

Mimicking Melanosomes: Polydopamine Nanoparticles as Artificial Microparasols

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General Methods

All reagents were purchased as listed below and used without further purification. Dopamine hydrochloride, Triton X-100, Crystal violet and, 2',7'-dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma-Aldrich (St. Louis, MO). AuNPs were purchased from BBI Solutions (Cardiff, United Kingdom). EpiLife medium, HKGS growth supplement, Nucblue and LysoTracker Red DND-99 were purchased from Life Technologies (Carlsbad, CA). Mesothelial cell growth medium was purchased from Zen-Bio Inc. (Research Triangle Park, NC). Image-iT DEAD Green, Alexa Fluor 555, and Hoechst 33342 were purchased from Thermo Fisher Scientific (Waltham, MA). MALDI TOF-Mass Spectra were obtained on a Bruker Biflex IV at the UCSD Chemistry and Biochemistry Molecular Mass Spectrometry Facility. Confocal Laser Scanning Microscopy images were obtained on an Olympus FV1000. Cell sectioning was performed by the UCSD School of Medicine Cellular & Molecular Medicine Electron Microscopy Core Facility. Cell Transmission Electron Microscopy (TEM) images were acquired on carbon grids (Ted Pella, Inc.) and obtained on a JEOL 1200 EX II TEM at 80 kV. Cell TEM was performed on samples by preparing them in two different ways, further outlined and indicated in the sections below. All other TEM images were acquired on carbon grids (Ted Pella) and obtained on a FEI Tecnai G2 Sphera at 200 kV. FTIR spectra were obtained using a Thermo Scientific Nicolet 6700. Optical density measurements were recorded using a Perkin Elmer EnSpire Multimode Plate Reader. Cells were irradiated at 365 nm using a hand held UV lamp (8W) (UVP, LLC). Dynamic Light Scattering (DLS) was performed on a DynaPro Nanostar (Wyatt Technology Corp.) with nanoparticles suspended in Milli-Q water and tested at room temperature. Zeta-potentials were obtained on a Zetasizer Nano (Malvern Instruments Ltd.) with nanoparticles suspended in Milli-Q water at room temperature. Scanning Electron Microscopy (SEM) images were acquired using a FEI XL30 at 10KV with nanoparticles on a silicon substrate and sputtered with gold. Energy-dispersive X-ray spectroscopy (EDX) was obtained on a EDX Silicon Drift Deter (SDD) system on FEI XL30 with nanoparticles distributed on a silicon substrate in the Nano3 cleanroom at UCSD.

Synthesis of MelNPs

MelNPs were prepared by spontaneous oxidation of dopamine. Briefly, 40 mL 100% ethanol and 90 ml deionized water were mixed in a 250 mL round flask. 2 mL of a 28-30% NH_4OH solution was added to the flask and stirred vigorously for 10 min to ensure adequate mixing. A dopamine hydrochloride solution of 400 mg dopamine hydrochloride (Sigma) in 10 mL deionized water was slowly added to the mixture, under vigorous stirring. The color of the solution turned to pale yellow as soon as the dopamine hydrochloride solution was added, and gradually changed to dark brown over the course of the reaction. After 8 h, MelNPs were retrieved by centrifugation (14000 rpm, 10 min) and washed with deionized water three times.

Synthesis of PDA@ SiO_2 Core-shell Nanoparticles

Polydopamine cores (PDA) were synthesized by oxidative self-polymerization of dopamine under basic conditions according to our previous work.¹ Silica shells (SiO_2) were coated on the surface of the PDA via the modified Stöber method. PDA cores (6.5 mg) were firstly dispersed in a mixed solution of 2-propanol (10 mL) and deionized water (1.75 mL) under magnetic stirring. A 28-30% NH_4OH solution (0.25 ml) was added and stirred for 10 min. The silica precursor, tetraethyl orthosilicate (TEOS, 80 μL), was continuously injected into the mixture to form SiO_2 shells on the surfaces of PDA cores due to the

hydrolysis and condensation of TEOS under alkaline conditions. The reaction was conducted at room temperature under constant stirring for an additional 70 min. The PDA@SiO₂ core-shell nanoparticles were finally collected by centrifugation (4000 rpm/10 min), washed with deionized water three times and redispersed in deionized water for future use.

Synthesis of SiO₂@PDA Core-shell Nanoparticles

The SiO₂ core was synthesized by the Stöber method.² First, an ammonia solution (3 mL) was added to a solution consisting of ethanol (160 mL) and deionized water (40 ml) with stirring for 10 min. The silica precursor, tetraethyl orthosilicate (TEOS, 5 mL) was continuously added drop by drop. The reaction was conducted at room temperature under constant stirring for 3 h. SiO₂ cores were collected by centrifugation (4000 rpm/10 min), washed with deionized water three times, and redispersed in deionized water for the next step. 5 mg SiO₂ cores were dispersed in 2.5 ml Tris buffer (10 mM, pH 8.5). Dopamine (5.0 mg) was added and self-polymerized on the surface of SiO₂ cores to form PDA shells under constant stirring for 8 h. The SiO₂@PDA core-shell nanoparticles were collected by centrifugation (4000 rpm/10 min), washed with deionized water three times and redispersed in deionized water for future use.

MALDI TOF-Mass Spectrometry

MALDI measurements were performed on a Bruker Reflex time of flight instrument, operating in positive linear mode. Ionization was achieved with a pulsed UV laser beam (nitrogen laser, $\lambda=337$ nm) were accelerated to 15 keV, UV laser light (approx. 50 μ J) was focused on the sample, using a focal diameter of approx. 100-300 μ m. Synthetic MelNPs were suspended in double-distilled water to reach a final concentration of 1 mg/mL. This suspension was deposited on the stainless steel sample holder and air-dried. A saturated solution of the matrix (2,5-dihydroxybenzoic acid, DHB) in 50/50 (v/v) water/acetonitrile was then added and allowed to air-dry before insertion into the mass spectrometer. Mass spectra were obtained by averaging the ions from 100 laser shots. Daily external calibration was provided by the [M + H] ions of angiotensin II (m/z 1046) and DHB (m/z 155). (**Figure S4**)

Confocal Laser Scanning Microscopy for Time-dependent Uptake in HEKa cells

HEKa cells were plated and incubated overnight in 35 mm glass-bottom round dishes in a 5% CO₂ atmosphere at 37 °C using EpiLife medium with HKGS growth supplement (Life Technologies). 2 mL of a 0.02 mg/mL MelNPs suspension was added and incubated with the cells for either 4 hours, 1 day, 2 days or 3 days. After washing with PBS buffer, the cells were incubated with the nuclear stain NucBlue (Life Technologies) for 25 min and then fixed with a 4% paraformaldehyde solution for 20 min. Finally, the cells were washed with PBS and imaged by confocal microscopy. (**Figure S6**)

Cell Pellet Embedding for Transmission Electron Microscopy. Utilized for Concentration-dependent Uptake Studies of MelNPs in HEKa cells

