Supplementary Materials

for

Designing Dual-functionalized Carbon Nanotubes with High Blood-brain-Barrier Permeability for Precise Orthotopic Glioma Therapy

Experimental Section

Materials

Multiwalled carbon nanotubes (MWCNTs) were obtained from Shenzhen Nanotech Port Co. Ltd., China. Oxaliplatin, polyethyleneimine (10000 Da, PEI), 1-ethyl-3-(3’-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich. Other chemicals used in this study are all of analytical grade. Milli-Q water used in this work was collected from ultrapure water purification system (Millipore). The amino acid sequence of TAT peptide is Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg.

Preparation of TBCNT@OXA: MWCNTs-COOH was synthesized as previously described.¹ For the synthesis of TAT- biotin-PEI copolymers, biotin and TAT were added into PEI solution (10 mg/ml) for 24 h along with EDC/NHS (4:1, m/m) as an amidation catalyst. The resulting suspension was purified by dialyzing with distilled water for 48 h (MW cutoff = 6000-8000 Da). Then, 20 mg MWCNTs-COOH was stirred with the TAT- PEI - biotin copolymers in water. The reaction product was purified by dialysis with the MW cutoff at 6000 Da) and collected TBCNT
Oxaliplatin was encapsulated into the TBCNT through nano-extraction as previously reported.\textsuperscript{2,3} Precisely, 10 mg TBCNT and 10 mL ethanol were added to a beaker, and then sonicated for 30 min. Afterwards, 10 mg oxaliplatin (in ethanol) was added drop by drop to the former solution and then stirred for 48 h at room temperature. The mixture was then extensively washed with ethanol and centrifuged at 10000 rpm/min to remove free oxaliplatin. TBCNT@OXA was then obtained by vacuum drying.

**Labeling of TBCNT@OXA by FITC**

In this study, 20 mg TBCNT@OXA in 10 mL water was sonicated to disperse completely, and then 1 mg FITC (in ethanol) was dropwise added to the former solution. Make it react overnight at room temperature in the dark. The resulting suspension was dialyzed in distilled water for 48 h (MW cutoff = 10 kDa) in the dark to remove the free FITC.

**Characterization of TBCNT@OXA**

Transmission electron microscopy (TEM, Hitachi H-7650), zetasizer particle size analysis (Malvern Instruments Limited), and X-ray photoelectron spectroscopy (XPS), and so on were employed to characterize the structure feature of TBCNT@OXA in this study.

**In Vitro Drug Release of TBCNT@OXA**

The drug release behavior of TBCNT@OXA was performed as previously published procedures.\textsuperscript{4} PBS (pH 7.4) and PBS (pH 5.3) was used as the drug release media to simulate the normal body blood and cancer cell microenvironment. TBCNT@OXA (10 mg) was dispersed into 10 mL released media, and then the solutions were put into glass tubes. These glass tubes were shaken at 37°C without
light. At specific time intervals, 200 μL of media was taken respectively out for measuring the released drug concentrations and then were returned to the fresh release media. The concentrations of Pt of all samples were determined by ICP-AES.

**MTT assay**

The cells were seeded in a 96-well tissue culture plates at a density of $2 \times 10^3$ cells per well in complete DMEM medium for 24 h. Then, the drugs were added respectively to the medium. After incubation for 72 h, MTT solution (5 mg/ml PBS, 30 μL/well) was added and then incubated for 4 h. The culture medium was then moved and replaced with 100 μL of DMSO for 10min with shake. The absorbance at 570 nm was recorded using a microplate reader SpectroAmaxTM 250). The results of IC$_{50}$ values were based on three independent experiments.

**Cellular uptake and Cellular Retention of TBCNT@OXA in vitro**

Fluorescence method was used to examine the cellular uptake. The cells were incubated with 5 μM FITC-labeled TBCNT@OXA and then the fluorescence intensity of FITC was detected via a microplate reader at the special times (Spectra Max M5, Bio-Tek, excitation wavelengths: 490 nm, emission wavelengths: 525 nm).

To compare quantitatively the cell uptake, the C6 cells were incubated with 5 μM drugs for 4 h, 6 h and 8 h in 10 cm dishes, respectively. At desired time points, the cells were digested and collected, and determined the Pt concentration by ICP-AES.  

The cells were seeded in a 6-well plate at a density of $20 \times 10^3$ cells/ml in medium, and cultured in a 5% CO2 at 37 °C for 24 h. Then, drugs were added to the medium and then incubated for 8 h. Then, the medium was moved and replaced
with 2 ml of medium without phenol. At specific time intervals, 100 μL of the medium were taken respectively out for measuring the drug concentrations. The concentrations of Pt of all samples were determined by ICP-AES.

