# *Supporting Information*

## Encapsulation of a small-molecule drug based on substituted 2-aminothiophenes in calcium carbonate carriers for therapy of melanoma

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## 1. Materials

### *1.1. For synthesis of compound 2-AT*

All the air-sensitive reactions were carried out under nitrogen atmosphere in standard Schlenk flasks. All the chemicals were of analytical grade and used without further purification. 4′-Methoxyacetophenone (99%), diethyl oxalate (≥99%), sodium methoxide (95%), cyclohexanone (≥99.0%), sulfur (99.98%), morpholine (≥99%), ethyl cyanoacetate (≥98%), propionic anhydride (≥99%), cyanoacetamide (99%), and potassium *tert*-butoxide (≥98%) were purchased from Sigma-Aldrich. Methanol (MeOH), ethanol (EtOH), and dioxane were dried over activated molecular sieves (3 Å) in Erlenmeyer flasks for two days prior to use. Chemically pure grade solvents were subjected to additional purification and drying. All other reagents were purchased from various commercial sources and used without additional purification.

### *1.2. For MPs synthesis*

Calcium chloride dihydrate ($CaCl_2 \times 2H_2O$), anhydrous sodium carbonate ($Na_2CO_3$), poly(sodium 4-styrenesulfonate) (PSS, $M_W$ = 70 000), and poly(allylamine hydrochloride) (PAH, $M_W$ = 17 000) were obtained from Sigma-Aldrich and used without further purification. Purified water with specific resistivity higher than 18.2 MΩ $cm^{-1}$ from a three-stage Milli-Q Plus 185 purification system was used.

### *1.3. For MPs 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 MPs radiolabeling*

Tin(II) chloride dihydrate ($SnCl_2 \times 2H_2O$, 98%) was purchased from Merck (Germany). Hydrochloric acid (> 35%, TraceSELECT) was purchased from Honeywell-Fluka (USA). Sodium hydroxide (BioXtra, ≥98%) and TWEEN® 80 were purchased from Sigma-Aldrich (Germany).

*1.5. For in vitro experiments*

Alpha Minimum Essential Medium (Alpha-MEM) was purchased from Biolot, Russia. Phosphate-buffered saline (PBS) and UltraGlutamine I were purchased from Lonza, Switzerland. Fetal bovine serum (FBS) was obtained from HyClone, USA. The Trypsin-EDTA solution was purchased from Capricorn Scientific, Germany. AlexaFluor 488-phalloidin (AF488), propidium iodide (PI, $M_w$ = 668.39, ≥94%), and calcein acetoxymethyl (Calcein AM) were purchased from Sigma-Aldrich.

*1.6. For in vivo experiments*

C57BL/6 mice 6-8 weeks old, a sterile syringe with a removable 29 G needle (Vogt Medical, Germany), isoflurane (Baxter, USA), zolazepam hydrochloride (Zoletil, Virbac, France), xylazine hydrochloride (Rometar, Bioveta, a.s., Czech Republic), and phosphate buffered saline (Sigma-Aldrich, Germany) were used.

*1.7. For histological analysis*

Formaldehyde, paraffin, 1% hydrochloric acid solution, methanol, potassium hexacyanoferrate (II) trihydrate, and PBS (phosphate buffered saline) 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).

## 2. Synthesis of MPs and 2-AT@MPs

### *2.1. Synthesis of compounds 1-4*

#### *2.1.1. Procedure for the synthesis of compound 1*

*(Z)*-2-hydroxy-4-(4-methoxyphenyl)-4-oxobut-2-enoic acid **1** was synthesized according to known protocols.[1]

*(Z)-2-hydroxy-4-(4-methoxyphenyl)-4-oxobut-2-enoic acid **1***. White crystals; 39.55 g, 88 % yield; m.p. 160-162°C. $^{1}$H NMR ($CDCl_3$, 400 MHz): $\delta_H$ 8.02 (m, 2H), 7.12 (s, 1H), 7.02 (m, 2H), 3.93 (s, 3H). $^{13}$C NMR ($CDCl_3$, 100 MHz): $\delta_C$ 164.8, 161.9, 132.1, 130.4, 125.6, 114.5, 94.4, 55.6.

#### *2.1.2. Procedure for the Synthesis of compound 2*

Ethyl 2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylate **2** was synthesized according to known protocols.[1]

*Ethyl 2-amino-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate **2***. Yellow crystals; 19.15 g, 85 % yield; m.p. 119-120°C. $^{1}$H NMR ($CDCl_3$, 400 MHz): $\delta_H$ 5.93 (s, 2H), 4.28 (q, $J$ = 7.1 Hz, 2H), 2.73 (m, 2H), 2.52 (m, 2H), 1.79 (m, 4H), 1.36 (t, $J$ = 7.1 Hz, 3H). $^{13}$C NMR ($CDCl_3$, 100 MHz): $\delta_C$ 166.1, 161.6, 132.5, 117.7, 105.9, 59.3, 26.9, 24.5, 23.3, 22.8, 14.4.

#### *2.1.3. Procedure for the Synthesis of compound 3*

A solution of the *(Z)*-2-hydroxy-4-(4-methoxyphenyl)-4-oxobut-2-enoic acid **1** (2.22 g, 0.01 mol) in ethanol (10 ml) was added to a solution of ethyl 2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylate **2** (2.25 g, 0.01 mol) in ethanol (10 ml). The resulting mixture was heated to 60°C and stirred for 1 hour. The solution was cooled to -27°C, the precipitate was filtered off and recrystallized. The physicochemical data of the obtained compound **3** correspond to the previously described.[1]

*2-{[3-(Ethoxycarbonyl)-4,5,6,7-tetrahydrobenzo[b]thiophen-2-yl]amino}-4-(4-methoxyphenyl)-4-oxobut-2-enoic acid **3**.* Red crystals; 3.86 g, 90 % yield; m.p. 186-187°C (acetonitrile). IR (nujol) *v*, $cm^{-1}$: 3412, 1712. $^{1}H$ NMR ($CDCl_3$, 400 MHz): $\delta_H$ 12.19 (s, 1H), 8.03 (m, 2H), 7.08 (s, 1H), 7.01 (m, 2H), 4.38 (q, *J* = 7.1 Hz, 2H), 3.91 (s, 3H), 2.81 (m, 2H), 2.77 (m, 2H), 1.88 (m, 2H), 1.82 (m, 2H), 1.40 (t, *J* = 7.1 Hz, 3H). $^{13}C$ NMR ($CDCl_3$, 100 MHz): $\delta_C$ 187.7, 164.4, 164.3, 162.4, 145.5, 144.5, 134.6, 131.2, 129.6, 128.7, 117.6, 114.3, 96.5, 61.3, 55.7, 26.5, 24.9, 22.7, 22.4, 14.2.

*2.1.4. Procedure for the Synthesis of compound 4*

A solution of compound **3** (3.86 g, 0.009 mol) in propionic anhydride (5 ml) was slowly heated to 140°C and stirred for 1 h. The precipitate formed after cooling down was filtered off, washed with absolute ether, and recrystallized from anhydrous toluene. The physicochemical data of the obtained compound **4** correspond to the previously described.[1]

*Ethyl 2-((5-(4-methoxyphenyl)-2-oxofuran-3(2H)-ylidene)amino)-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate **4**.* Red crystals; 3.33 g, 90 % yield; m.p. 146-147°C (toluene). IR (nujol) *v*, $cm^{-1}$: 1797, 1697, 1610. $^{1}H$ NMR ($CDCl_3$, 400 MHz): $\delta_H$ 7.80 (m, 2H), 7.00 (m, 2H), 6.77 (s, 1H), 4.38 (q, *J* = 7.1 Hz, 2H), 3.90 (s, 3H), 2.79 (m, 2H), 2.75 (m, 2H), 1.87 (m, 2H), 1.82 (m, 2H), 1.40 (t, *J* = 7.1 Hz, 3H). $^{13}C$ NMR ($CDCl_3$, 100 MHz): $\delta_C$ 166.5, 164.1, 163.6, 161.3, 149.7, 146.2, 138.2, 136.0, 131.6, 128.7, 119.5, 114.8, 96.4, 61.2, 55.9, 25.7, 25.2, 22.9, 22.3, 14.3.