HEKa cells were plated in 35 mm round plastic dishes and incubated in a 5% CO₂ atmosphere at 37 °C using EpiLife medium with HKGS growth supplement (Life Technologies) until reaching ~80% confluency. Different concentrations of MelNPs in growth media were added and incubated for 4 hours. The cells were washed three times with PBS to remove free MelNPs, then fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH = 7.4 (SC buffer) on ice for more than 2 hours. After washing three times

with 0.1 M SC buffer for 5 min, the cells were post-fixed with 1% osmium tetroxide in 0.1 M SC buffer for 1 hour on ice. The cell pellets were washed 3 times with 0.1 M SC buffer for 5 min, followed by a quick rinse in distilled H₂O. Cell pellets were stained with 2% uranyl acetate (UA) for 1 hour on ice, then dehydrated in a graded series of ethanol (50%, 70%, 90% and 2 times 100%) for 5-8 min, and dried in acetone at room temperature. The cell pellets were infiltrated by a 50:50 dry acetone/Durcupan solution for 1-2 hours on a shaker, followed by 100% Durcupan overnight and two further treatments of 100% durcupan the next day. Finally, cell pellets were embedded in durcupan and incubated at 60 °C for 36-48 hours. Ultrathin sections (around 60 nm) were cut and examined by electron microscopy (**Figure S5**).

Flat Embedding for Preparation of Cells in Monolayer Culture for Transmission Electron Microscopy of HEKa Cells for Treatment with Nanoparticles

Round glass coverslips (18 mm diameter/1 mm thickness, Chemglass Life Science Inc.) were placed in a 12-well plastic plate, and then immersed in 70% ethanol solution for overnight sterilization under UV, till dryness. HEKa cells were plated in a 12-well plastic plate on coverslips and incubated in a 5% CO₂ atmosphere at 37 °C using EpiLife medium with HKGS growth supplement (Life Technologies) until reaching ~80% confluency. Then 0.02 mg/mL MelNPs, PDA@SiO₂ core-shell nanoparticles, SiO₂@PDA core-shell nanoparticles and AuNPs with similar particle numbers were added to the medium and incubated for either 4 hours, 1 day, 2 days or 3 days. The cells were washed 3 times with PBS to remove excess nanoparticles, and then fixed with 2% glutaraldehyde and 2mM CaCl₂ in 0.1 M sodium cacodylate buffer at pH = 7.4 (SC buffer) on ice for more than 60 min at 4 °C. The cell monolayer covered coverslips were washed by 0.1 M SC buffer for 2 min (5 times), and then were incubated in 1% OsO₄ in 0.1 M SC buffer for 30 min to 1 hour on ice. After the incubation, the coverslips were washed by 0.1M SC buffer for 2 min (5 times) again, and followed by Milli-Q water for 2 min (2 times). Then all coverslips were incubated with 2% UA for 30 min to 1hr on ice, and were rinsed in Milli-Q water. The monolayer cells were dehydrated with gradient ethanol (20% 50% 70% 90% 2X100%) for 1 min respectively, and then were dried with acetone for 1 min (2 times) on ice. The monolayer cell coverslips were immersed in a solution of 50/50 Durcupan /Acetone for 1 hour with gentle rotation at room temperature, and then incubated with 100% Durcupan for another 1 hour for full infiltration. Then the coverslips were embedded in aluminum foil dishes and incubated in 60 °C oven for 24 to 36 hours. TEM sections were cut with a diamond knife at a 50-60 nm thickness. All sections were post-stained with uranyl acetate and lead for better contrast. The images were collected on a FEI Tecnai Spirit scope at 80kV. (Main Text **Figure 2, Figure S7, Figure S11, Figure S13**)

Confocal Laser Scanning Microscopy for Time-dependent Uptake of MelNPs in MeT-5A cells

MeT-5A cells were plated in 35 mm round glass-bottom dishes in a 5% CO₂ atmosphere at 37 °C using mesothelial cell growth medium (Zen-Bio Inc.) overnight. 2 mL of a 0.02 mg/mL MelNP suspension were added and incubated with the cells for either 4 hours, 1 day, 2 days or 3 days. After washing with PBS buffer, the cells were stained with the nuclear stain Nucblue (Life Technologies) for 20 min. The cells were fixed with a 4% paraformaldehyde solution, then washed with PBS and imaged by confocal microscopy. (**Figure S9**)

Transmission Electron Microscopy for Time-dependent Uptake of MelNPs in MeT-5A cells

MeT-5A cells were plated in 35 mm round plastic dishes and incubated in a 5% CO₂ atmosphere at 37 °C

using mesothelial cell growth medium (Zen-Bio Inc.) until reaching ~80% confluency. Then, 2 mL of a 0.02 mg/mL suspension of MelNPs were added to the medium for different time incubation (1 day, 2 days and 3 days). The cells were washed with PBS three times to remove free MelNPs, and then were processed with a similar protocol as described for the flat embedding procedure above. (**Figure S9**)

Confocal Laser Scanning Microscopy for Colocalization of MelNPs and LysoTracker

HEKa cells were plated in 35 mm round glass-bottom dishes in a 5% CO₂ atmosphere at 37 °C using EpiLife medium with HKGS growth supplement (Life Technologies) overnight. 2 mL of a 0.02mg/mL MelNP suspension was incubated with the cells for either 4 hours, 1 day, 2 days or 3 days. After washing to remove free MelNPs the cells were stained with the nuclear stain Nucblue (Life Technologies) and the lysosome stain LysoTracker Red DND-99 (Life Technologies), fixed with a 4% paraformaldehyde solution, washed with PBS, then imaged via confocal microscopy (Main Text **Figure 2**)

Crystal Violet Stain for HEKa cells

HEKa cells were plated in 35mm round glass-bottom dishes in a 5% CO₂ atmosphere at 37 °C using EpiLife medium with HKGS growth supplement (Life Technologies) overnight. 2 milliliters of a 0.02 mg/mL MelNPs suspension was incubated with the cells for 3 days, after which they were washed with PBS buffer to remove free MelNPs. Cells treated with and without MelNPs were irradiated by UV for 5 min, then incubated under normal cell culture conditions for another 24 hours. The cells were fixed in a 4% paraformaldehyde solution for 15 min at room temperature, then stained with 0.1%w/v crystal violet for 20 min at room temperature. After pouring off the crystal violet solution, the cells were gently washed with deionized water until the water ran clear, then dried at room temperature overnight. 1mL of 100% methanol was added to re-solubilize the cells, whose OD absorption at 540 nm was measured using a plate reader. (Main Text **Figure 3**)

Confocal Laser Scanning Microscopy for ROS detection

HEKa cells were plated in 35mm round glass-bottom dishes in a 5% CO₂ atmosphere at 37 °C using EpiLife medium with HKGS growth supplement (Life Technologies) overnight. 2 milliliters of a 0.02 mg/mL MelNPs, PDA@SiO₂ core-shell nanoparticles, SiO₂@PDA core-shell nanoparticles and AuNPs were incubated with the cells for 3 days. After washing with PBS buffer to remove free MelNPs, 2ml 0.01 mM 2',7'-dichlorofluorescein diacetate (DCFH-DA) PBS solution was added to the cells and incubated for 20 min. After washing with PBS buffer, cells with and without treated MelNPs were irradiated by UV for 5 min, then stained by the nuclear stain Nucblue for 25 min at room temperature and fixed with a 4% paraformaldehyde solution for 20 min at room temperature. The cells were washed with PBS and imaged by confocal microscopy. (Main Text **Figure 4, Figure S14**).

