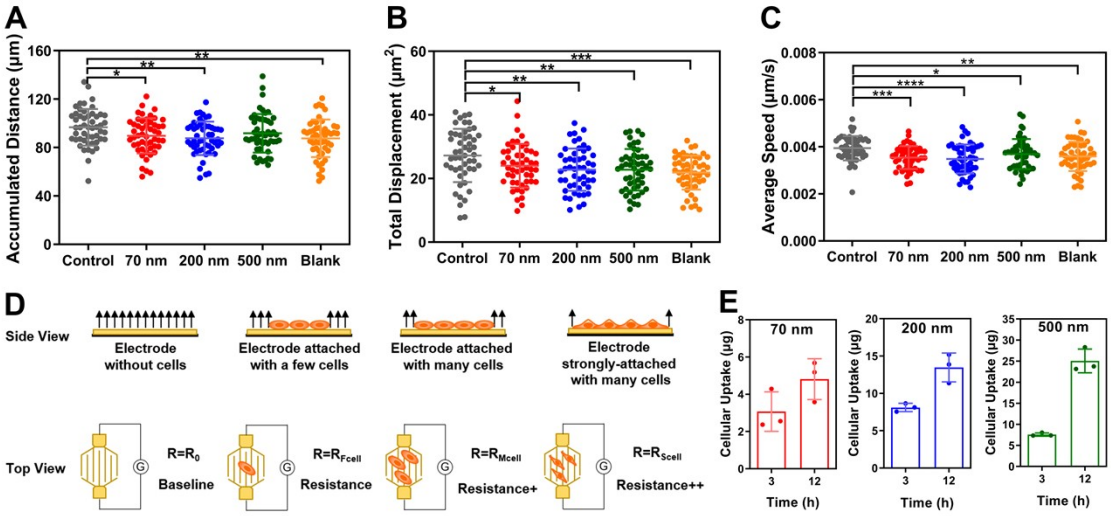

Figure S8. Accumulated distance (A), total displacement (B) and average speed (C) of MDA-MB-231 cells incubated on the ECM coated with polystyrene nanoparticles (NPs) calculated by high content analysis system (n = 51). (D) The scheme showing the working principle of RTCA system. (E) Cellular uptake of three kinds of NPs (n = 3). (Data were presented as mean±SD. Statistical significances were calculated by Student's t-test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.)