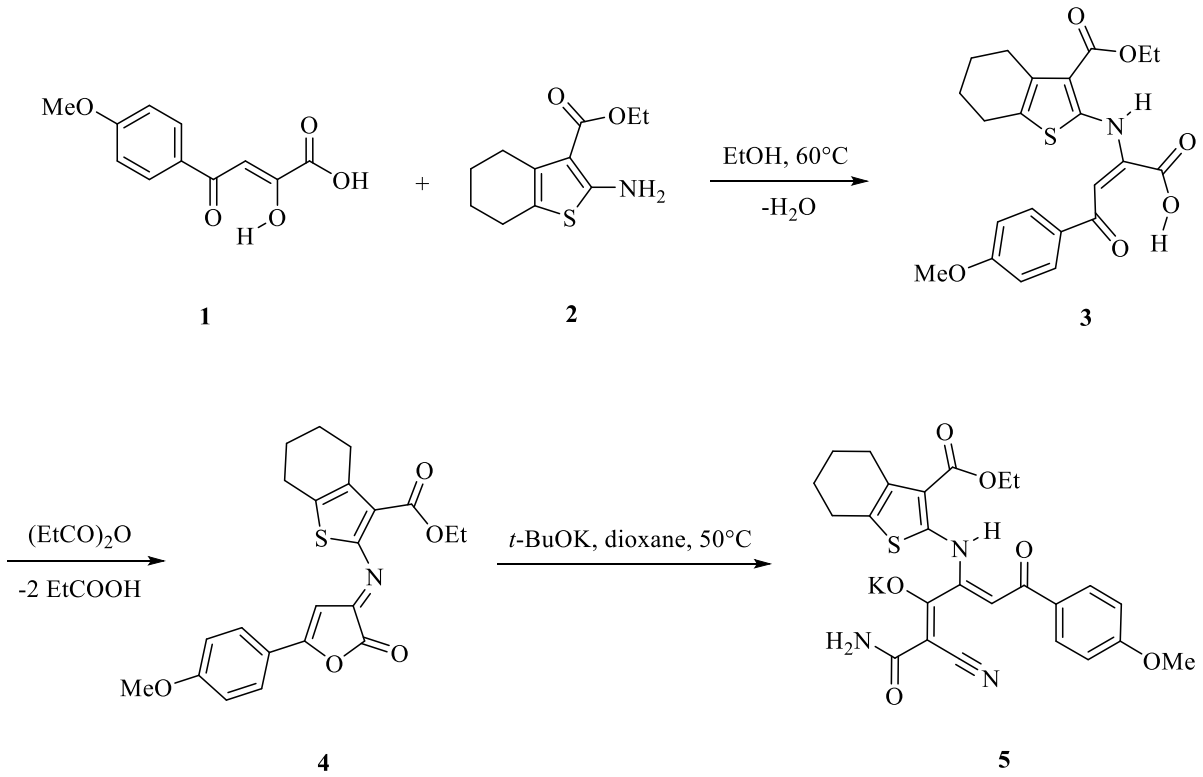


**Figure S1**. Scheme of multistep synthesis of **2-AT** (compound **5**).

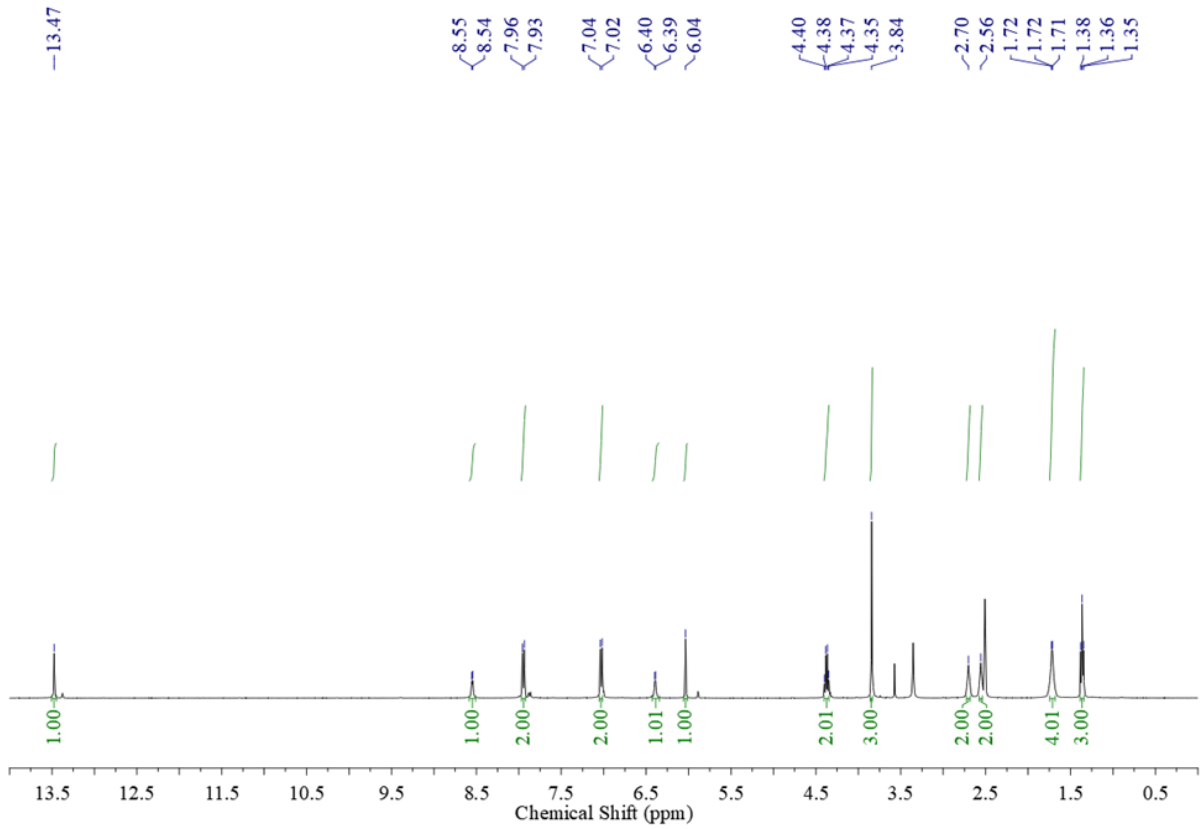


**Figure S2**. $^{1}$H NMR spectrum of **2-AT** (DMSO-$d_6$, 400 MHz).

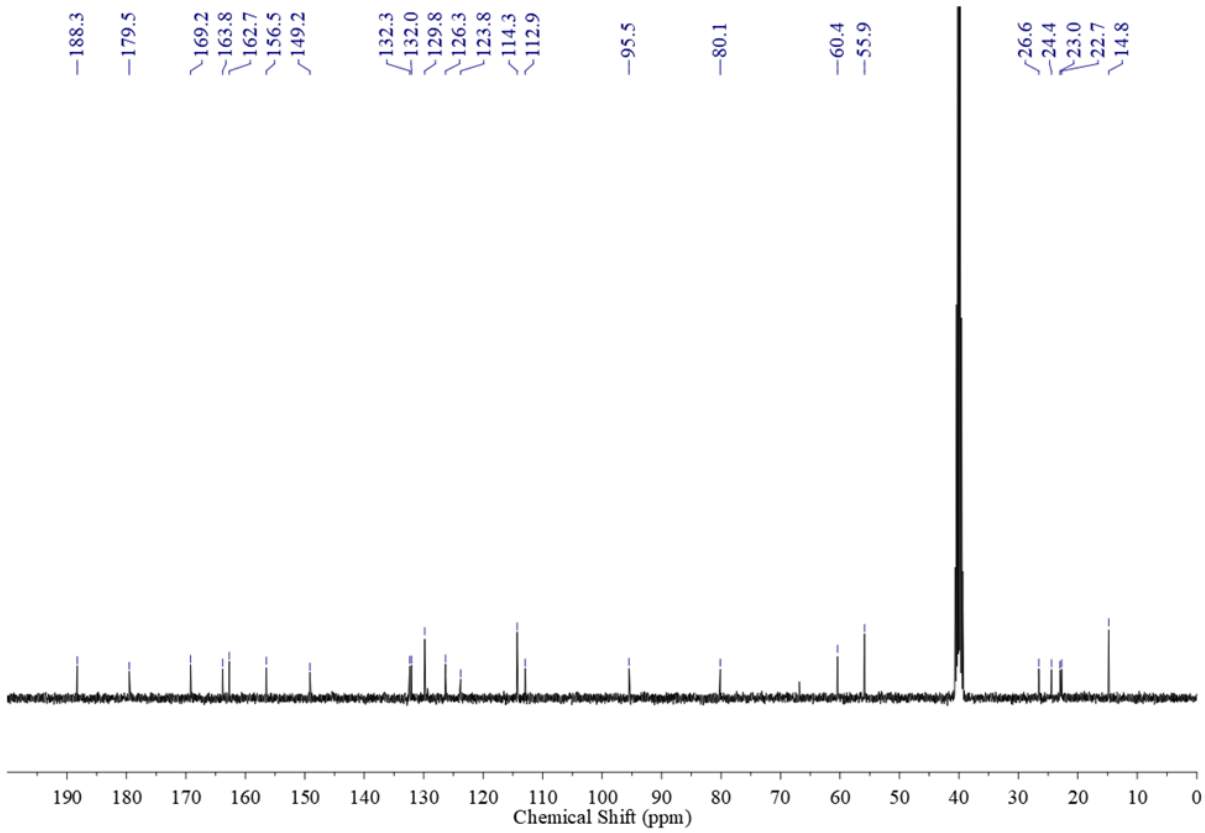


**Figure S3**. $^{13}$C NMR spectrum of **2-AT** (DMSO-$d_6$, 100 MHz).

*2.2. Synthesis of MPs*

To obtain **MPs**, 615 $\mu$L of 1 M $CaCl_2$ aqueous solution was mixed with 2.5 mL of water. Afterwards, 615 $\mu$L of 1 M $Na_2CO_3$ aqueous solution was added to the mixture and the obtained solution was vigorously stirred at 1000 rpm for 30 sec at room temperature. The resulting micrometric $CaCO_3$ cores were washed 3 times with water at 4000 rpm for 1 min using microspin FV-2400 (Biosan). To achieve the core-shell structure, Layer-by-Layer technology was used. For this, 1 mL of PAH (2 mg/mL) was added to the washed **MPs** and shaken for 10 min at room temperature. Afterwards, the **MPs** were washed 2 times with water *via* centrifugation with FV-2400 (Biosan) for 20 sec. Then, 1 mL of PSS (2 mg/mL) was added to the **MPs**, sonicated for 5 sec and shaken for 10 min at room temperature. This procedure was repeated 6 times for the formation of core-shell particles: **MPs@(PAH/PSS)$_3$**.

*2.3. Synthesis of 2-AT@MPs*

To obtain **2-AT@MPs**, the co-precipitation method was used. For this, 615 $\mu$L of 615 $\mu$L of 1 M $CaCl_2$ aqueous solution was mixed with 2.4 mL of water. Then, 100 $\mu$L of **2-AT** in DMSO (70, 100 and 200 mg/mL) was added to the solution. Next, 615 $\mu$L of 1 M $Na_2CO_3$ aqueous solution was added to the reaction. The components were stirred together at room temperature for 30 sec. The resulting **2-AT@MPs** was washed multiple times with Milli-Q water and purified by centrifugation for 1 min at 4000 rpm after the reaction. To achieve the core-shell structure, Layer-by-Layer technology was used. For this, 1 mL of PAH (2 mg/mL) was added to the washed **2-AT@MPs** particles and shaken for 10 min at room temperature. Afterwards, the **MPs** particles were washed 2 times with water *via* centrifugation with FV-2400 (Biosan) for 20 sec. Then, 1 mL of PSS (2 mg/mL) was added to the **2-AT@MPs** particles, sonicated for 5 sec and shaken for 10 min at room temperature. This procedure was repeated 6 times for the formation of core-shell particles: **2-AT@MPs@(PAH/PSS)$_3$**.

*2.4. Synthesis of Cy5@MPs*

*2.4.1. Fluorescence labeling of bovine serum albumin (BSA)*

BSA 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 $\mu$g 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. 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.

*2.4.2. Cy5 labeling of MPs*

To obtain **Cy5@MPs**, 2 mL of Milli-Q water, 615 $\mu$L of 1 M $CaCl_2$ aqueous solution, 615 $\mu$L of 1 M $Na_2CO_3$ aqueous solution, and 500 $\mu$L 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.

## 3. MPs and 2-AT@MPs characterization

*3.1. Scanning electron microscopy (SEM)*

Morphology and size of the developed **MPs** and **2-AT@MPs** were characterized by scanning electron microscopy (SEM, Quanta 200, FEI, Netherlands) with an accelerating voltage of 10 kV.

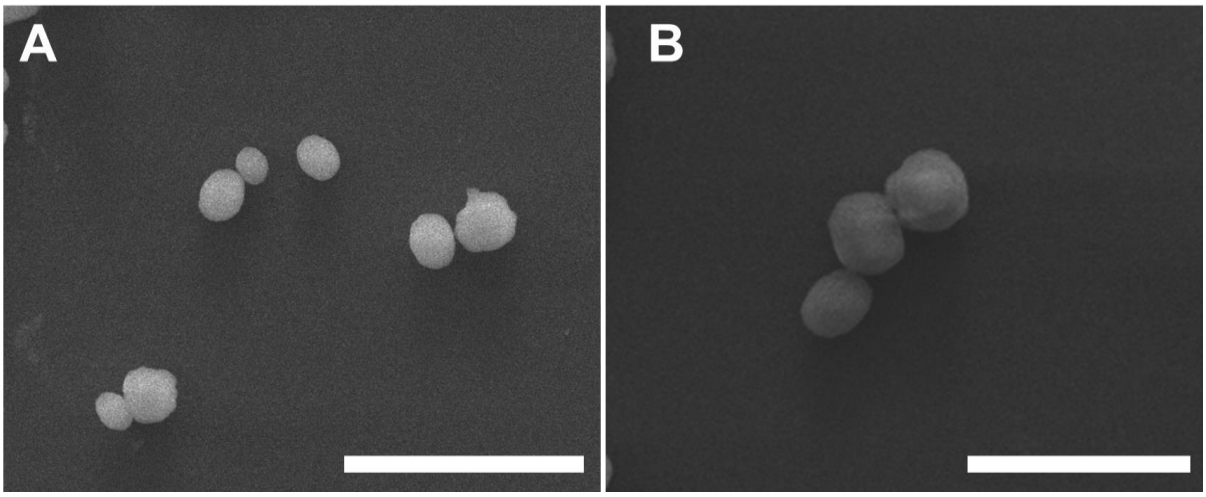


**Figure S4.** (**A**) SEM images of **MPs** and (**B**) **2-AT@MPs**. Scale bars = 20 $\mu$m.

*3.2. Transmission electron microscopy (TEM)*

Morphology and size of the developed **MPs** and **2-AT@MPs** were characterized by transmission electron microscopy (TEM). To obtain TEM images, 5 $\mu$L 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).