Confocal Laser Scanning Microscopy for DNA Damage Stain

HEKa cells were plated in 35 mm round glass-bottom dishes in a 5% CO₂ atmosphere at 37 °C using EpiLife medium with HKGS growth supplement (Life Technologies) overnight. 2 mL of a 0.02 mg/mL MelNPs suspension was incubated with the cells for 3 days, after which they were washed with PBS buffer to remove free MelNPs. Cells, treated with and without MelNPs, were irradiated by UV for 5 min, then incubated under normal cell culture conditions for another 24 hours. To test cell viability, Image-iT DEAD Green (Thermo Fisher Scientific) was added to the cells and allowed to incubate for 30 min under

normal cell culture conditions. The cells were then fixed in a 4% paraformaldehyde solution for 15 min, permeabilized by a Triton X-100 solution (15 μ l in 6 mL PBS) for 15 min, and blocked by a BSA solution (0.25 g in 25 mL PBS) for 1 hour. Subsequently, a solution of the primary antibody pH2ax was added to the cells and incubated for 1 hour. The Alexa Fluor 555 conjugated secondary antibody/ Hoechst 33342 nuclear counterstain (Thermo Fisher Scientific) (3 μ l/1 μ l respectively in 6 mL BSA blocking buffer) was added to the cells and incubated for another hour. Finally, the cells were washed with PBS buffer and imaged by confocal microscopy. (Main Text **Figure 5** and **Figure S16**)

IR images for MeINPs with gradient concentrations

To test for heat generation under UV irradiation, aqueous solutions of MeINPs ranging from 2 mg/mL to 0.002 mg/mL were prepared. These solutions were irradiated for 5 min. IR spectra were obtained immediately after UV irradiation. Pure distilled water was used as a reference. (**Figure S15**)

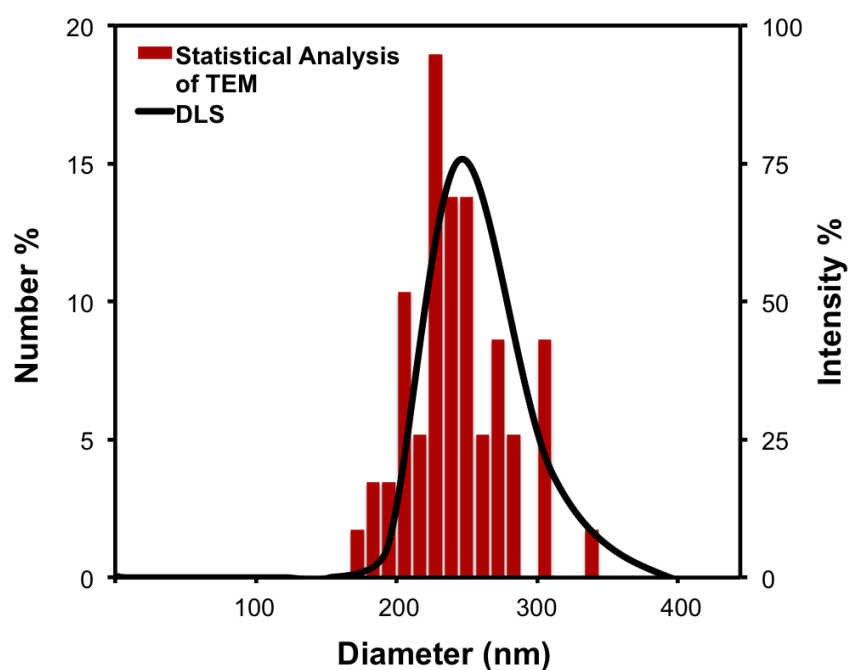


Figure S1. Average size (number %) of synthetic MeINPs by analysis of TEM images (red columns) and DLS measurement (black curve).

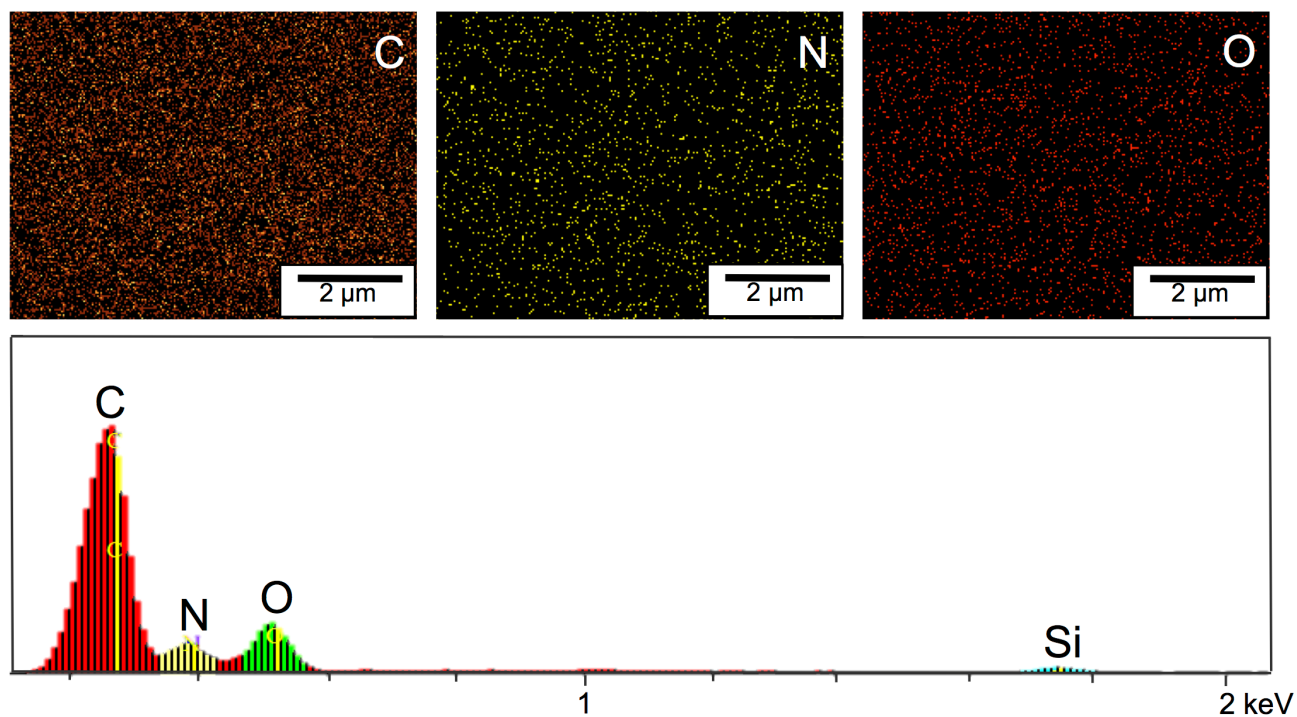


Figure S2. EDX mapping images and spectrum of MelNPs.: Carbon (C, Orange), Nitrogen (N, yellow), Oxygen (O, red). MelNPs solution (~10 mg/ mL) was dropped on silicon substrate and dried under room temperature for test without gold coating.