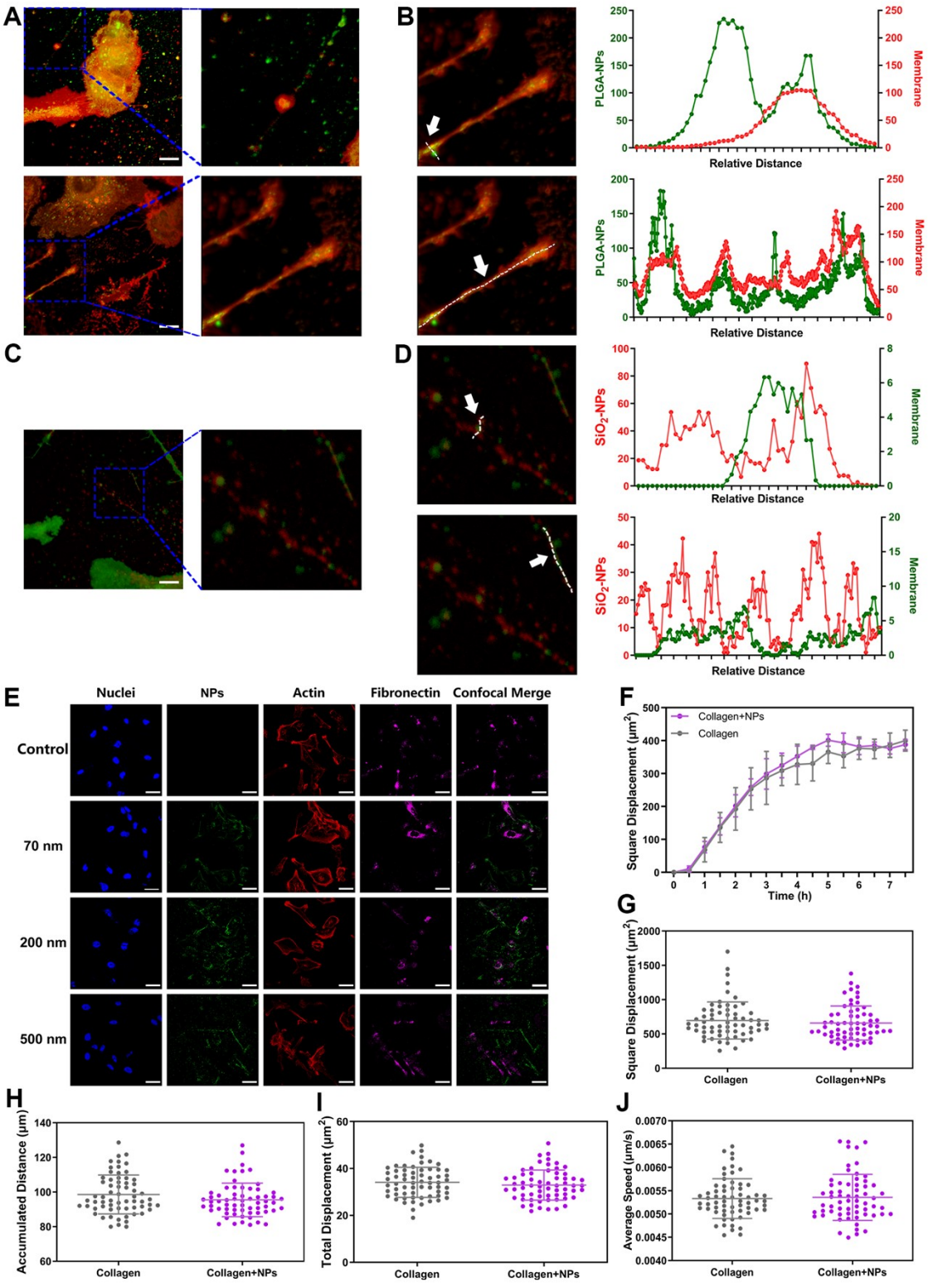

Figure S9. (A) Representative CLSM images showing the combination between PLGA-NPs and RFs of MDA-MB-231 cells. Scale bar, 10 μm. (B) Co-localization analysis of PLGA-NPs and membrane structures at different positions across the RFs or along the RFs (white lines, as arrows show). (C) Representative CLSM images showing the combination between SiO_2-NPs and RFs. Scale bar, 10 μm. (D) Co-localization analysis of SiO_2-NPs and membrane structures at different positions across the RFs or along the RFs (white lines, as arrows show). (E) Representative CLSM

images showing co-localization between polystyrene nanoparticles (NPs) and fibronectin. Scale bar, 50 μm. (F) Square displacement of MDA-MB-231 cells incubated on the collagen coated with NPs at each time point calculated by high content analysis system (n = 3). Mean square displacement over 8 h (G), accumulated distance (H), total displacement (I) and average speed (J) of MDA-MB-231 cells incubated on the collagen coated with NPs calculated by high content analysis system (n = 63). (Data were presented as mean±SD. Statistical significances were calculated by Student's t-test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.)