*3.3. Dynamic laser scattering (DLS)*

BKwinner803 Photon Correlation DLS Nano Particle Size Analyzer (Jinan Winner Particle Instrument Stock Co., Ltd., China) was used to measure the hydrodynamic diameters ($D_h$) of the **MPs** and **2-AT@MPs**.

*3.4. Powder X-Ray Diffraction (PXRD)*

The crystalline phase of **MPs** and **2-AT@MPs** was studied using PXRD analysis. Diffraction patterns were recorded on a Shimadzu 7000-maxima X-ray diffractometer with a 2 kW characteristic CuKα (Kα1 λ = 1.54059 Å, angular range 2θ = 5°-60°) X-ray radiation source and Bragg-Brentano goniometer geometry. The angular resolution during the analysis was 0.01 deg at a scanning speed of 1 deg/min.

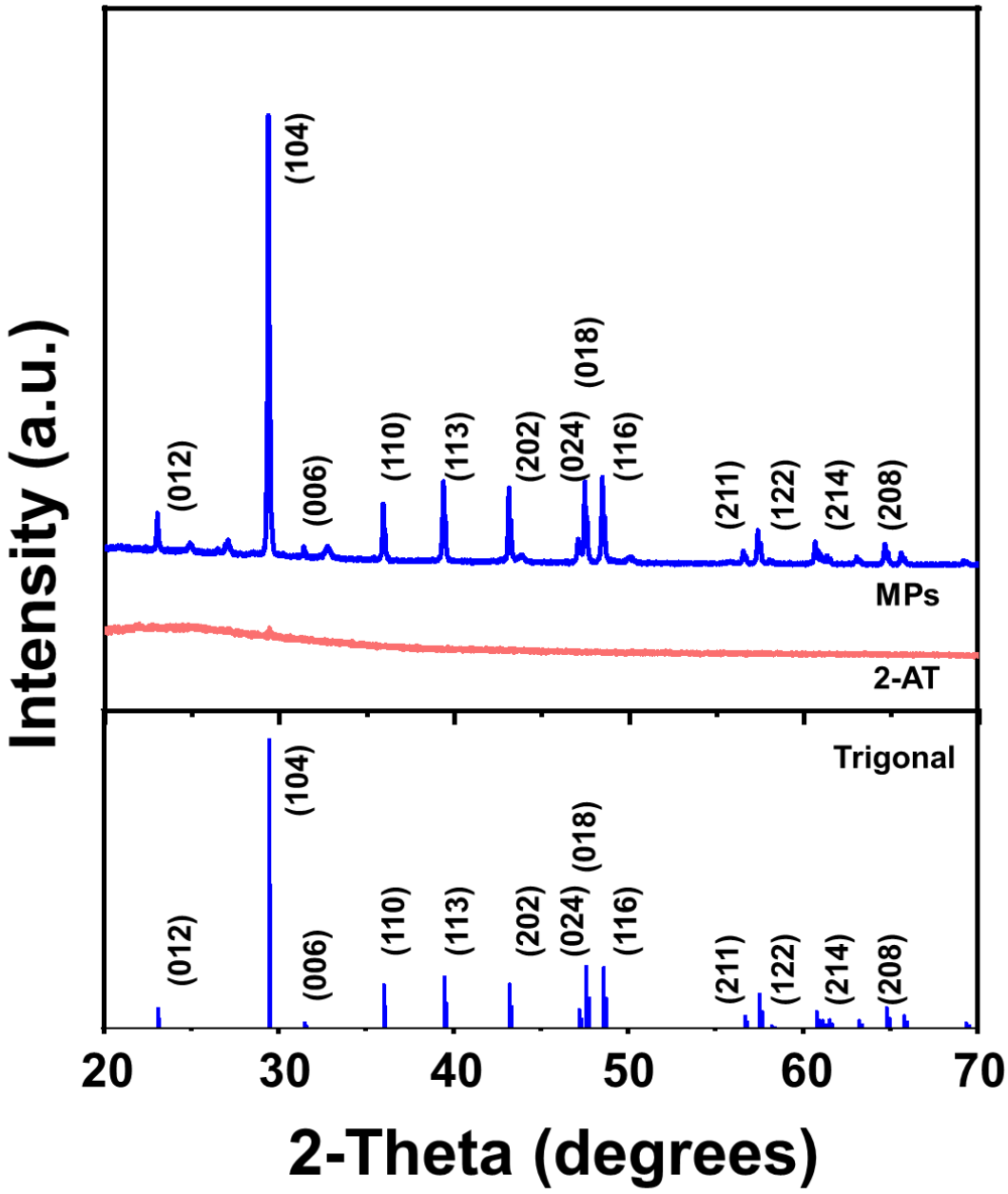


**Figure S5**. PXRD spectra of $CaCO_3$ **MPs** and **2-AT**. The trigonal phase of $CaCO_3$ is shown below.

*3.5. Fourier-transform infrared (FTIR) spectroscopy*

The Fourier transform infrared spectra were obtained by Shimadzu IRTracer-100 combined with Specac Quest, a single-reflection ATR accessory, by scans in the range from 400 to 4000 $cm^{-1}$ with a resolution of 1 $cm^{-1}$.

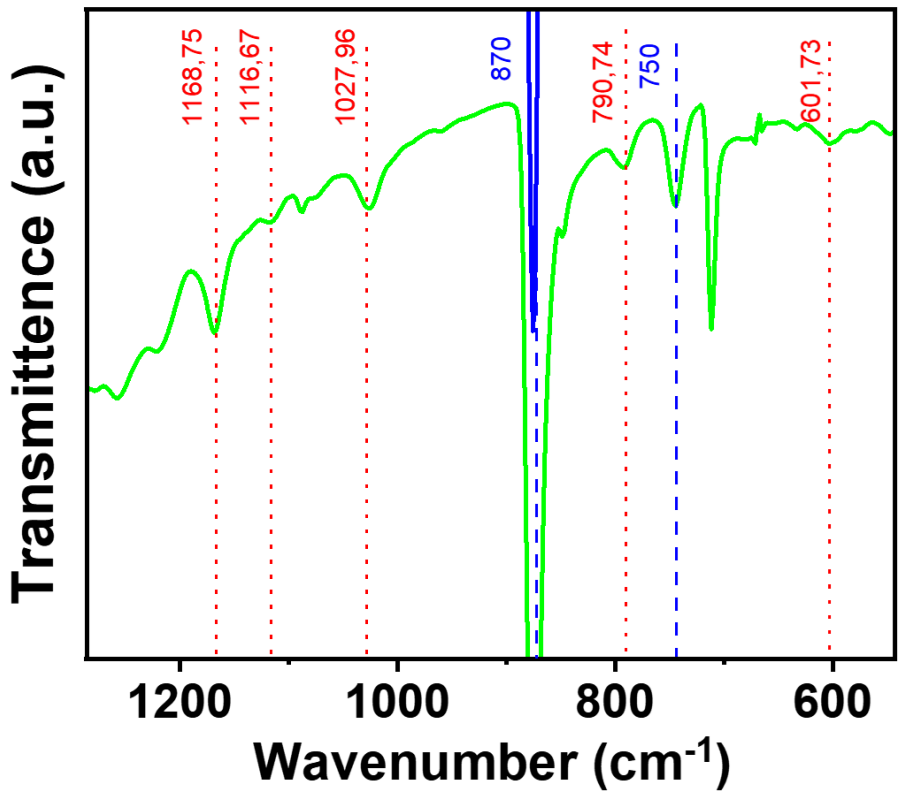


**Figure S6.** FTIR spectra of 2-AT@MPs from 1300 to 500 $cm^{-1}$.

*3.6. Energy Dispersive X-Ray Analysis (EDX)*

An X-MAX energy-dispersive X-ray detector (Carl Zeiss, Germany) was used to analyze the elemental composition of the particles.

## 4. 2-AT loading and release efficiency

*4.1. Estimation of 2-AT loading into MPs*

At the final stage of **2-AT@MPs** synthesis, after each wash step supernatants were collected to calculate the amount of non-binded drug by recording the absorbance using a NanoPhotometer spectrophotometer C40-Touch in a quartz cuvette (**Figure S6**). The absorption wavelength was λ = 343 nm. To estimate the amount of **2-AT** loaded into **MPs**, a calibration curve of absorbance dependence on the drug concentration was plotted (**Figure S7**).