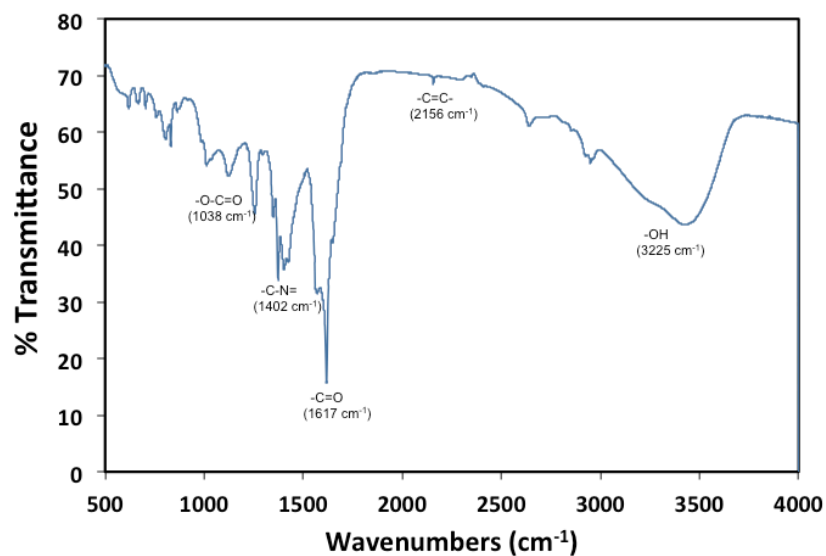


Figure S3. FTIR measurement of MelNPs.

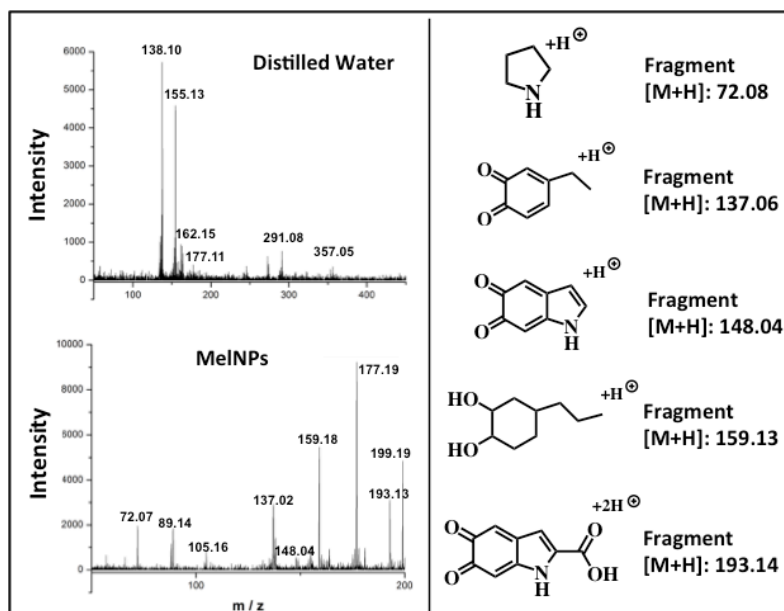


Figure S4. MALDI-TOF MS spectrum of distilled water and MelNPs, and structures consistent with masses observed in the spectrum.

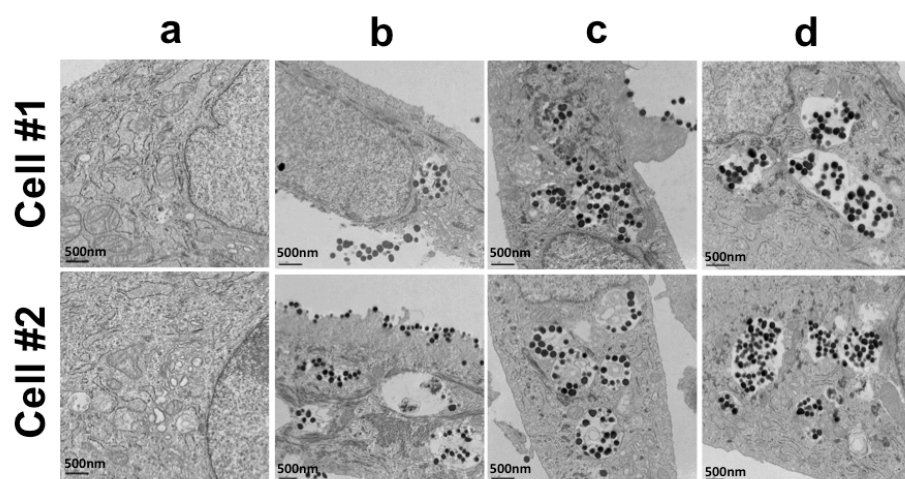


Figure S5. Stained TEM images for HEKa cells incubated with different concentrations of MelNPs for 4 hours: a) 0 mg/mL; b) 0.4 mg/mL; c) 0.1 mg/mL; d) 0.02 mg/mL. Cell sections were obtained by sectioning pellets, as described above.

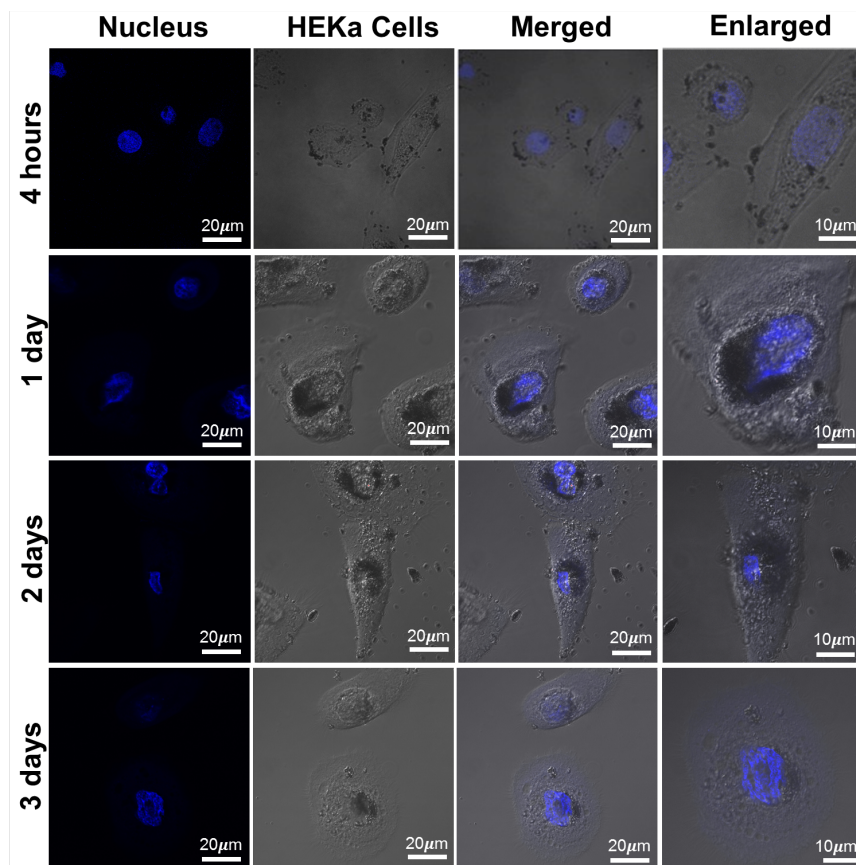


Figure S6. Confocal images for HEKa cells incubated with 0.02 mg/mL MeINPs for 4 hours, 1 day, 2 days and 3 days, respectively. Nuclei of HEKa cells were stained by Nucblue (blue); MeINPs were black in HEKa cells under bright field microscopy.