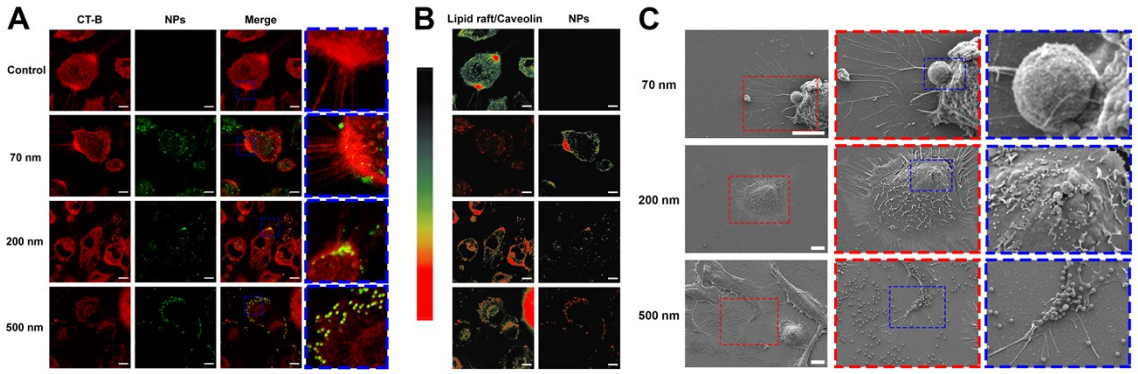

Figure S10. (A) Representative CLSM images showing co-localization between lipid rafts/caveolae (labeled by CT-B) and NPs on the cellular surface. Scale bar, 10 µm. (B) Surface plot showing the heterogeneous distribution of the co-localized points between lipid rafts/caveolae and NPs on the cellular surface. Scale bar, 10 µm. (C) Representative SEM images showing the heterogeneous binding of polystyrene nanoparticles (NPs) to the MDA-MB-231 cells' membrane. Scale bar, 10 µm.

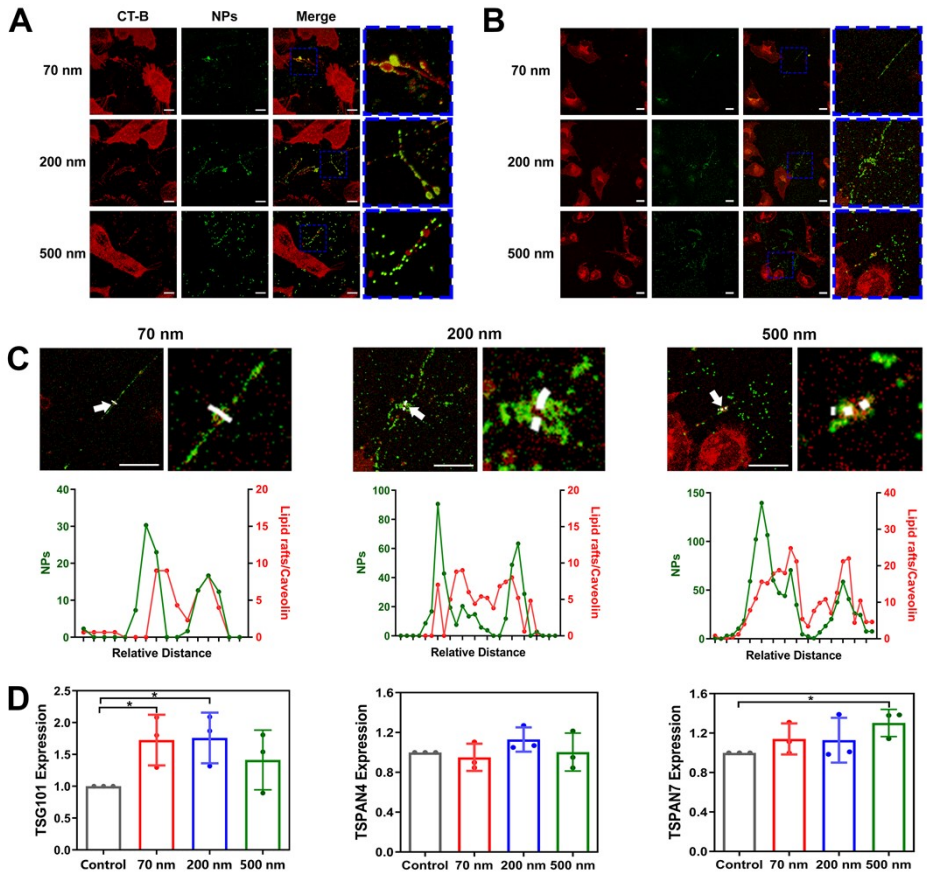

Figure S11. (A) Representative CLSM images showing co-localization between lipid rafts/caveolae (labeled by CT-B) and polystyrene nanoparticles (NPs) across migrasomes of MDA-MB-231 cells. Scale bar, 10 μm. (B) Representative CLSM images showing co-localization between lipid rafts/caveolae (labeled by Filipin) and NPs across migrasomes. Scale bar, 10 μm. (C) Co-localization analysis of NPs and lipid rafts/caveolae (labeled by Filipin) at different positions across migrasomes (white lines, as arrows show). Scale bar, 10 μm. (D) Gray analysis of TSPAN4, TSPAN7 and TSG101 expression in the NPs-treated ECM (n=3). (Data were presented as mean±SD. Statistical significances were calculated by Student's t-test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.)