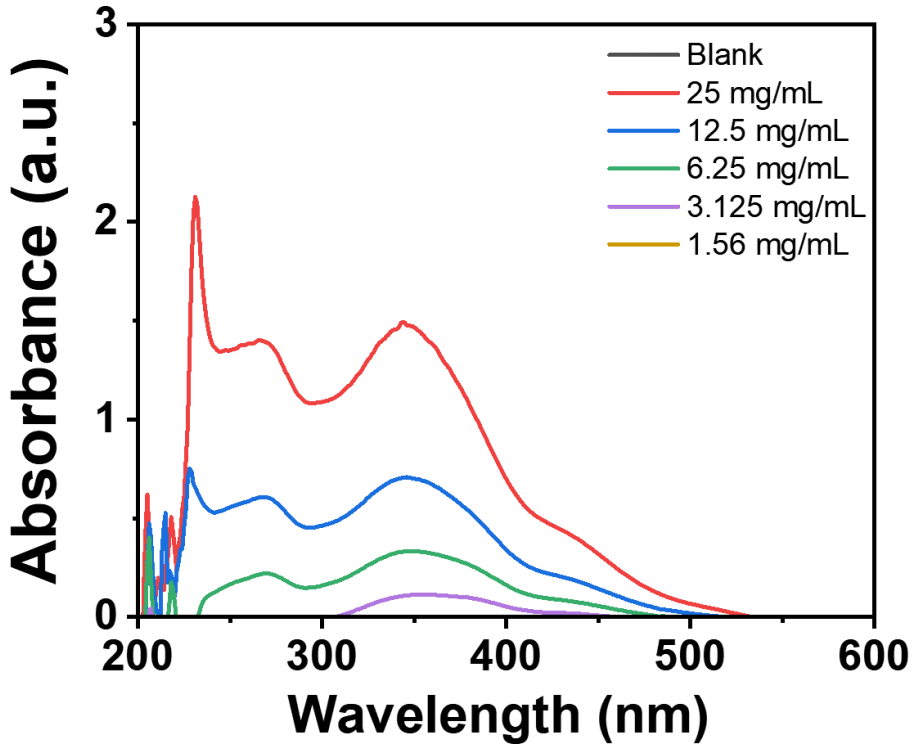


**Figure S7**. UV-VIS absorbance spectra of **2-AT** in DMSO solutions at different concentrations (1.56-25 mg/mL).

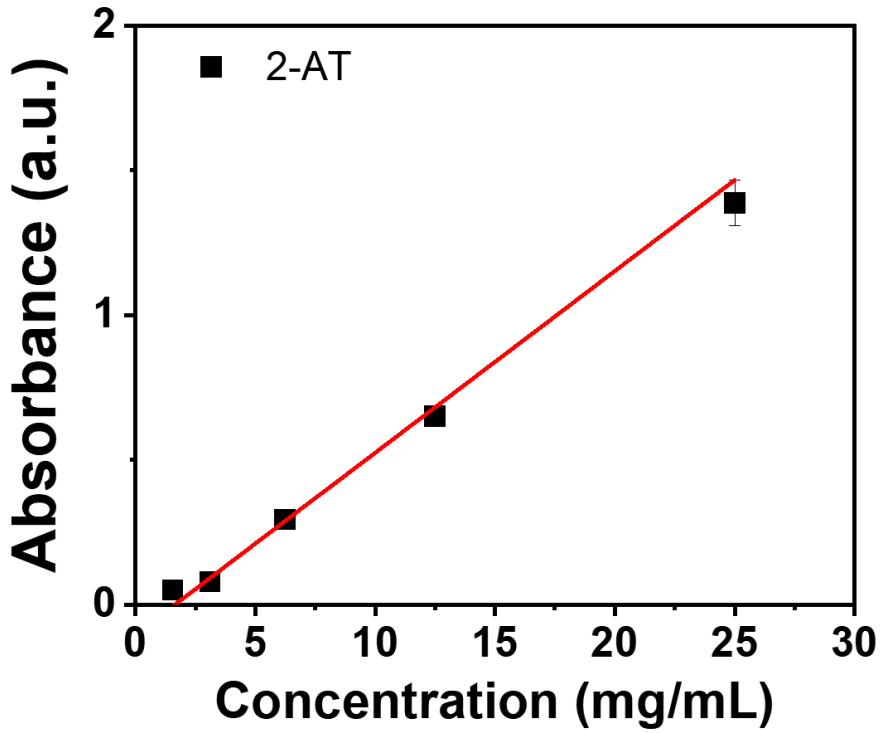


**Figure S8**. Calibration curve for **2-AT**. Absorbance (a.u.) *versus* concentration (mg/mL).

Therefore, after measurements of absorbance, we can calculate the concentration of the drug. Then, the percentage of bound drug (%) can be calculated as:

$$Bound\ (\%)\ = \frac{amount\ of\ unbound\ drug\ in\ sup\ (mg)}{amount\ of\ added\ drug\ (mg)} * 100\%$$

*4.2. Estimation of drug release from 2-AT@MPs*

To obtain the release curves of **2-AT**, **2-AT@MPs** were resuspended in the aqueous solutions with different pH values (pH 5.8, 7.4, 8.4). At a certain time point, particles were centrifuged with 4000 rpm for 3 min, and then, the absorbance spectrum of the supernatant was measured. After that, **2-AT@MPs** were resuspended in a new buffer solution and left in a shaker. Knowing the calibration curve for **2-AT**, the drug release was calculated using the following formula:

$$Cumulative\ release\ ratio\ (\%)\ = \sum_{time\ point} \frac{m_{released}}{m_{loaded}} * 100\%$$

**5. *In vitro* stability of MPs and 2-AT@MPs**

*In vitro* stability of **MPs** and **2-AT@MPs** was investigated using SEM, CLSM and DLS. For this, **MPs** and **2-AT@MPs** were incubated in 0.9% NaCl and 0.1% BSA at pH 5.8, pH 7.4 and pH 8.4. The $D_h$ change was analyzed at different moments of time (1, 2, 5, 8, and 12 d).

*5.1. Dynamic laser scattering (DLS)*

Analysis of the hydrodynamic diameter ($D_h$) of **MPs** and **2-AT@MPs** were performed using a BKwinner803 Photon Correlation DLS Nano Particle Size Analyzer (Jinan Winner Particle Instrument Stock Co., Ltd., China).

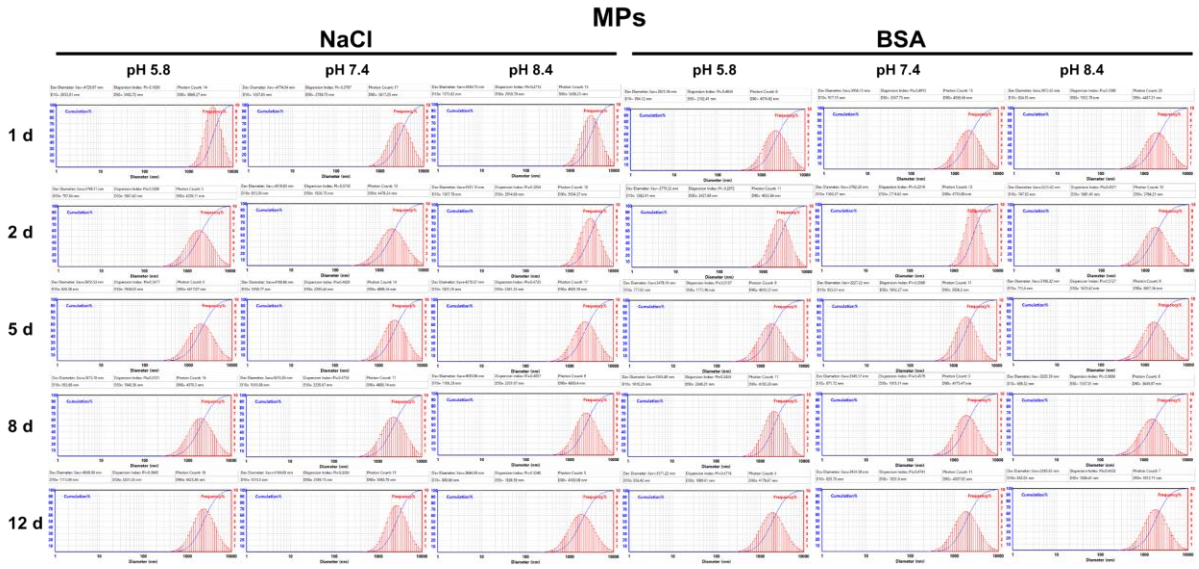


**Figure S9**. Hydrodynamic diameters ($D_h$) of **MPs** incubated in 0.9% NaCl and 0.1% BSA at pH 5.8, pH 7.4 and pH 8.4 during 12 d.

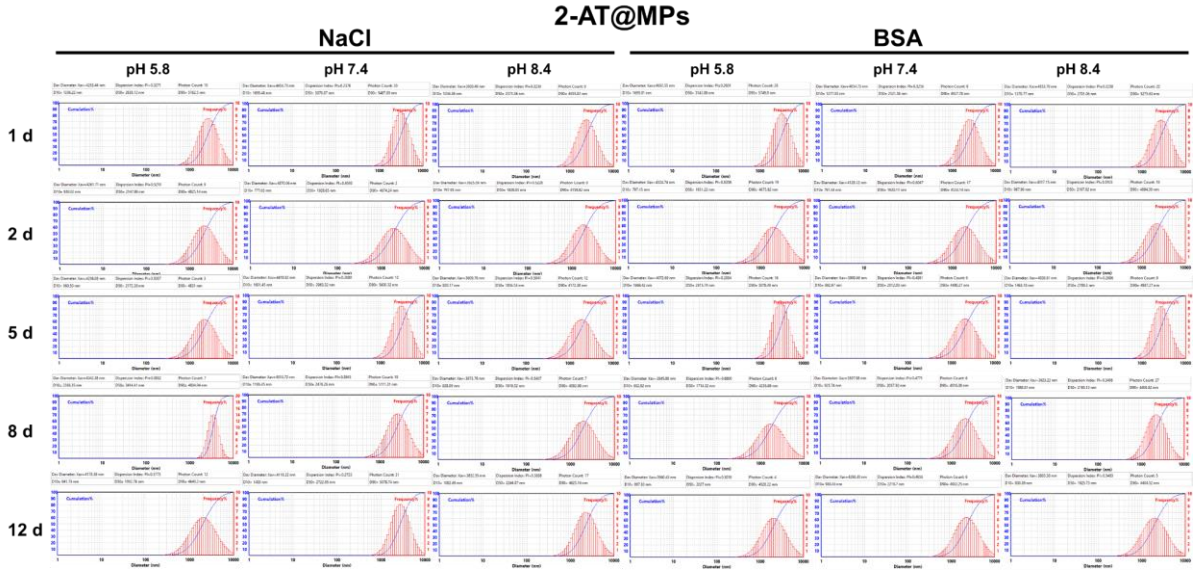


**Figure S10**. Hydrodynamic diameters ($D_h$) of **2-AT@MPs** incubated in 0.9% NaCl and 0.1% BSA at pH 5.8, pH 7.4 and pH 8.4 during 12 d.

*5.2. Scanning electron microscopy (SEM)*

To obtain SEM images, **MPs** and **2-AT@MPs** were dropped onto a silicon plate and dry. Then, samples visualized using SEM (Quanta 200, FEI, Netherlands).