**Flow Cytometric Analysis**

Flow cytometric analysis was used to analyze cell cycle distribution of cancer cells treated with TBCNT@OXA. Briefly, cells were seeded in dishes plates (6 cm) at density of 2×10^4 cells /mL and incubated for 24 h. After treated or untreated with drugs for 24 h, cells were digested with trypsin-EDTA solution (0.25% trypsin, 1mM EDTA) and collected by centrifugation and fixed with 70% ethanol at -20 °C for 12h. Then, the cells were centrifuged and stained with propidium iodide (PI) which contains 1.21mg/mL Tris, 700U/mL RNase, 50.1µg/mL PI for 1 h in darkness. The stained was measured by flow cytometry according to the protocol provided by the Epics XL-MCL flow cytometer (Beckman Coulter, Miami, FL). MultiCycle software (Phoenix Flow Systems, San Diego, CA) was used to analyze cell cycle distribution.

**Measurement of Intracellular ROS Generation**

DHE assay was used to detected the effects of TBCNT@OXA on ROS generation in cancer cells as previously described. In briefly, cells were collected by centrifugation and suspended in PBS and incubated in the dark with the fluorescent DHE (10 μM) as a probe for 30 min at 37 °C. The cells were then seeded in 96-well tissue culture plates at the density of 10×10^3 cells /well. And then added the drugs into the well, fluorescence was monitored using a microplate reader (SpectraMax M5, MD, USA) with 300 nm excitation and 610 nm emission settings. For confocal microscopy images of ROS, the cells were seeded in a 6-well plate at a density of 20 × 10^3 cells per well in complete DMEM medium, and cultured
in a 5% CO2 at 37 °C for 24 h and were then incubated in the dark with DHE for 30 min at 37 °C. Then, the drugs were added into the plate. And then, the confocal microscopy images were taken by a confocal microscope.

**Construction of BBB model in vitro and the Examination of BBB permeability**

The establishment of BBB model was used the previous method. HBMEC cells were widely used as a model for mimicking brain capillary endothelial cells and evaluating the BBB penetrating capability in vitro. The HBMEC cells (8×10^4 cells/insert) were seeded on culture insert (Corning, 0.3 μm pore size), while C6 cells (5000 cells/compartment) were planted on the basolateral compartment. The tightness of the monolayer was evaluated by measuring value of the transendothelial electrical resistance (TEER) using a TEER instrument (Millipore, ERS-2) and the value should go over 250 Ω·cm^2. The BBB model was used to measure the BBB permeability of drug. Drugs were mixed into the insert. After 24 h, the culture medium in the basolateral compartment was used to determine the penetration ratio by ICP-MS. The concentration of oxaliplatin and TBCNT@OXA used to detect the BBB permeability was 10 μM.

**Tumor spheroids**

C6 spheroids were cultured in vitro using liquid overlay system. And then C6 cells (5000 cells/well) were seeded into 96-well ultra-low attachment surface culture plate (Costar). Subsequently, plates were gently agitated at set intervals on the first day, and C6 spheroids were allowed to grow for 4 days.

**Penetrating ability to C6 spheroids**

To compare the penetration ability of CNT@OXA and TBCNT@OXA, C6 spheroids were treated with the FITC-labelled three nanoparticles for 12 h. Thereafter, the tumor spheroids were scanned at the different layers from the top
of the spheroid to the middle using a confocal laser scanning fluorescent microscope (Zeiss LSM700).

**Inhibitory effect**

To compare the inhibitory effect of oxaliplatin and the three nanoparticles, C6 spheroids were treated with oxaliplatin and TBCNT@OXA respectively. The size of a tumor spheroid was used to evaluate the inhibitory effect under an invert microscope at day 0, 1, 2, 3, and 5. Briefly, the major (d<sub>max</sub>) and minor (d<sub>min</sub>) diameters of each spheroid were determined, and spheroid volume was calculated by using the following formula: \( V = \frac{\pi \times d_{\text{max}} \times d_{\text{min}}}{6} \). The volume change ratio of C6 spheroid was calculated with the formula: \( R = \frac{V_{\text{dayi}}}{V_{\text{day0}}} \times 100\% \). Each assay was repeated in triplicate, and sextuplicate determinations were set for each dose level.

**Western Blot Analysis:** The receptor expression levels of the tested cells were analyzed by western blotting as previously described.\(^8\)

**Construction of Orthotopic Xenografts Tumor Model of Glioma**

C6 cells and SD mice were performed to establish intracranial orthotopic xenografts tumor model of glioma. The mice were anesthetized with an intraperitoneal injection of diazepam (20 mg/kg) followed by intramuscular injection of ketamine (40 mg/kg). The stereotactic head frame was used to immobilize the head of mice. Then, a burr hole was made 1 mm anterior to the bregma and 3 mm right of the midline. 10 \( \mu \)L C6 cells (1\( \times 10^7 \) cells/ml) were injected into the brain at a depth of 5 mm from the dura by a microsyringe. The incision was sutured closed with 5/0 prolene. Postoperatively, the mice were given a subcutaneous injection of 5 mg/kg caprofen. MRI was used to verify the successful establishment of the tumor model.
*In vivo* Antitumor Ability of TBCNT@OXA

The antitumor ability of TBCNT@OXA were analyzed as previously described.\(^9\)

The orthotopic xenografts tumor rats were divided into 3 group (n = 5 per group). The rats were deal with CNT@OXA and TBCNT@OXA (4 mg/kg) with injection them into the veins of the tails every other day.