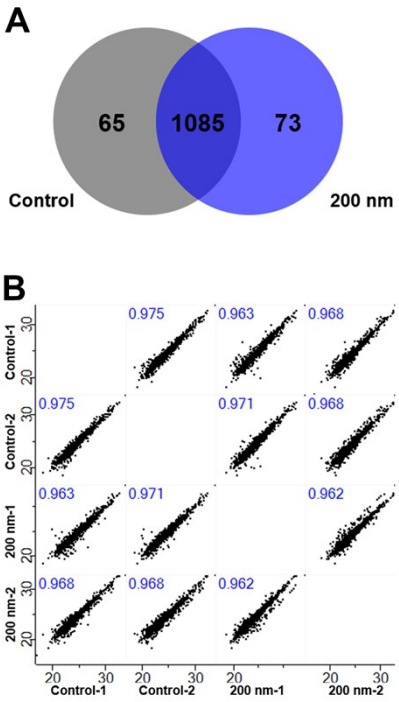

Figure S12. (A) Venn diagram illustrating the expression changes of proteins in the ECM over control group after treatment of 200 nm polystyrene nanoparticles. (B) Scatter plot illustrating the correlation coefficient of protein expression between duplicated groups or between control group and 200 nm NPs-treated group.

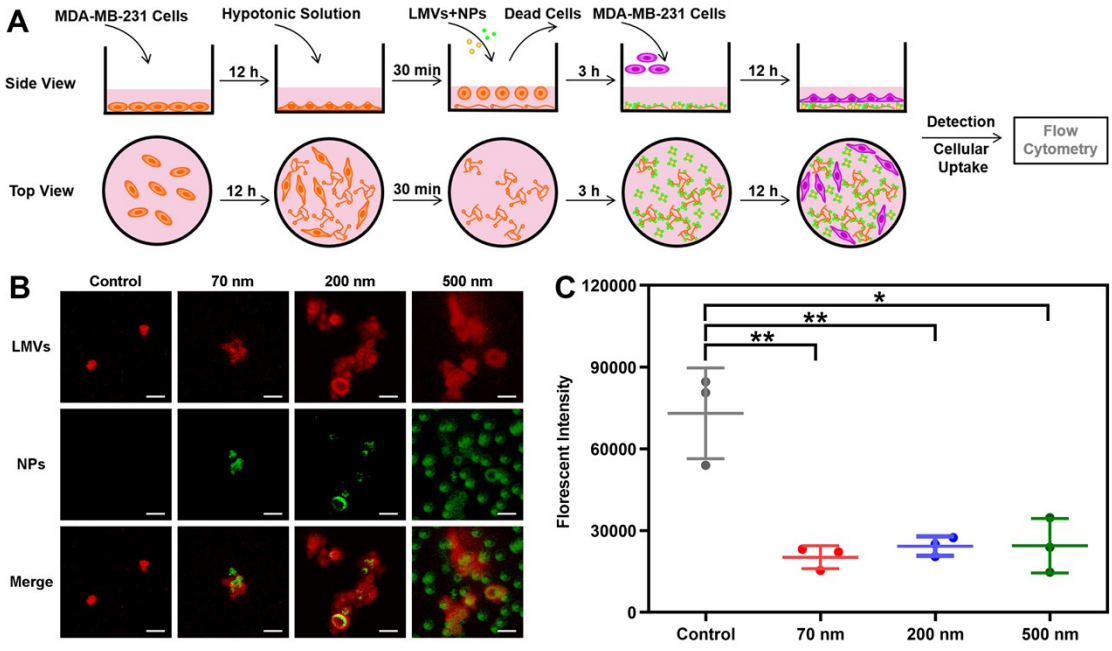

Figure S13. (A) The scheme of simulating nano-migrasome interaction and testing the endocytosis of LMVs. (B) Representative CLSM images showing LMVs incubated with polystyrene nanoparticles (NPs). Scale bar, 5 μm. (C) MDA-MB-231 cells' uptake of LMVs tested by FCM (n = 3). (Data were presented as mean±SD. Statistical significances were calculated by Student's t-test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.)