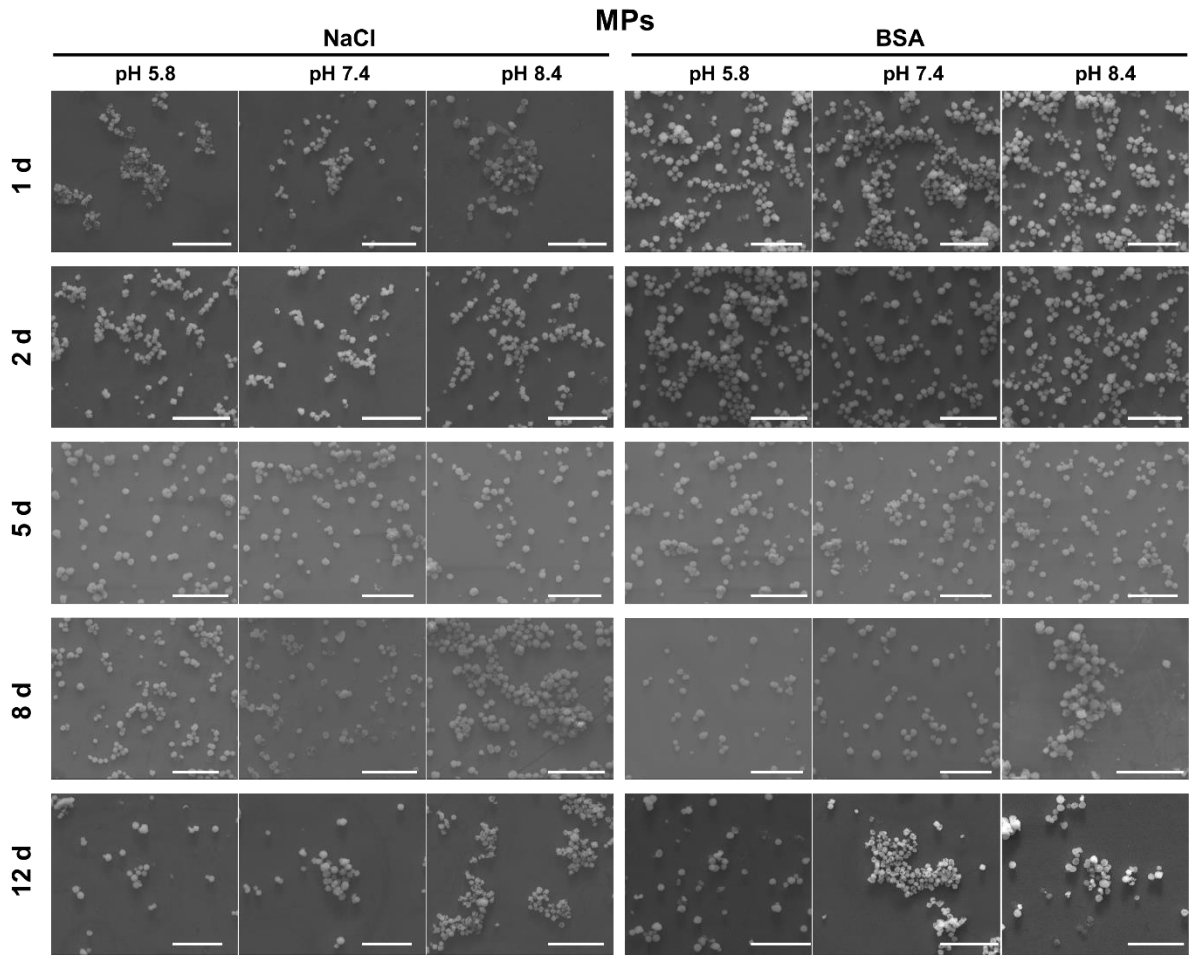


**Figure S11**. SEM images of **MPs** after incubation in 0.9% NaCl and 0.1% BSA solutions at pH 5.8, 7.4 and 8.4 for different time periods (1, 2, 5, 8 and 12 d). Scale bars = 20 $\mu$m.

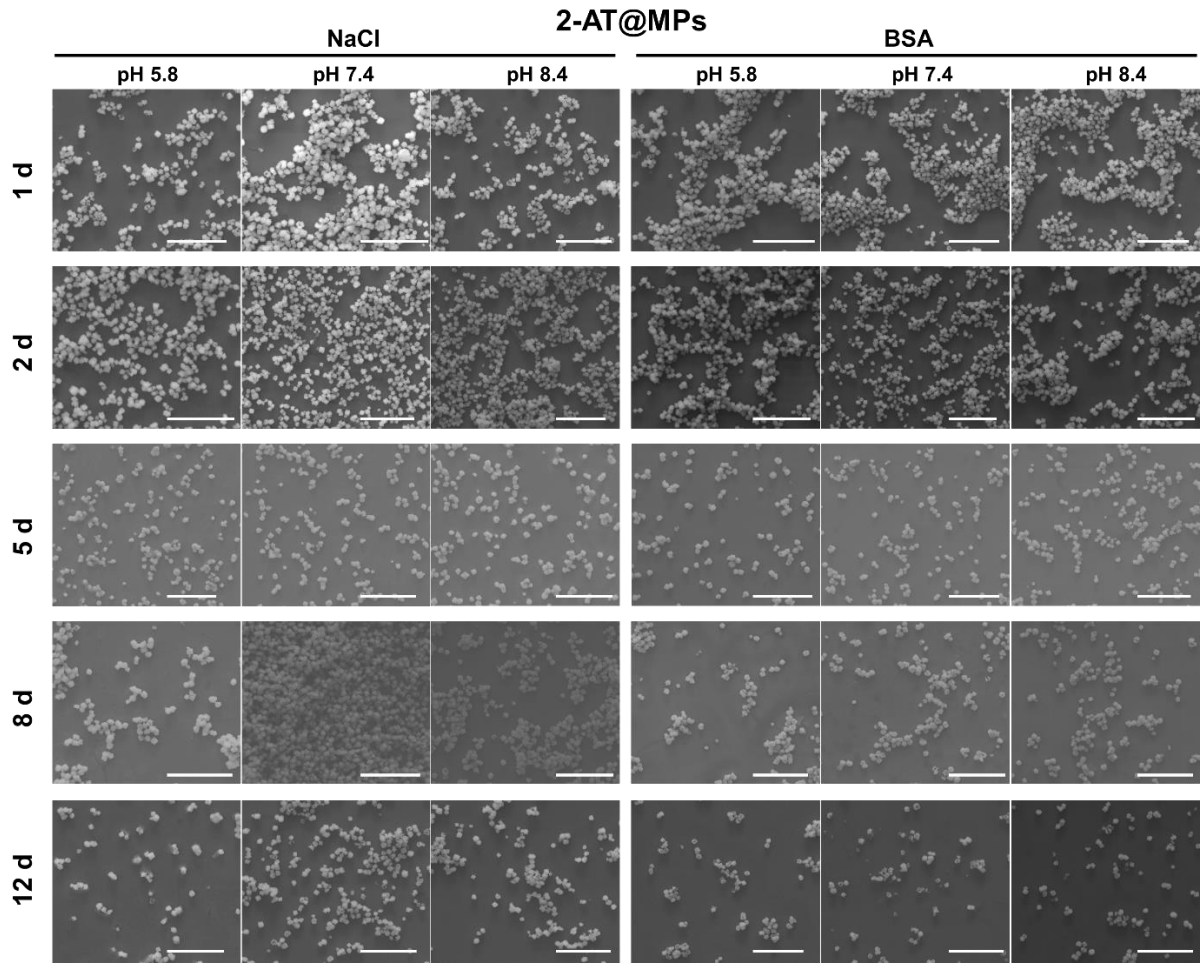


**Figure S12**. SEM images of **2-AT@MPs** after incubation in 0.9% NaCl and 0.1% BSA solutions at pH 5.8, 7.4 and 8.4 for different time periods (1, 2, 5, 8 and 12 d). Scale bars = 20 $\mu$m.

*5.3. Confocal laser scanning microscopy (CLSM)*

Confocal laser scanning microscopy (CLSM) images of **2-AT@MPs** were taken with a Leica TCS SP8 (Germany). Objective HC PL FLUOTAR 10x/0.30 PH2 (**Figure S13**).

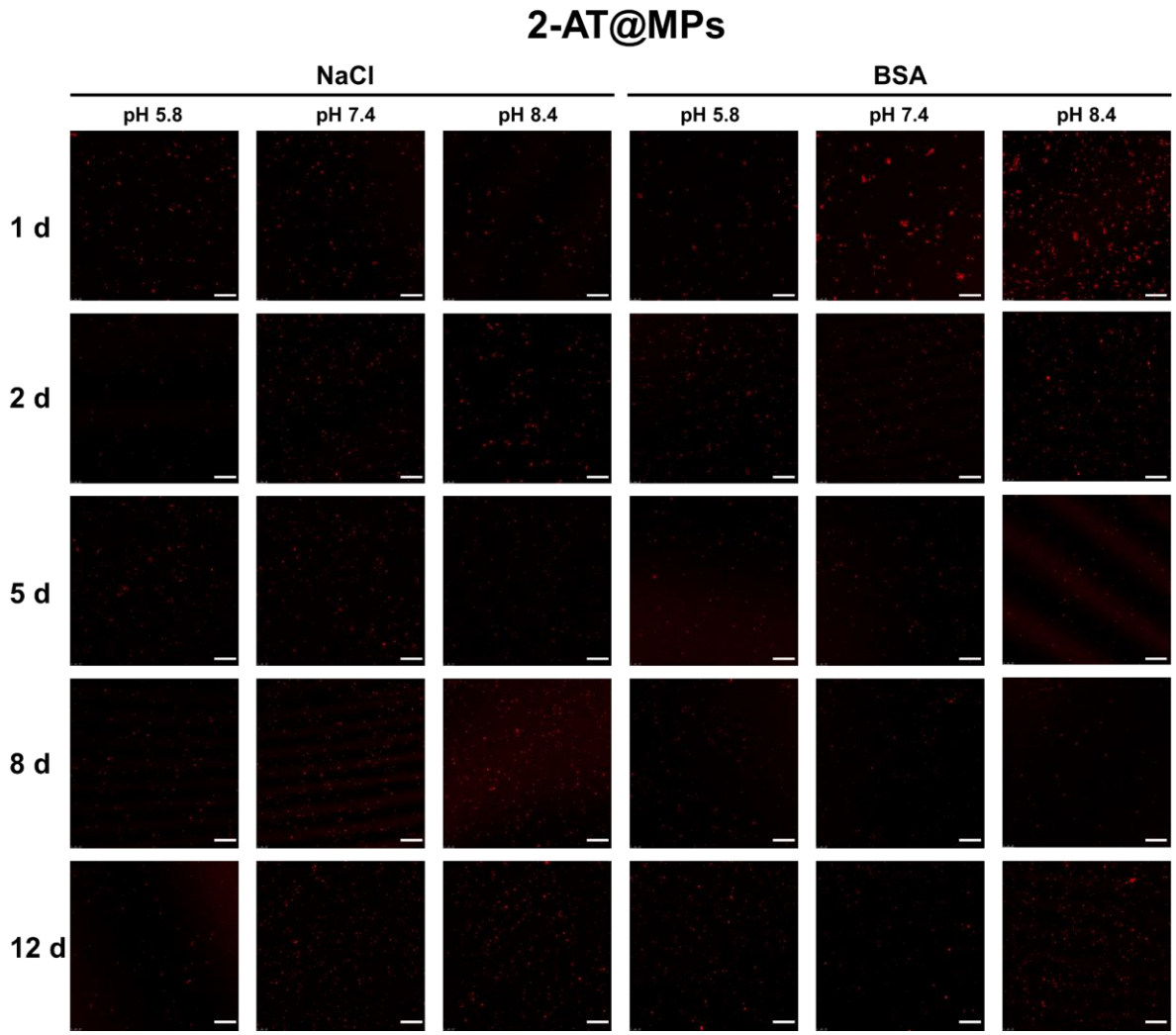


**Figure S13**. CLSM images of **2-AT@MPs** after incubation in 0.9% NaCl and 0.1% BSA solutions at pH 5.8, 7.4 and 8.4 for different time periods (1, 2, 5, 8 and 12 d). Scale bars = 50 $\mu$m.

## 6. *In vitro* studies

### *6.1. Cell culture*

For *in vitro* studies, highly metastatic mouse melanoma B16-F10 cells were used. The cells were cultured in $\alpha$MEM (Alpha Modification of Eagle's Medium) with an addition of 10% FBS (fetal bovine serum). The cells were incubated in a humidified atmosphere, containing 5% $CO_2$, at 37°C. For *in vitro* experiments, the cells were passaged several times after defrosting.

*6.2. Cellular uptake*

To visualize cellular uptake of **MPs** by B16-F10, the cells were seeded into 24-well plates ($1.0\times10^5$ per well). Next day, the Cy5-labeled **MPs** (**Cy5@MPs**) were added to the cells at different concentrations (0.031-16 mg/mL), and the cells were left overnight. Next day, the cells were fixed. Then, their cytoskeletons were stained with phalloidin conjugated with AF488, and the cell nuclei were stained with PI. For this, the cells were washed 2 times with PBS and permeabilized with 10 vol % of formalin for 40 min. Afterwards, the cells were washed again with PBS, and 1 $\mu$M of AF488 was added. The cells were left for another 1 h. After that, 20 $\mu$M of PI was added for 20 min. Afterwards, the cells were washed again with PBS. Cellular uptake of **MPs** was visualized using a 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 Cy5-labeled **MPs,** 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.