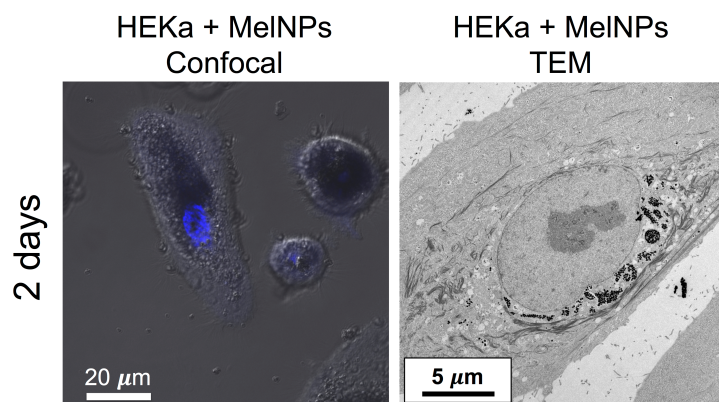


Figure S7. Confocal image (left) and stained TEM image (right) of HEKa cells after incubating with 0.02 mg/mL MeINPs for 2 days. Nuclei of HEKa cells were stained by Nucblue (blue); MeINPs were black in HEKa cells under bright field microscopy. Cell sections for TEM were obtained by flat embedding cells in monolayer cell culture, not from cell pellets – See section above for detailed description of procedure used.

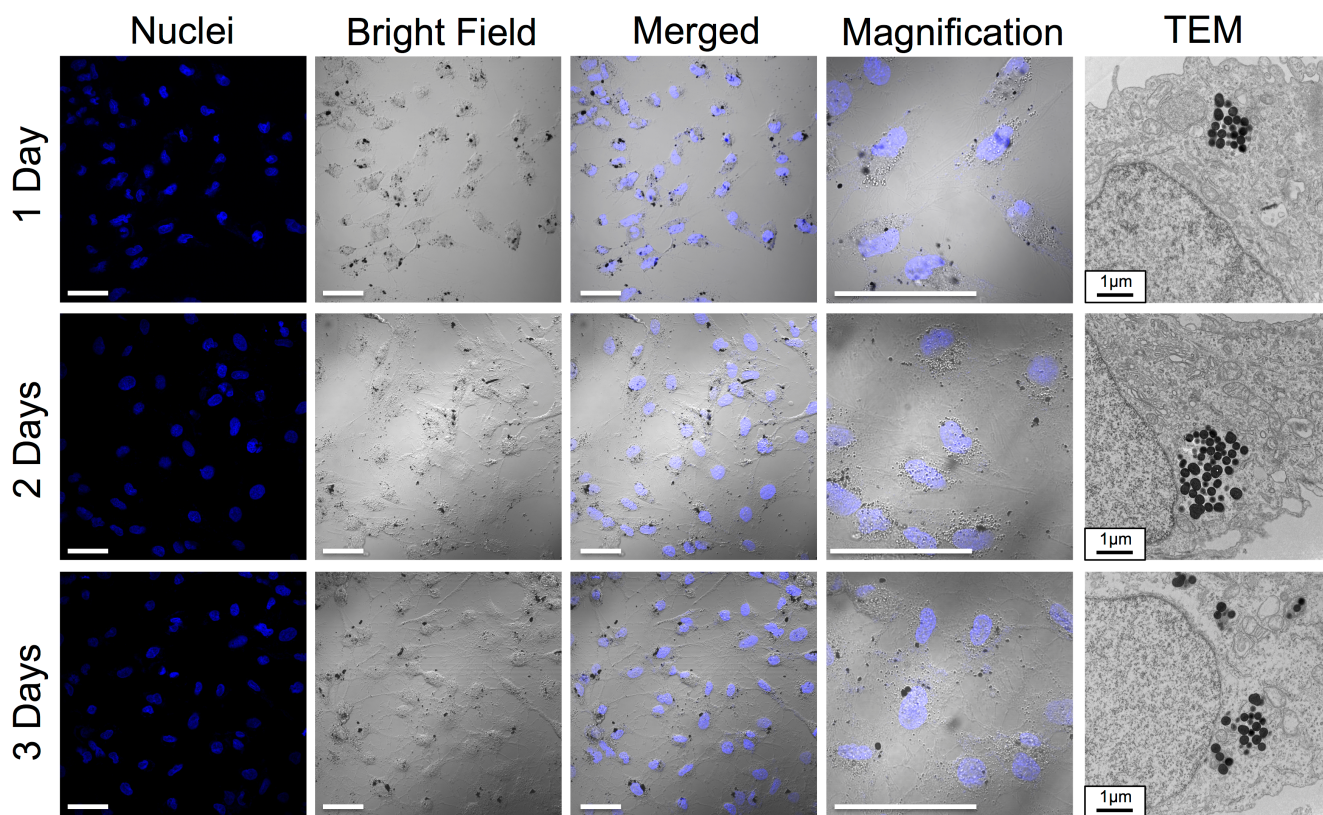


Figure S8. Confocal images and stained TEM images of the time-dependent uptake of 0.02 mg/mL MeINPs in MeT-5A cells at 1, 2 and 3 days respectively. Nuclei of MeT-5A cells were stained by Nucblue (blue); MeINPs were black in HEKa cells under bright field microscopy. Cell sections for TEM were obtained by sectioning pellets, as described above.

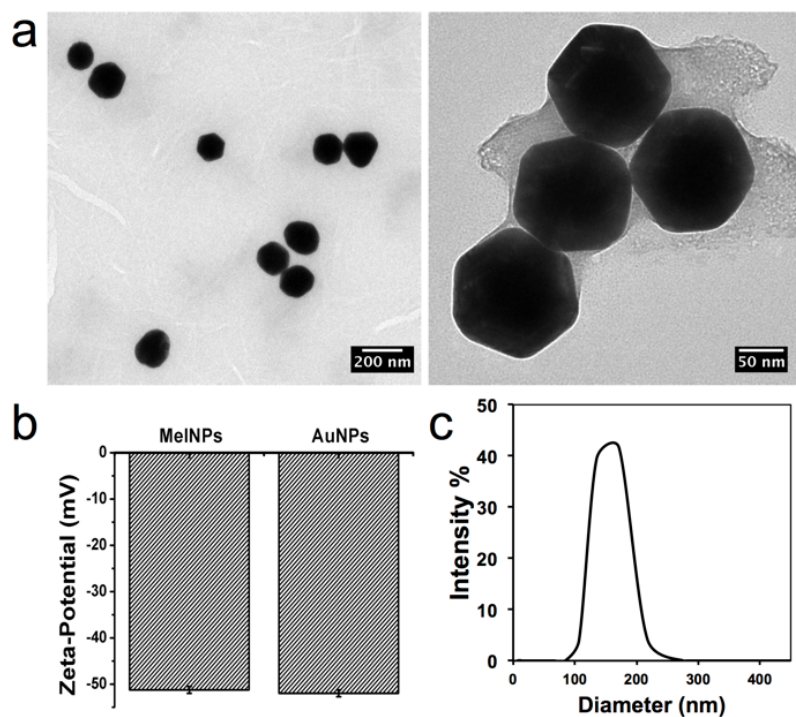


Figure S9. Characterization of AuNPs. a) TEM images of AuNPs with diameters around 150 nm; b) Zeta-potentials of MeINPs and AuNPs; c) DLS measurement of AuNPs (average diameter = 180 nm).