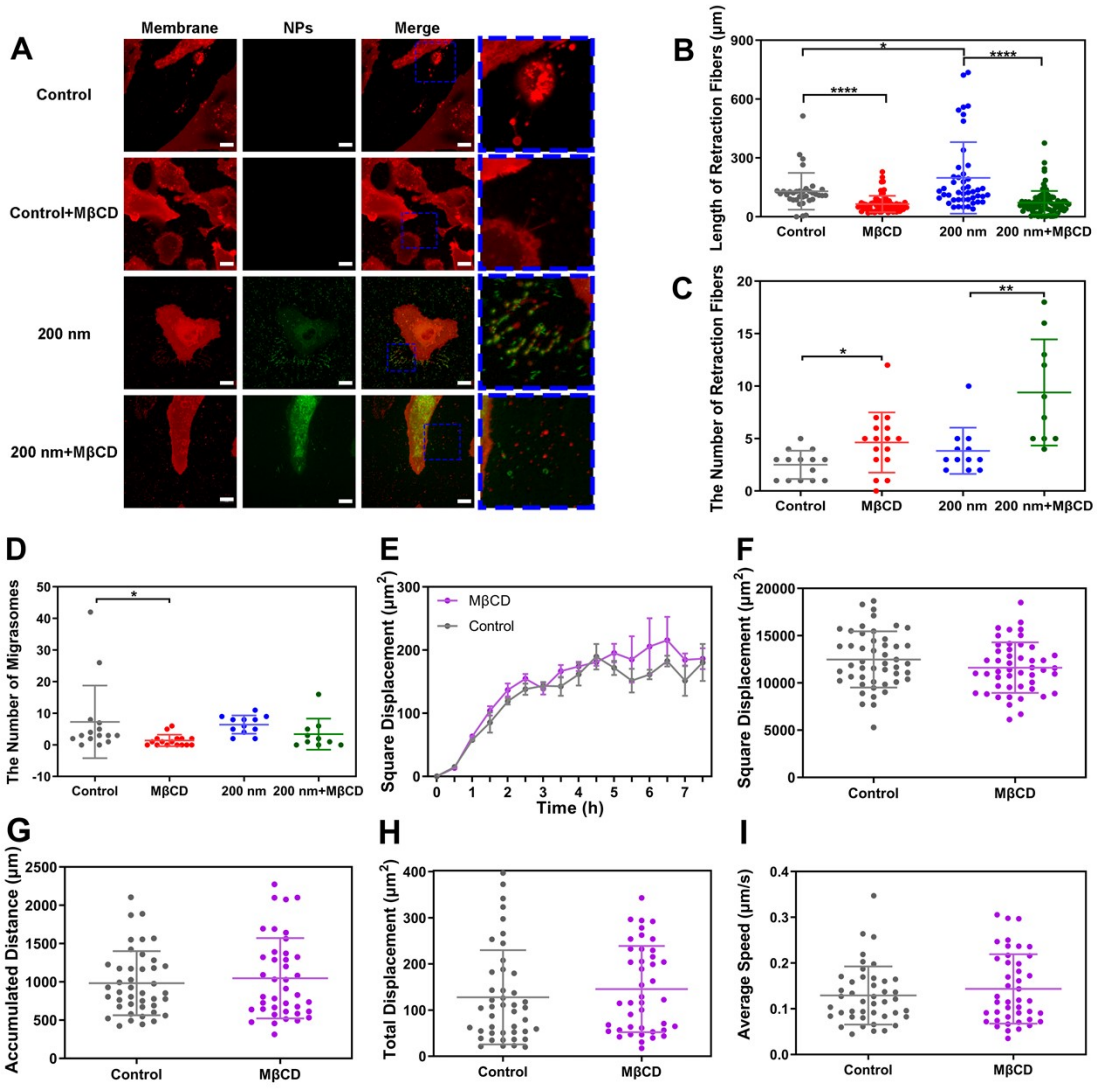

Figure S14. (A) Representative CLSM images showing the changes of RFs and migrasomes after MβCD treatment with or without the existence of polystyrene nanoparticles (NPs). Scale bar, 10 μm. (B) The length of RFs before and after MβCD treatment. (C) The number of RFs before and after MβCD treatment. (D) The number of migrasomes before and after MβCD treatment. (E) Square displacement of MDA-MB-231 cells incubated on the MβCD-treated ECM at each time point calculated by high content analysis system (n = 3). Mean square displacement over 8 h (F), accumulated distance (G), total displacement (H) and average speed (I) of MDA-MB-231 cells incubated on the MβCD-treated ECM calculated by high content analysis system (n = 48). (Data were presented as mean±SD. Statistical significances were calculated by Student's t-test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.)