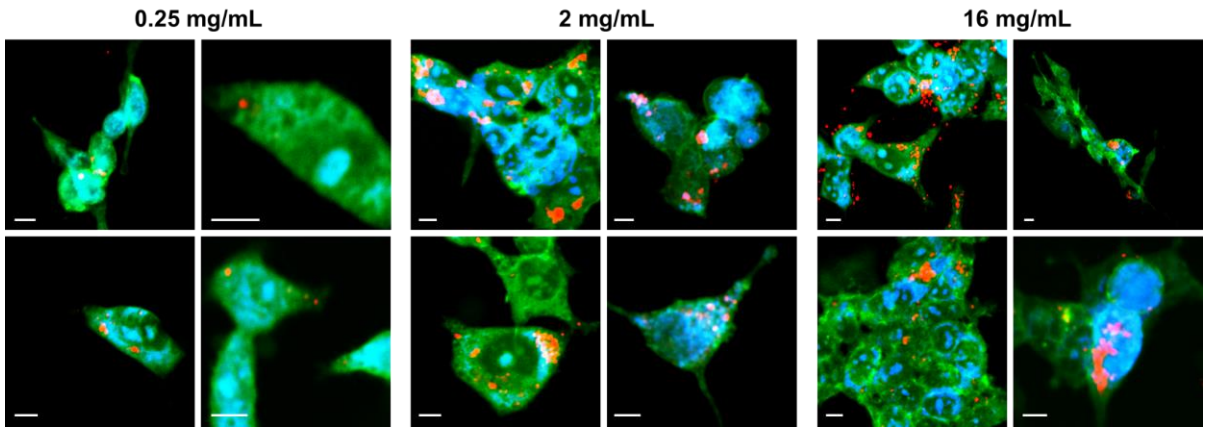


**Figure S14**. CLSM images of B16-F10 cells incubated with **Cy5@MPs** at different concentrations (0.25, 2, 16 mg/mL). Scale bars = 10 $\mu$m.

*6.3. Spheroid distribution*

Spheroids B16-F10 ($7.0\times10^3$ cells per drop) were obtained using the hanging droplet method.[3] The 40 droplets containing 30 $\mu$L of cell suspension 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. Then it was incubated for 120 h at 37°C, 5% $CO_2$. Every 48 h, every drop was washed with 30 $\mu$L of medium and further incubated in a fresh cell culture medium. The formed spheroids were washed with PBS and 10 $\mu$L of Rhodamine B (RhB) was added for staining. Afterwards, the spheroids were washed with PBS, and **Cy5@MPs** were added to the spheroids. At 1 h and 24 h, the spheroids were fixed with formalin. After the fixation, the spheroids were transferred to a PBS solution and observed under CLSM to evaluate the penetration ability of **Cy5@MPs**. To visualize RhB-stained spheroids, an argon laser emitting at 514 nm was used. To visualize the **Cy5@MPs**, 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.

*6.4. Toxicity studies*

*6.4.1. LIVE/DEAD assay*

To study the toxicity of the **MPs** at different concentrations (0.25, 0.5, 1, 2, 4, 8, and 16 mg/mL), LIVE/DEAD assay was used. For this, B16-F10 cells were seeded into a 24-well plate ($3.0\times10^4$ cells per well). On the same day, the **MPs** were added to the cells. The next day, the B16-F10 cells were stained with 0.5 $\mu$M Calcein AM (live staining) and 3 $\mu$M 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.

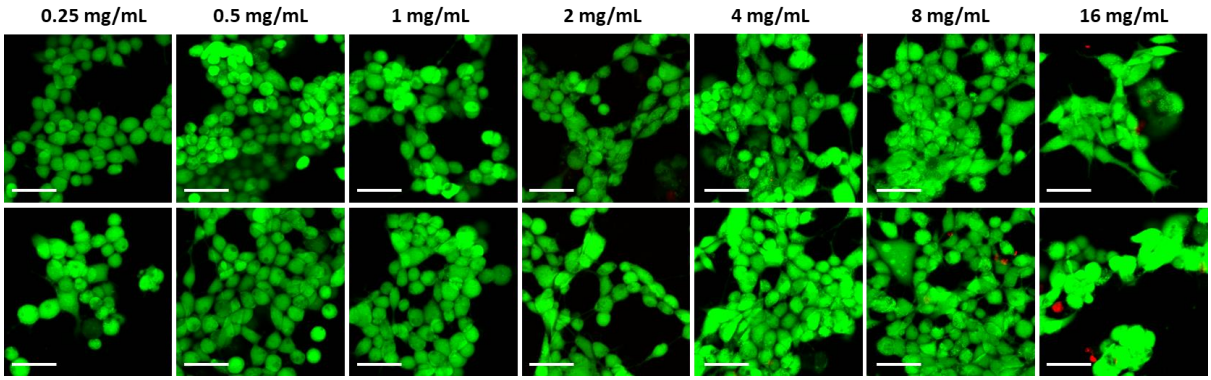


**Figure S15**. CLSM images of B16-F10 cells co-stained with calcein AM (green) and PI (red) after incubation with MPs at different concentrations (0.25-16 mg/mL). Scale bars = 50 $\mu$m.

*6.4.2. Cytotoxicity of 2-AT*

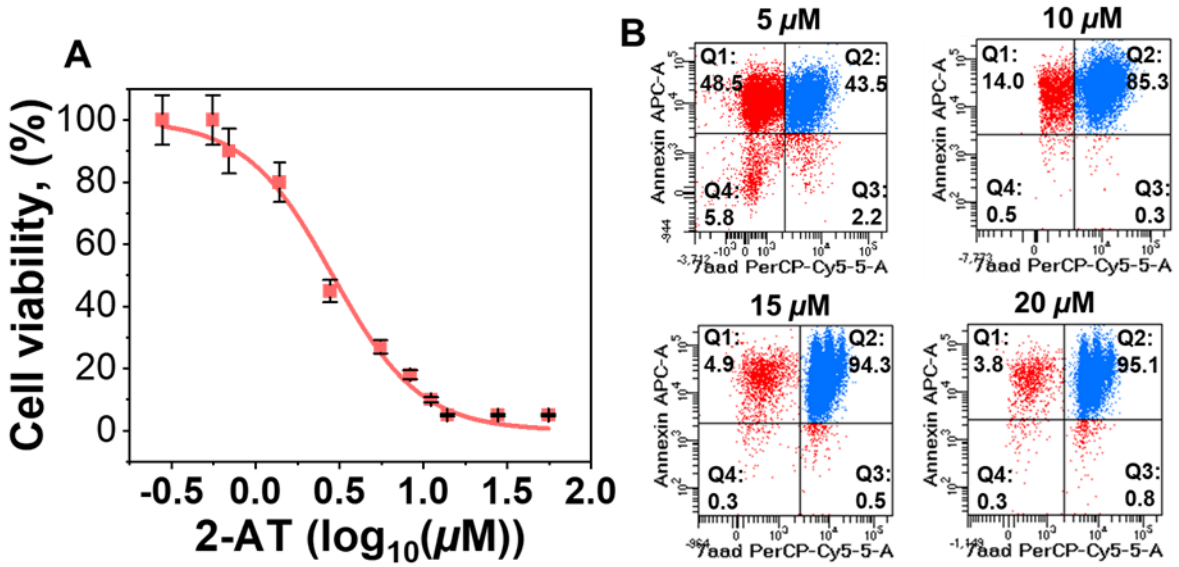


**Figure S16.** (A) Cells were treated with **2-AT** to assess their cytotoxic activity determined by AlamarBlue assay. The concentration range of tested compounds was 0.28–55 $\mu$M that corresponds to $\log_{10}$ = -0.56–1.74. (B) Apoptosis and necrosis analysis by flow cytometry using Annexin-V-APC/7-AAD double staining determined after 48 h incubation of B16-F10 cells with different

concentrations of **2-AT** (5, 10, 15 and 20 $\mu$M which corresponds to 2.7, 5.4, 8.0 and 10.7 $\mu$g/mL). These results were presented in our previous work.[4]

**Table S1**. The effect of synthesized compound **2-AT** on the survival of cancerous cells (B16-F10 melanoma) and normal non-cancerous fibroblasts (MEF NF2).[4]

| № | Compound | Substituent | | | $IC_{50}$ ± S.D. ($\mu$M) B16-F10 | $IC_{50}$ ± S.D. ($\mu$M) MEF NF2 |
|---|---|---|---|---|---|---|
| | | R | $R^1$, $R^2$ | $R^3$ | | |
| **1** | **2-AT** | 4-$MeOC_6H_4$ | -$(CH_2)_4$- | $CONH_2$ | 2.41 ± 0.3 | > 100 |

Cell survival was determined using Alamar Blue assay. The $IC_{50}$ values are represented as mean ± SD of three independent experiments performed in triplicate.

*6.4.3. AlamarBlue test*

AlamarBlue assay was used to examine the viability of cells after the incubation with several concentrations of **MPs**, **2-AT** and **2-AT@MPs**. The alamarBlue analysis includes a fluorometric/colorimetric growth indicator based on the determination of metabolic activity.[5] For this test, B16-F10 cells were seeded into a 96-well plate ($1.0\times10^4$ cells per well) and incubated overnight at 5% $CO_2$, 37°C. Before samples were added to the cell cultures, calculations were made (**Table S2**).

**Table S2.** Relationship between the amount of **2-AT** and the concentration of **MPs**.

| **2-AT**, $\mu$g per well* | **MPs** mg/mL |
|---|---|
| 0.004 | 0.004 |
| 0.007 | 0.008 |
| 0.015 | 0.016 |
| 0.03 | 0.031 |
| 0.06 | 0.062 |
| 0.12 | 0.125 |
| 0.23 | 0.250 |
| 0.47 | 0.500 |
| 0.94 | 1.000 |
| 1.87 | 2.000 |
| 3.75 | 4.000 |
| 7.5 | 8.000 |
| 15 | 16.000 |

*The volume of the well is 100 $\mu L$

After that, **MPs**, **2-AT** and **2-AT@MPs** were added to the wells with B16-F10 cells in the concentrations shown in **Table S2** and incubated for 24 h. After that, the cell medium was removed, and alamarBlue (10% v/v) was added to each well. All the wells were incubated for 4 h at 5% $CO_2$ and 37°C, and rechecked for color

changes. Absorbance was analyzed on a spectrophotometric microplate reader at wavelengths of 570 and 600 nm. Untreated cells were used as controls. The results were evaluated according to the formula:

$$Cell\ viability\ (\%) = \frac{(O2 \times A1) - (O1 \times A2)}{(O2 \times P1) - (O1 \times P2)} \times 100\%$$

Where:

O1 = molar extinction coefficient (E) of the oxidized alamarBlue at 570 nm (80586)

O2 = E of the oxidized alamarBlue at 600 nm (117216)

A1 = absorbance of the test wells at 570 nm

A2 = absorbance of the test wells at 600 nm

P1 = absorbance of the positive growth control wells (cells plus alamarBlue but no test agent) at 570 nm

P2 = absorbance of the positive growth control wells (cells plus alamarBlue but no test agent) at 600 nm