**Statistical Analysis**

Results were expressed as the mean ± SD, which were tested from at least 3 parallel experiments. Difference with *p < 0.05* (*) or *p < 0.01* (**) was considered statistically significant.

References:

Figure S1. The 1H-NMR spectrum of TAT, biotin, PEI and TAT-PEI-Biotin.

Figure S2. TEM images of MWCNTs.
Figure S3. TEM images of MWCNT-COOH.

Figure S4. The BBB model *in vitro*.
Figure S5. The cellular uptake comparison of TBCNT@OXA in C6 cells and CHEM-5 cells.

Figure S6. Quantitative analysis of cellular uptake of oxaliplatin, CNT@OXA and TBCNT@OXA in C6 cells and CHEM-5 cells at different time. The cells treated with 5 μM drug.
Figure S7. Quantitative analysis of the cellular retention of oxaliplatin and TBCNT@OXA in C6 cells. The cells were treated with drug (5 μM) for 6 h, then the medium was removed and replaced with fresh medium (2 mL) and the amount of drug inside the cells was determined after different periods of time.

Figure S8. Expression level of FAR on cancer cells and normal cells. The effects of biotin on the cellular uptake of TBCNT@OXA. The cells were preprocessed biotin for 2 h and then treated with 10 μM TBCNT@OXA for 6 h.
**Figure S9.** Biotin obviously inhibited the cell uptake and cell viability of TBCNT@OXA (1 μM).

**Figure S10.** Intracellular uptake of TBCNT@OXA in C6 cells with different endocytosis-inhibited conditions. Before the incubation of TBCNT@OXA, cells were treated with specific endocytosis-inhibitors at different periods of time at 37 °C.
**Figure S11.** Inhibitory effect of oxaliplatin, CNT@OXA and TBCNT@OXA (5 μM) to C6 brain tumor spheroids.

**Figure S12.** *In vitro* drug release of TBCNT@OXA (1mg/ml) in PBS at pH 7.4 and pH 5.3. Each value is represented as mean ±SD (n=3).
Figure S13. The image of establishment of orthotopic glioma model.
Figure S14. T2 MR images of tumors for healthy mice.

Figure S15. The changes of orthotopic tumor volume growth rate in CNT@OXA and TBCNT@OXA groups. The tumor volumes were detected by MRI.
**Figure S16.** The values of slow ADC

**Figure S17.** The body weight of orthotopic glioma mice treated with saline, CNT@OXA or TBCNT@OXA and the images of the mice on the last day. Values represented means ± SD of triplicates.
**Figure S18.** Safety Evaluation of TBCNT@OXA *in vivo*. (A) H&E staining of normal organs from mice after treated with CNT@OXA and TBCNT@OXA for 21 days. (B–D) Blood biochemistry analysis of in mice treated with CNT@OXA and TBCNT@OXA at 21 days of treatment.

**Figure S19.** Flow cytometry analysis of C6 cells treated with oxaliplatin and TBCNT@OXA for 72 h.
Figure S20. The images of DHE fluorescence in C6 cells treated with TBCNT@OXA at different periods.

Figure S21. (A) The antioxidants NAC obviously reduce the production of ROS induced by TBCNT@OXA. Cells were firstly treated with NAC (10 mM) for 2 h and then exposed to TBCNT@OXA at indicated concentrations and immediately read in a microplate. (B) The antioxidants NAC obviously reduced the cell cytotoxicity induced by TBCNT@OXA. Cells were treated with NAC (10 mM) for 2 h and then exposed to TBCNT@OXA for 24 h. Cell viability were determined by MTT assay. Each value was represented as mean ±SD (n=3). * P < 0.05 vs. control, ** P < 0.01 vs. control.
Table S1. Loading capacity of CNT@OXA, BCNT@OXA, TCNT@OXA and TBCNT@OXA for oxaliplatin.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Loading capacity for oxaliplatin (%)</th>
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<tbody>
<tr>
<td>CNT@OXA</td>
<td>25.4±3.4</td>
</tr>
<tr>
<td>BCNT@OXA</td>
<td>20.6±1.4</td>
</tr>
<tr>
<td>TCNT@OXA</td>
<td>20.3±0.9</td>
</tr>
<tr>
<td>TBCNT@OXA</td>
<td>19.7±1.3</td>
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