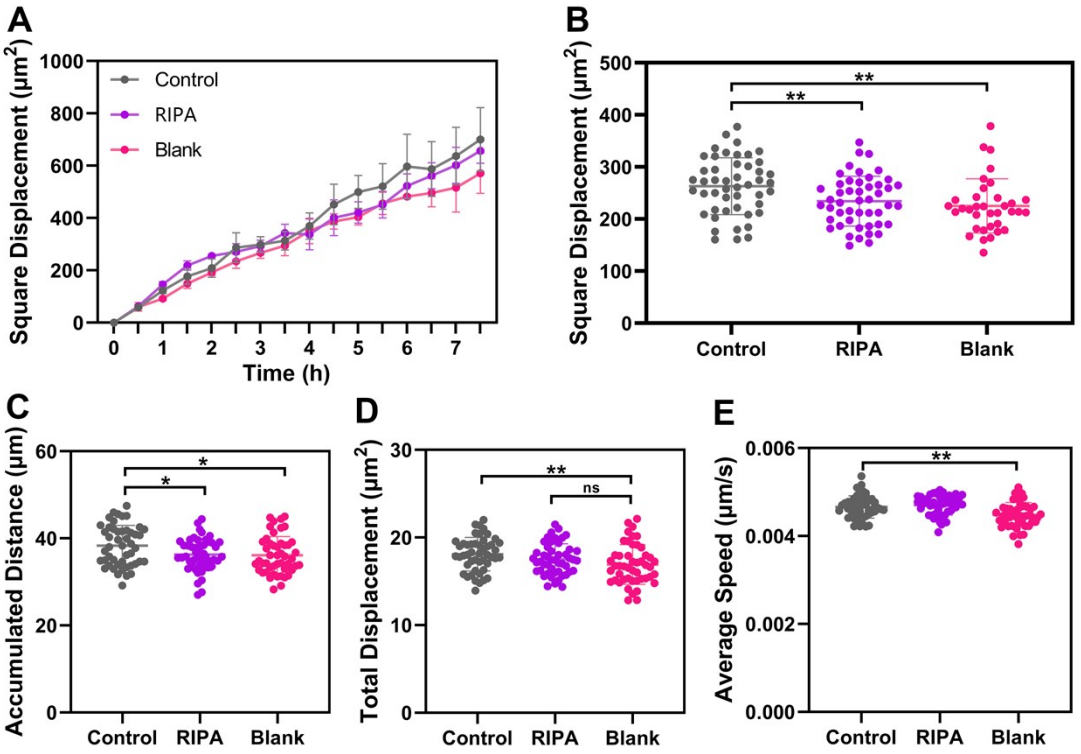

Figure S15. (A) Square displacement of MDA-MB-231 cells incubated on the RIPA lysis buffer-treated ECM at each time point calculated by high content analysis system (n = 3). Mean square displacement over 8 h (B), accumulated distance (C), total displacement (D) and average speed (E) of MDA-MB-231 cells incubated on the RIPA lysis buffer-treated ECM calculated by high content analysis system (n = 48). (Data were presented as mean±SD. Statistical significances were calculated by Student's t-test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.)