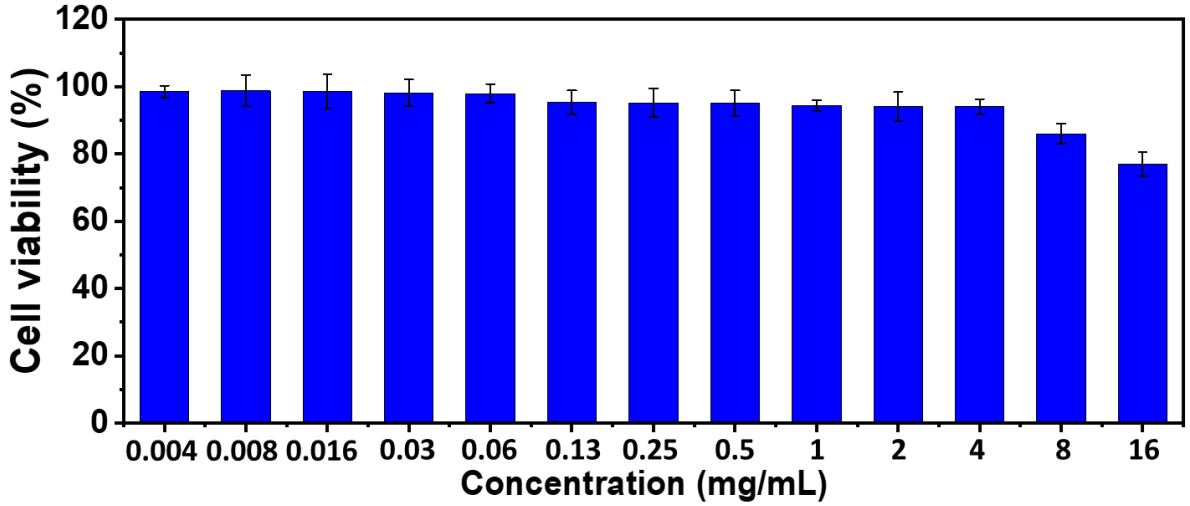


**Figure S17.** Cytotoxicity of **MPs** examined on B16-F10 cells after 48 h of incubation. The results are shown as an average value ± standard deviation (n = 4).

## 7. Hemolysis assay

Hemolysis assay was performed to study the influence of **MPs** and **2-AT@MPs** on human red blood cells (RBCs). For this, 1 mL of 3.8% sodium citrate solution was added to 10 mL of blood to avoid blood clotting. Then, blood was added to a 7 mL centrifuge tube containing 7 mL of ficoll solution (density ~ 1.077). Next, the reaction solution was centrifuged for 30 min at 312 × g. As a result of the interaction of ficoll and blood, the RBCs sank to the bottom of the tube, separating from the plasma. The resulting cell pellet was washed in a sodium phosphate buffer (pH 7.4) five times. Then, the human RBCs were diluted to a final concentration (5%, v/v) in PBS. **MPs** and **2-AT@MPs** in PBS were further added to human RBCs at different concentrations (0.25 - 16 mg/mL) to a final volume of 1 mL. Afterwards, the human RBCs were incubated for 2 h, then centrifuged for 5 min at 300 × g. The quantitative determination of hemoglobin concentration in the supernatant was carried out using a spectrophotometer at an absorption wavelength of 540 nm. As a positive control, a buffer for lysis of erythrocytes was added to the human RBCs, and PBS was added as a negative control. The percentage of hemolysis was calculated using the formula:

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

## 8. Animals

Healthy mice of the C57BL/6 strain (male, age 8 weeks, weight 18-22 g, Rappolovo, St. Petersburg, Russia) were used for *in vivo* studies. All animal studies were approved by the local authorities and were performed in accordance with the guidelines for animal housing and care (European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes). Mice were maintained at room temperature (22-24°C) and humidity

(40-60%) under a 12-hour light/dark cycle in standardized cages with free access to food and water for at least 15 days prior to the experiments.

To evaluate the results of all experiments, mice were sacrificed by cervical dislocation, as this method minimizes animal suffering and does not result in chemical contamination of samples and organs for further study.

## 9. Mice tumor model

B16-F10 cells were used for an in vivo subcutaneous melanoma model. Cells were cultured in $\alpha$-MEM medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Biochrom) and maintained at 37°C in a humidified incubator with 5% $CO_2$, expanded over several passages, trypsinized in the exponential growth phase, washed with PBS and diluted in αMEM medium to a concentration of $1.0\times10^6$ cells per 100 $\mu$L. Cells were routinely examined for Mycoplasma contamination. C57BL/6 mice (males, eight weeks old, 18-22 g, Rappolovo, St. Petersburg, Russia) were then subcutaneously injected with the cell suspension in the volume of 100 $\mu$L (0 d) into the right hind limb. Seven days after tumor injection (7 d), the mice were examined and animals with a sufficient tumor size (approximately $0.05 \pm 0.01$ $cm^3$) were used for further experiments.

## 10. Radiolabeling of MPs

### *10.1. Radiolabeling of MPs with $^{99m}Tc$*

The **MPs** were radiolabeled using a chelater-free method.[6] Briefly, 10 mg of $SnCl_2$ were dissolved in 800 $\mu$L 2% HCl solution and were added with 1 $\mu$L of Tween 80 to the particles' pellet (10 mg). The suspension was carefully resuspended, and 200 $\mu$L (12 MBq) of $^{99m}TcO_4^-$ (solution in 0.9% NaCl) was added to the resulting suspension. The samples were incubated in a thermomixer for 1 h (700 rpm, 24°C). Then, the pH of the reaction was gently adjusted to 6.5-7.2 with

0.1 M NaOH. The final $^{99m}$Tc-labeled **MPs** (**$^{99m}$Tc@MPs**) were washed 3 times (4000 rpm, 1 min) and resuspended in 1 mL of 0.9% NaCl solution.

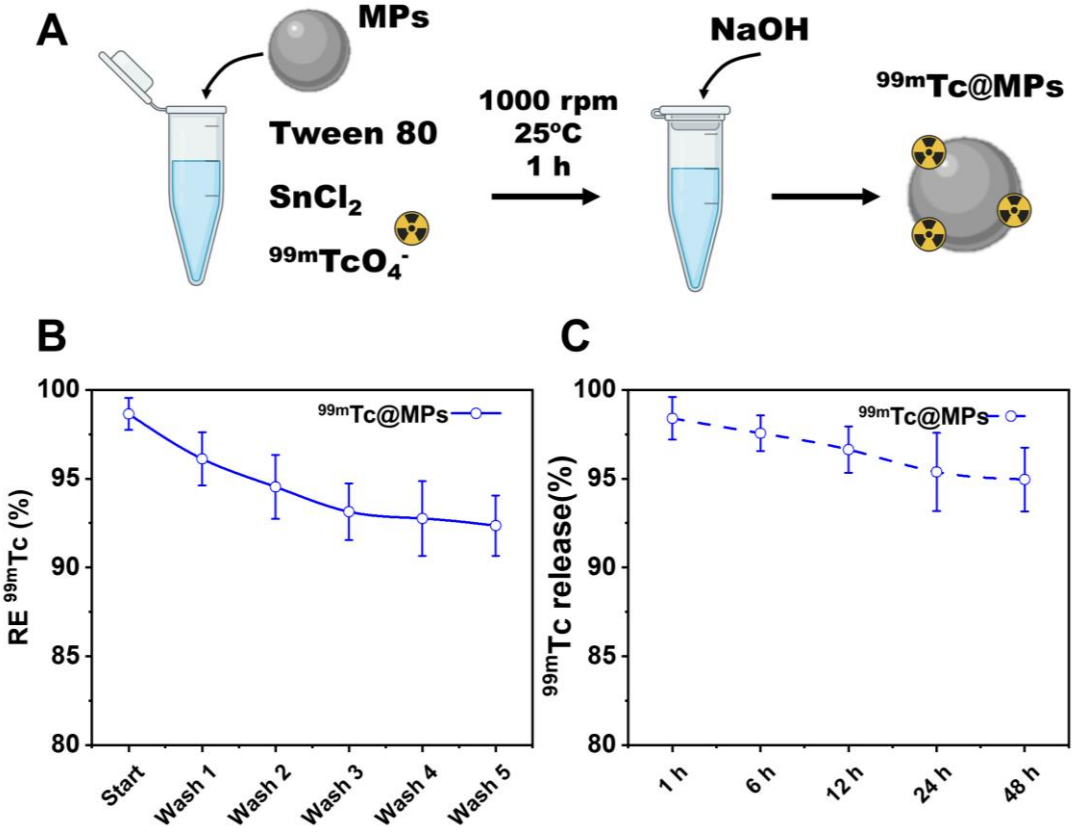


**Figure S18**. Radiolabeling of **MPs** with $^{99m}$Tc: (**A**) Schematic illustration of radiolabeling. (**B**) Radiolabeling efficiency (RE, %) of **$^{99m}$Tc@MPs**. (**C**) Release of $^{99m}$Tc from **$^{99m}$Tc@MPs** for 48 h. The results are shown as average value ± standard deviation (n = 4).

*10.3. In vitro radiochemical stability (isotope retention)*

The studies concerning the retention of isotopes within the **$^{99m}$Tc@MPs** were carried out as follows. The obtained **$^{99m}$Tc@MPs** (100 MBq) were washed 5 times with 0.9% NaCl solution by centrifugation at 4000 rpm for 1 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). The measurements of the radioisotope retention level were carried out for 2 d. For this, the samples

were centrifuged (4000 rpm, 1 min) 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 $^{99m}$Tc 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).

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

## 11. Biodistribution studies

### *11.1. SPECT*

Single-photon emission computed tomography (SPECT) was used to observe the biodistribution of **$^{99m}$Tc@MPs** (10 MBq, 50 $\mu$L) in the mice. The 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** were redispersed in 200 $\mu$L of sterile PBS to obtain the required concentration of radioactivity (100 MBq/mL), and 50 $\mu$L 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.

*11.2. IVIS bioluminigraph*

To confirm the biodistribution and retention of **MPs** inside tumors, the **Cy5@MPs** were intratumorally injected to tumor-bearing C57BL/6 mice. At 24 h and 48 h, the mice were sacrificed, and then the main organs (heart, lung, liver, spleen, kidney) and tumors were taken for analysis. The fluorescent signals from organs and tumors were monitored using IVIS Lumina II (IVIS Lumina II, PerkinElmer Inc, USA) in epi-fluorescence 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 area of interest (ROI) of each animal was detected by IVIS with the selected settings in a specific area.