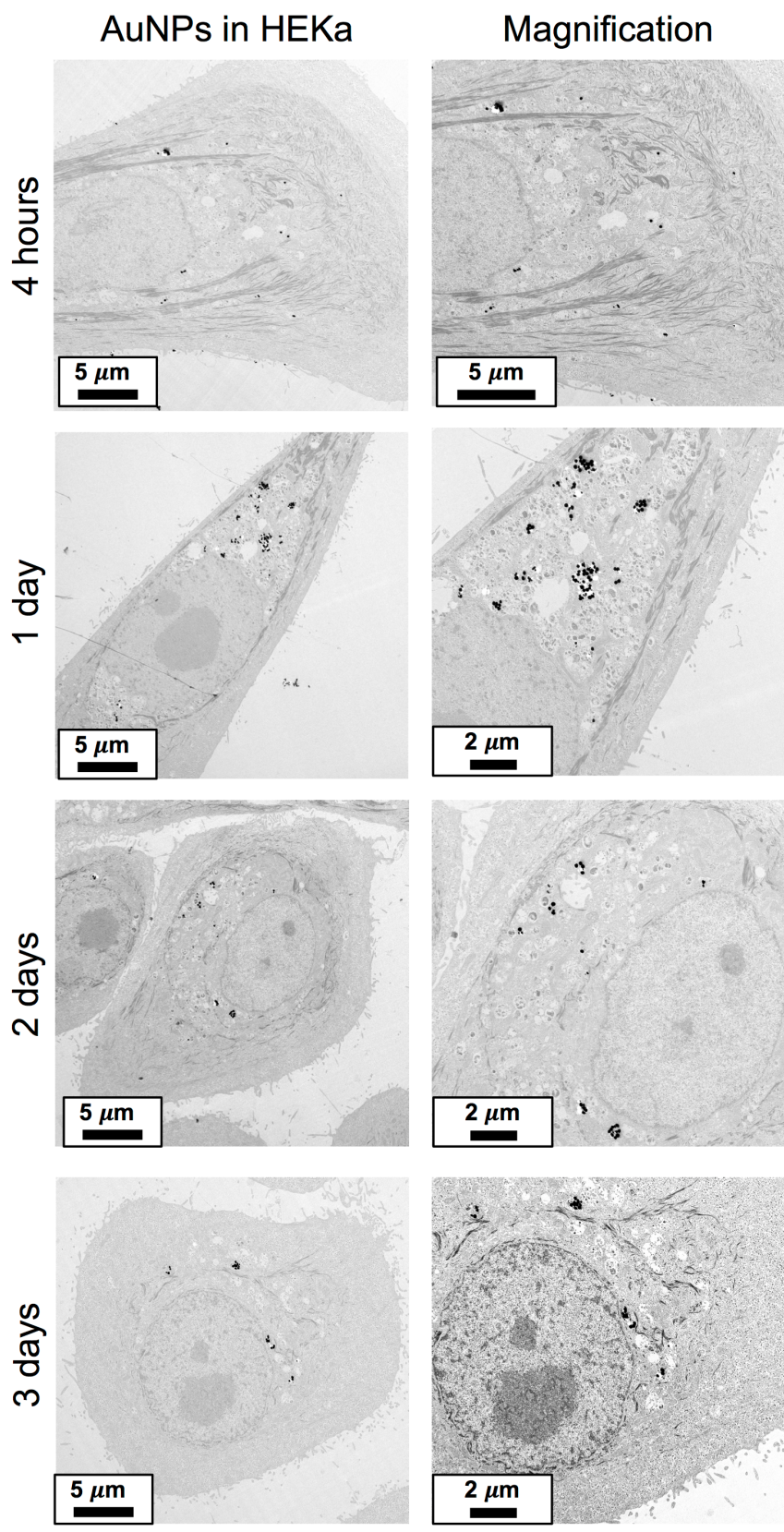


Figure S10. Stained TEM images obtained by the flat embedding method for HEKa cells incubated with AuNPs for 4 hours, 1 day, 2 days, and 3 days, showing the random distribution of AuNPs in HEKa cells.

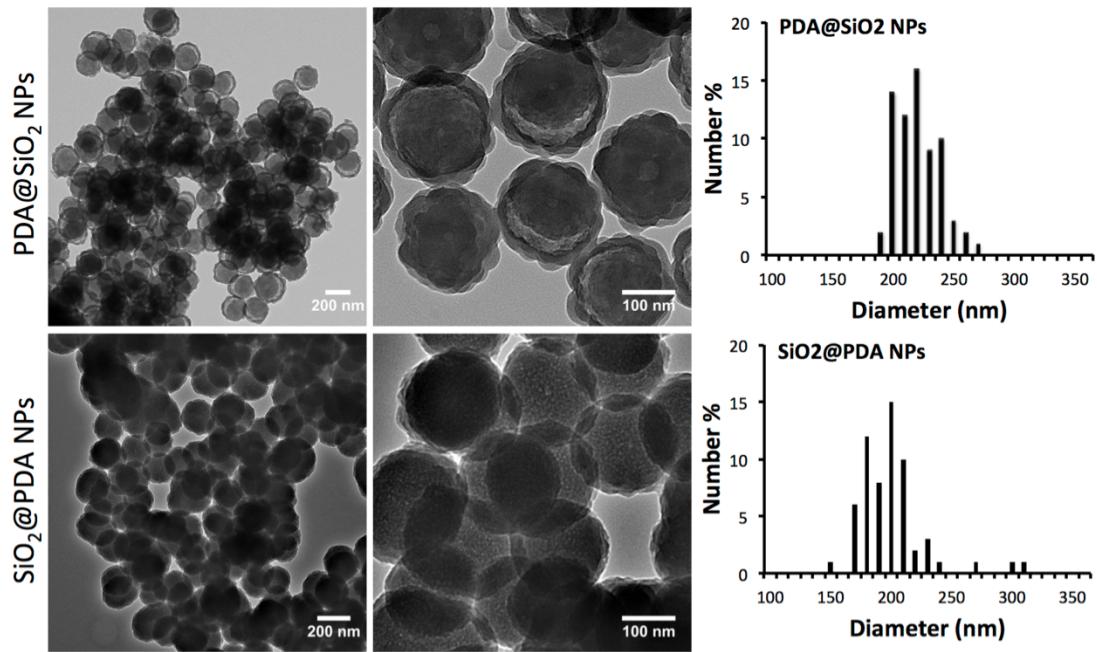


Figure S11: Unstained TEM and higher magnification images and size distributions of PDA@SiO₂ and SiO₂@PDA core-shell nanoparticles by DLS.