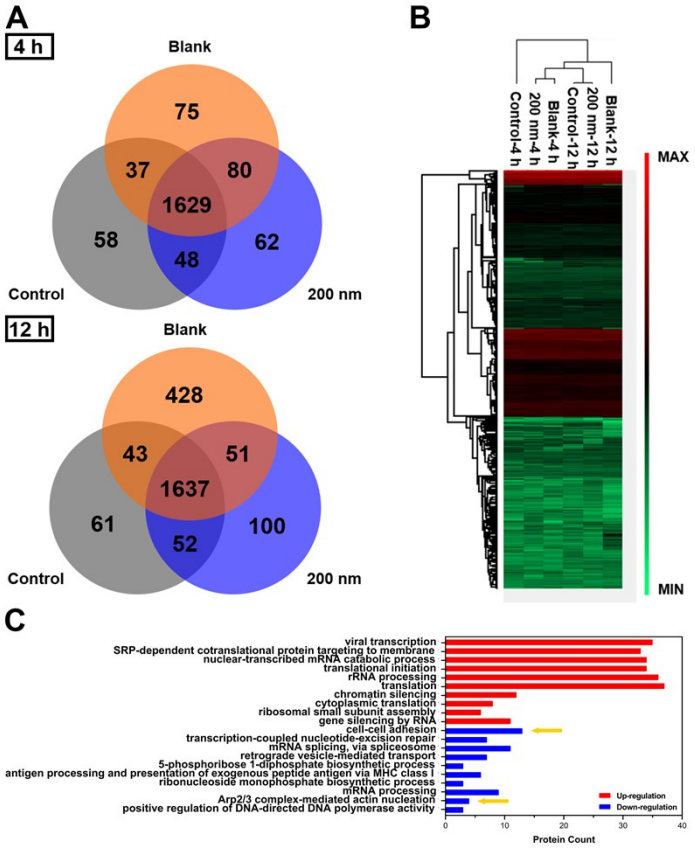

Figure S16. (A) Venn diagrams illustrating the expression changes of proteins after cells were incubated on the ECM or the 200 nm polystyrene nanoparticles (NPs) coated ECM for 4 h and 12 h. (B) The gene functional annotation clustering of upregulated and downregulated cellular proteins after MDA-MB-231 cells were incubated on the ECM or the 200 nm NPs coated ECM for 4 h and 12 h. (C) The diagram illustrating the DAVID cluster analysis of cellular proteins after cells were cultured on NPs coated ECM for 4 h.

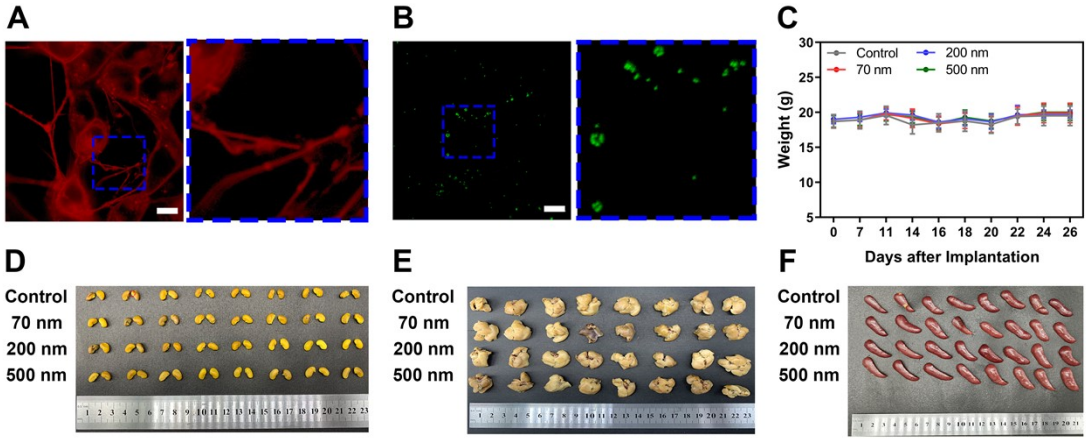

Figure S17. (A) Representative CLSM images showing extracellular membrane/lipid structures of 4T1 cells. Scale bar, 10 μm. (B) Representative CLSM images showing NPs binding with extracellular membrane/lipid structures of 4T1 cells. Scale bar, 10 μm. (C) Body weight changes of 4T1 tumor cells-bearing mice treated with different NPs (n = 8). (D) The image of kidneys obtained from mice treated with different NPs. (E) The image of livers obtained from mice treated with different NPs. (F) The image of spleens obtained from mice treated with different NPs. (Data were presented as mean±SD. Statistical significances were calculated by Student's t-test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.)

Supplementary Movie 1 Dynamic movement process of MDA-MB-231 cells incubated with polystyrene nanoparticles and the corresponding trajectories identified by high content analysis system. Scale, 100 μm.

Supplementary Movie 2 Dynamic process of the interaction between polystyrene nanoparticles and MDA-MB-231 cells as well as the ECM during the incubation. Scale, 100 μm.

Supplementary Movie 3 Dynamic movement process of MDA-MB-231 cells when they were cultured on polystyrene-nanoparticles-coated ECM and the corresponding trajectories identified by high content analysis system. Scale, 100 μm.

Supplementary Movie 4 Dynamic changes of RFs located at the rear of MDA-MB-231 cells and the binding process between polystyrene nanoparticles and RFs. Scale, 10 μm.

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