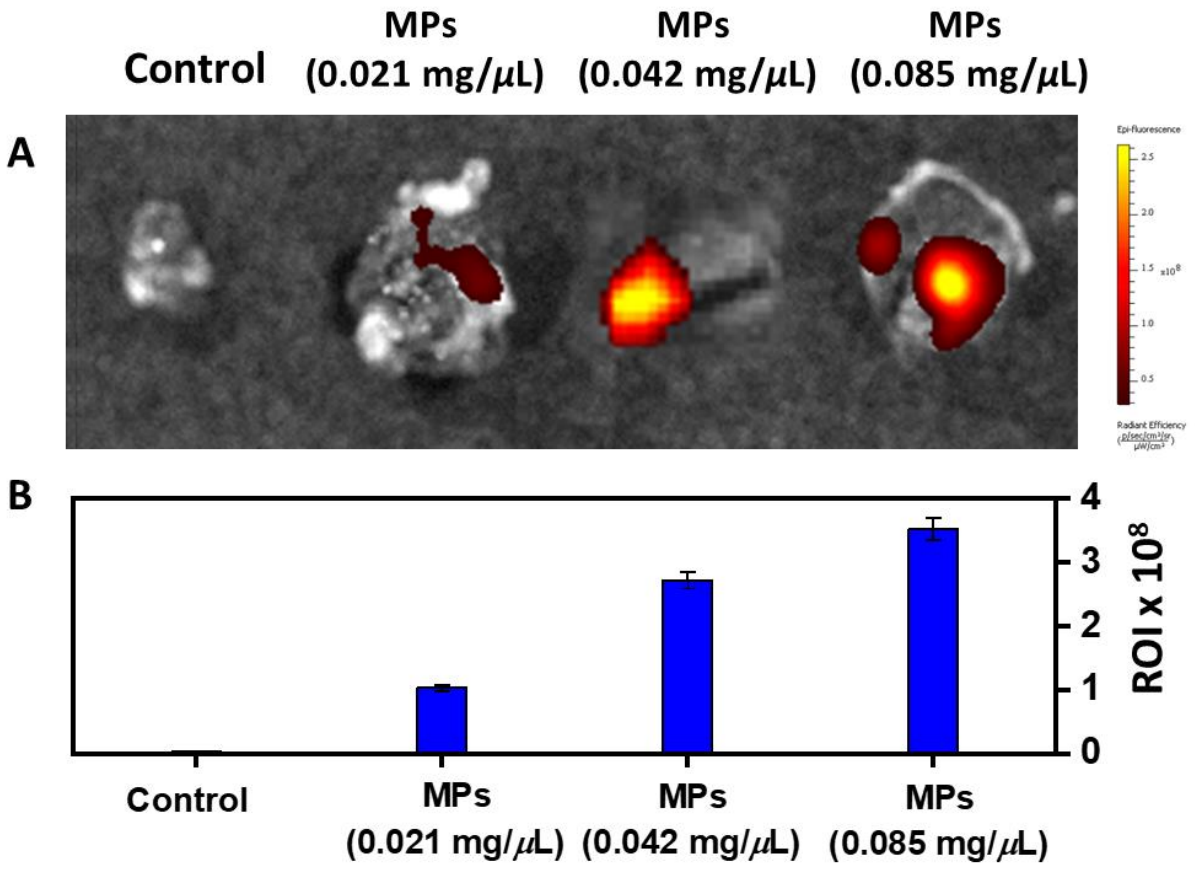


**Figure S19**. (**A**) *Ex vivo* fluorescent imaging of tumors after intratumoral injections of **Cy5@MPs**. (**B**) Quantitative fluorescent signals from regions of interest (ROI).

*11.3. Direct radiometry*

Direct radiometry analysis of the biodistribution of the **$^{99m}$Tc@MPs** in the tumor-bearing C57BL/6 mice was performed using a TRIATHLER portable spectrometric radiometer (Hidex Oy, Finland). Each animal was injected intratumoral with **$^{99m}$Tc@MPs** (10 MBq, 50 $\mu$L). The animals were sacrificed 48 h after the **$^{99m}$Tc@MPs** administration. Then, the main organs (heart, lung, liver, spleen, and kidney) 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 (% dose/g).

## 12. Histological analysis

*12.1. Preparation of the histological slices*

For preparation of histological slices mice were euthanized. Next, biopsy samples were isolated from the main organs (heart, lung, liver, spleen, kidney) and tumors. The tissue samples were soaked in PBS and transferred to a buffered formalin solution for 3 d. The samples were then dehydrated with an isopropanol solution on a Leica TP1020 Tissue Processor. After 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 $\mu$m sections using a HM 340E rotary microtome (Thermo Scientific) and placed on glass slides for further staining.

*12.2. Fluorescent staining*

Histological tissue sections obtained from euthanized mice after the injection of **Cy5@MPs**, were placed on glass slides. Then, the samples were placed in a solution of PI (10 mg/mL) for 5 min to fluorescently stain the cell nuclei. The sections were then washed twice in PBS solution and fixed with glycergel according to the standard protocol. The fixed samples were then visualized under CLSM. All the slides were

evaluated by CLSM to observe the localization of **MPs** in tumors. To visualize the tumor tissues, a helium neon laser emitting at 543 nm was used. To visualize **Cy5@MPs,** 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.

## 13. Therapy studies

### *13.1. Histological analysis with H&E staining*

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

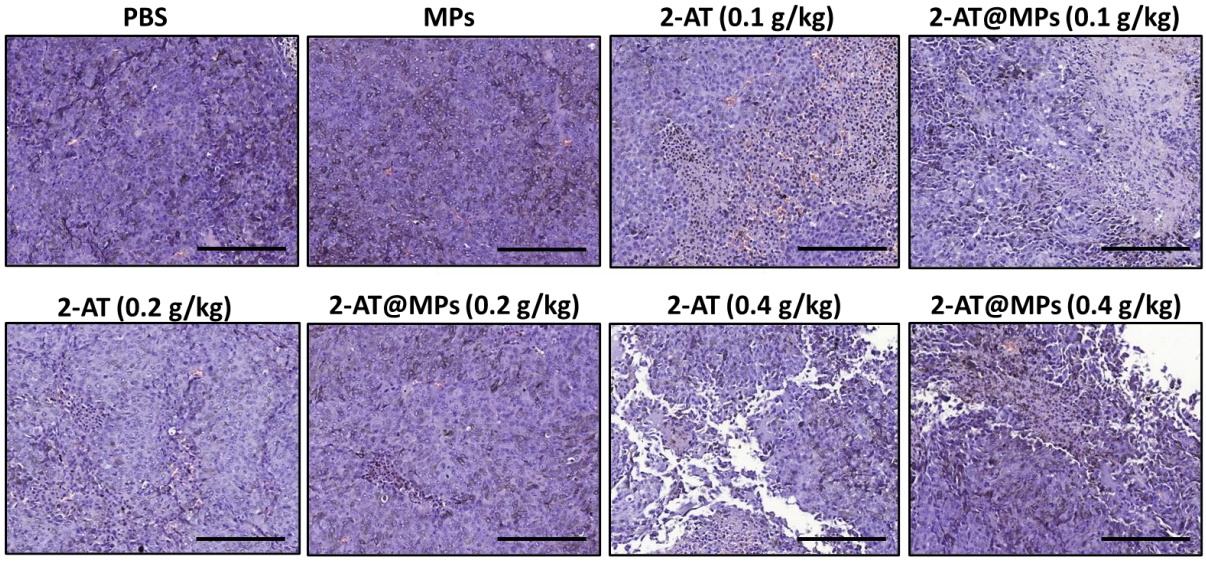


**Figure S20**. H&E histological staining of tumor tissues after the treatment. Scale bars = 200 $\mu$m.

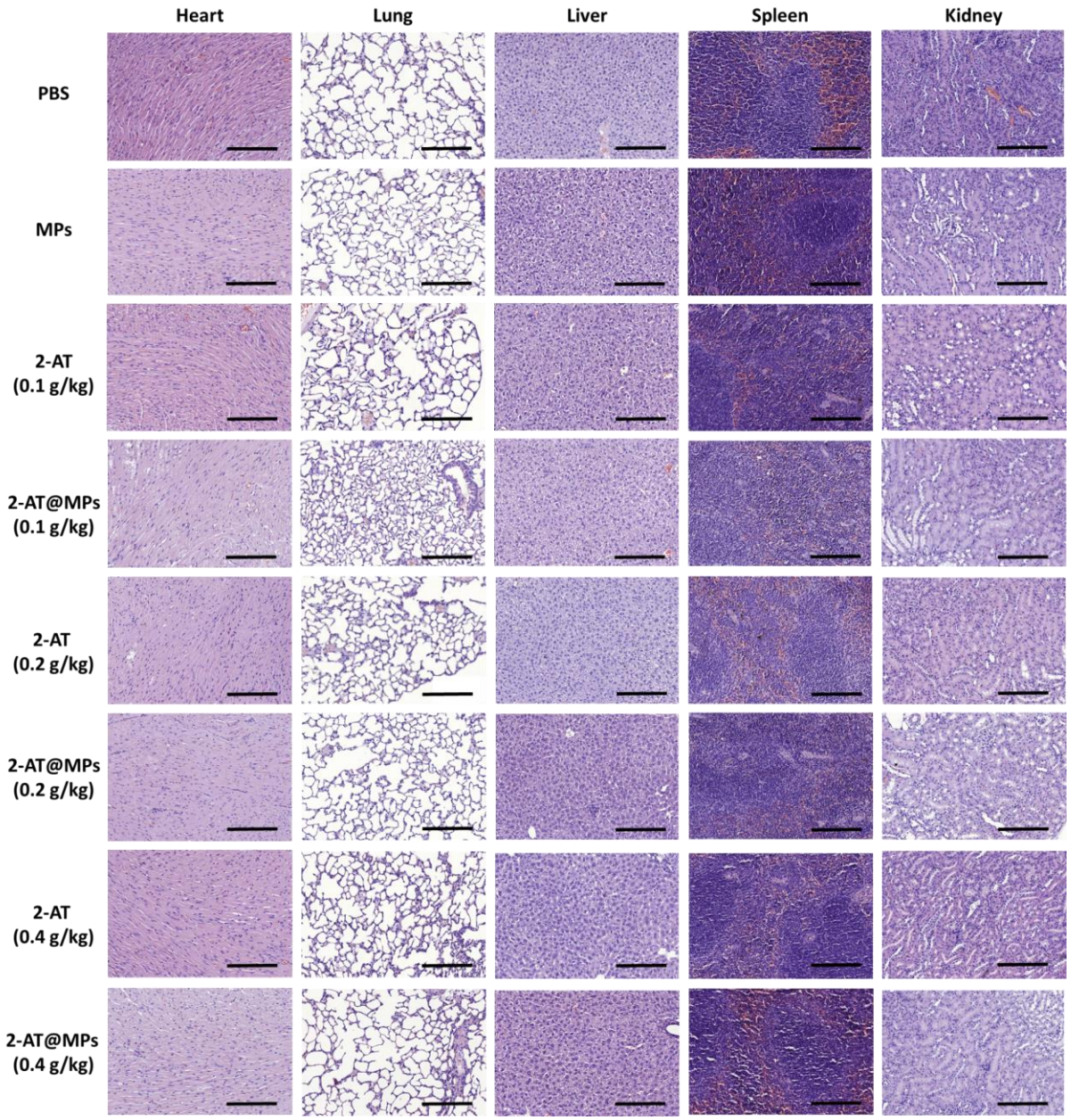


**Figure S21**. H&E-stained main organs (heart, lung, liver, spleen, and kidney) of mice treated with **PBS**, **MPs** ($C_{MPs}$ = 0.085 mg/$\mu$L), **2-AT** ($C_{2\text{-}AT}$ = 0.1, 0.2, and 0.4 g/kg), **2-AT@MPs** ($C_{2\text{-}AT}$ = 0.1, 0.2, and 0.4 g/kg, $C_{MPs}$ = 0.085 mg/$\mu$L) on the 14$^{th}$ day of therapy. Scale bars = 200 $\mu$m.

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