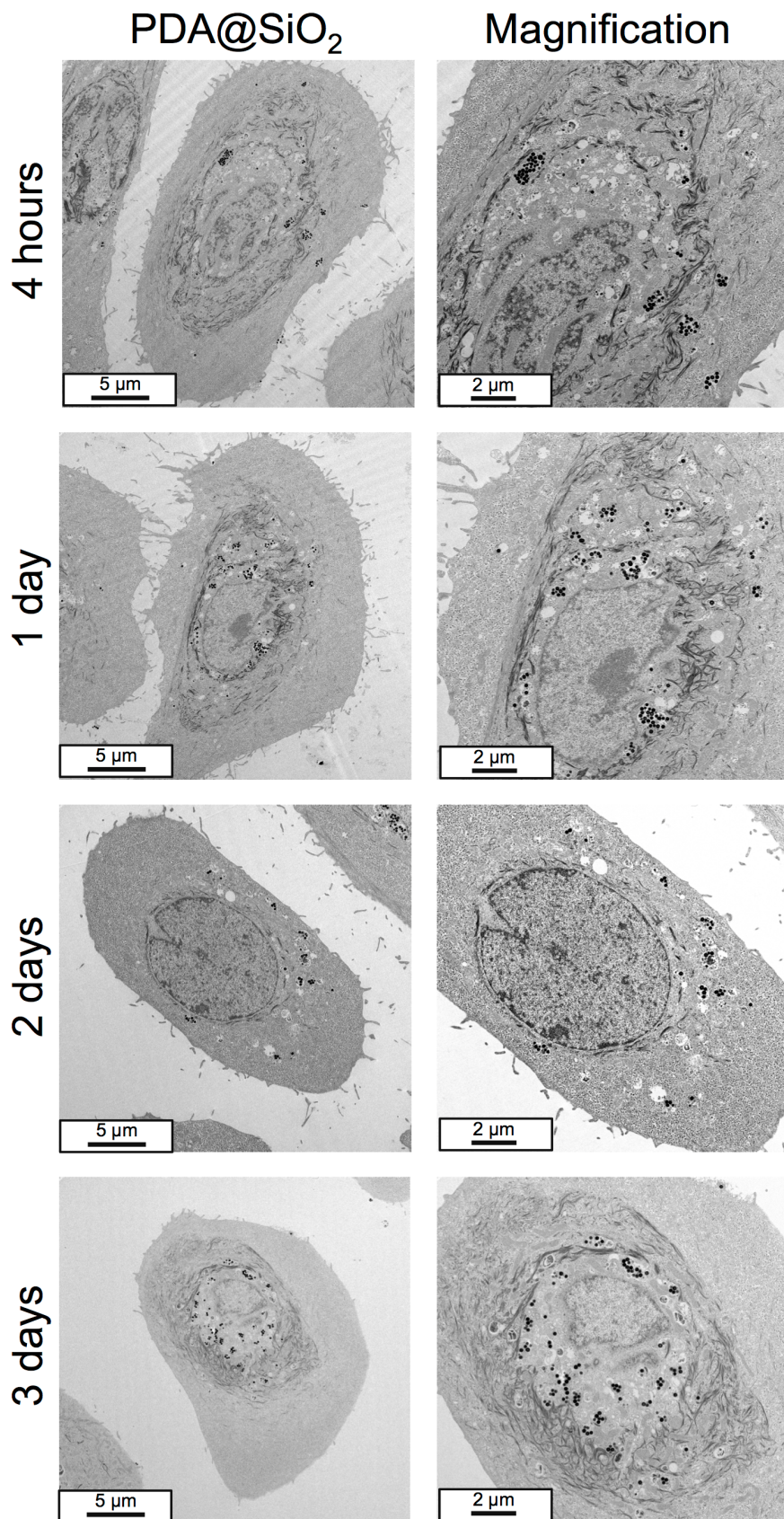


Figure S12. Cell TEM images for the distributions of PDA@SiO₂ core-shell nanoparticles in HEK293 cells at different time points. Cell sections for TEM were obtained by flat embedding cells in monolayer cell culture, not from cell pellets – See section above for detailed description of procedure used.

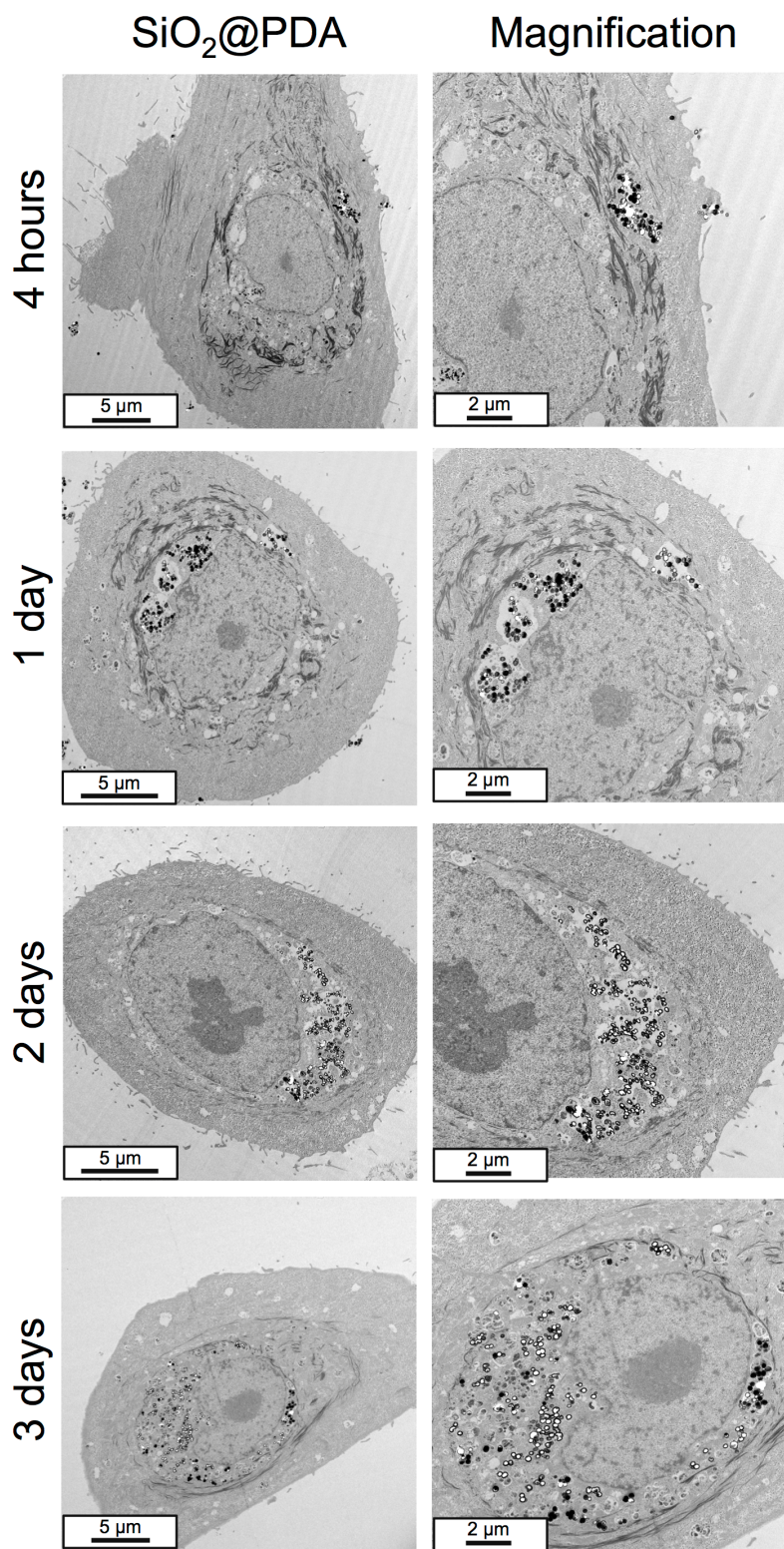


Figure S13. Cell TEM images for the distributions of SiO₂@PDA core-shell nanoparticles in HEK293 cells at different time points. Bright spaces ringed by dark PDA is believed to be the missing rigid core from the original particles being removed during sample slicing with the diamond knife. Occurring due to differences in rigidity of the cell and the silica core (dimensions are consistent with this conclusion). Cell sections for TEM were obtained by flat embedding cells in monolayer cell culture, not from cell pellets – See section above for detailed description of procedure used.

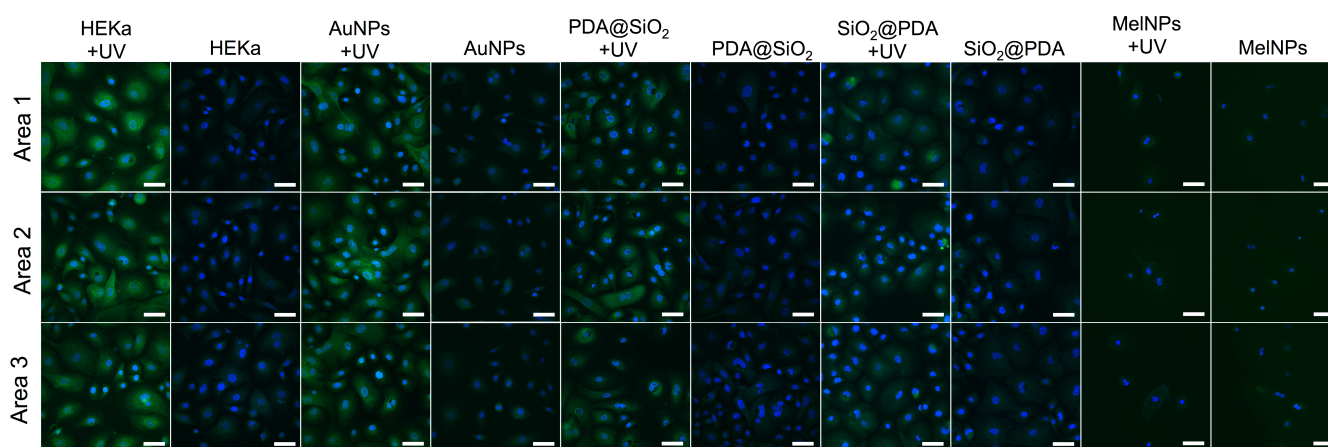


Figure S14. Confocal imaging for ROS detection in HEKa cells, HEKa cells with MeINPs, SiO₂@PDA core-shell nanoparticles, PDA@SiO₂ core-shell nanoparticles and AuNPs incubation for 3 days before and after UV irradiation. The nuclei were stained with NucBlue (blue); ROS generated in HEKa cells were detected with DCFH-DA (green). Scale bars are 50 μ m;

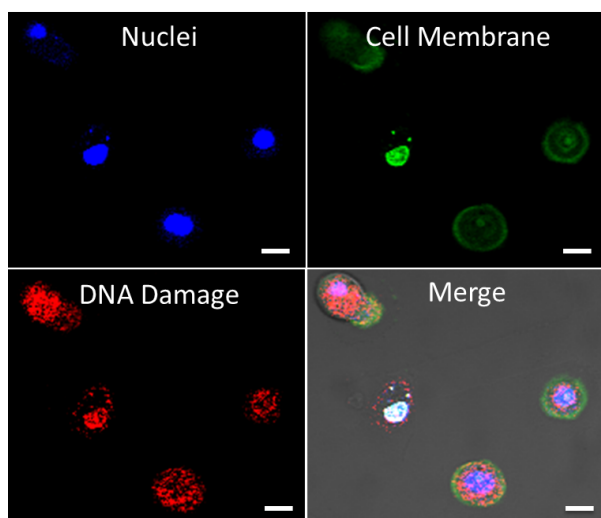


Figure S15. Confocal microscopy images of HEKa cells irradiated by UV. Nuclei were stained by Hoechst 33342 and are shown as blue. Cell membranes were stained by Image-iT DEAD Green and are shown in green. Damaged DNA was stained with an antibody against phosphorylated H2ax labeled with Alexa Fluor 555 and are shown in red. Scale bars are 40 μ m.

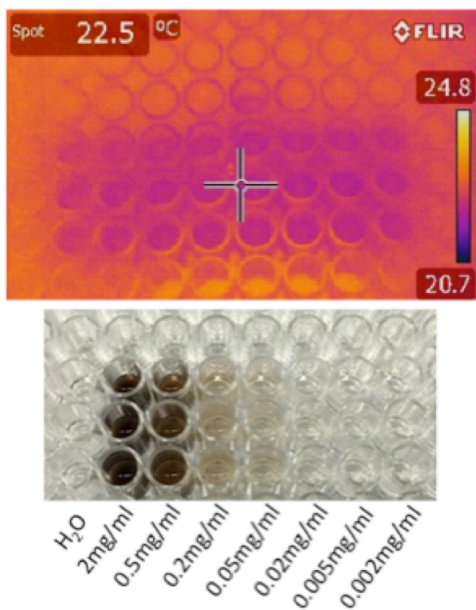


Figure S16. IR image (top) of MelNPs solutions over a 1000-fold concentration range (bottom) after irradiation for 5 minutes. The uniform color shows that no heat is generated upon UV irradiation.

References:

1. Xiao, M.; Li, Y.; Allen, M. C.; Deheyn, D. D.; Yue, X.; Zhao, J.; Gianneschi, N. C.; Shawkey, M. D.; Dhinojwala, A., Bio-Inspired Structural Colors Produced via Self-Assembly of Synthetic Melanin Nanoparticles. *ACS nano* **2015**, *9*, 5454-5460.
2. Stöber, W.; Fink, A.; Bohn, E., Controlled growth of monodisperse silica spheres in the micron size range. *Journal of colloid and interface science* **1968**, *26*, 62-69.