Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B.

Supporting Information

Tailored mesoporous silica nanosystem with enhanced permeability of blood-brain barrier to antagonize glioblastoma

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Methods

In vitro cellular uptake of BSeC@MSNs-RGD. U87 and C6 cells were put into 96-well plates (10×10^3 cells/well). After 24 h, the different concentrations of BSeC@MSNs-RGD were added into the culture medium. At different time points, 0.1% Triton X-100 in NaOH solution was added to lysis the cells. The absorption of BSeC@MSNs-RGD was measured by UV-vis Reader (Spectra Max M5, Tecan) with the wavelength at 348 nm based on the specific absorbance of BSeC. The concentration of BSeC in the cells was calculated according to the standard curve.

RGD competing assay and endocytosis inhibitors blocking assay. U87 cells (8×10^4 cells/ml) were added into a 96-well plate and cultured allowed to growing for 24 h. Then, U87 cells were pre-treated with RGD (0~2.5 mg/ml) or endocytosis inhibitors for 2 h and expose to BSeC@MSNs-RGD (20 μM) for 6 h. Finally the cells were washed with PBS and read under a UV-vis spectrometer at 348 nm.

MTT assay. Cells were seeded in a 96-well plate (2×10^4 cells/ml) and cultured for 24 h. And the cells were exposed to the drugs with different concentration for 72 h. Thereafter, MTT solution (20 μL/well) was added and incubated for another 4 h. 150 μL/well DMSO replaced the medium to dissolve the formazan crystals and measured at 570 nm using a microplate spectrophotometer (VSERSA Max).

Flow cytometric analysis. We further using flow cytometry detected the influence of cell cycle by BSeC@MSNs-RGD in U87 and C6 cell lines. In short, after treated by BSeC@MSNs-RGD for 72 h, cells were trypsinized by pancreatic enzymes and washed with PBS. Thereafter, 70% ethanol fixed the collected cells at -20 ℃ for 12 h. Then, the cells were collected by centrifuge, and stained with PI working solution (1.21 mg/mL Tris, U/mL RNase, 50.1 μg/mL PI, pH 8.0) for 1 h in darkness. Epics XL-MCL flow cytometer (Beckman Coulter, Miami, FL) was the instrument that analyzed the stained cells and 10,000 cells per

sample were recorded. The result was analyzed by Multi Cycle software (Phoenix Flow Systems, San Diego, CA).

Measurement of ROS generation. DCF fluorescence assay and DHE fluorescence assay were traditional method to analyze the effects of BSeC and BSeC@MSNs-RGD on ROS generation.[36] In brief, the cells were trypsinized, dispersed in the culture medium without phenol red and incubated with DCFH-DA or DCF-DA with final concentration of 10 mM at 37 ℃ for 30 min. The cells were then put into 96-well plates. And the different concentration of BSeC and BSeC@MSNs-RGD were added into the well. ROS level was examined on a fluorescence reader (SpectraMax M5, MD, USA) to measure the fluorescence intensity. The excitation and emission wavelengths of DHE were 300 and 610 nm and that of DCF were 479 and 599 nm.

Caspase activity assay. Lysis buffer (Beyotime) incubated the cells to obtain cellular proteins after U87 cells exposure to BSeC@MSNs-RGD for 72 h. The cell lysates were put into 96-well plates and then incubated with specific caspase-3, -8, -9 substrates (Ac-LEHD-AMC) at 37 ℃ for 2 h. The fluorescence intensity was used to determine caspase activity with excitation wavelengths and emission wavelengths 380 nm and 440 nm, respectively.

Western blot analysis. U87 cells after exposure to BSeC@MSNs-RGD for 72 h were incubated with lysis buffer (Beyotime) for 10 min to obtain cellular proteins. BCA assay was used to examine the protein concentration. SDS-PAGE was the method that separated the equal amount of proteins for each lane in10% tricine gels. And then the proteins transferred to nitrocellulose membrane at 110 V for 1 hand blocked the membranes in 5% non-fat milk in Tris-Buffered Saline Tween-20 buffer (TBST). After washed with TBST three times for 10min, the membranes incubated with the first primary antibodies at 4 ℃ with continuous agitation for 12 h and then scoured with secondary antibodies at 4 ℃ for 2 h.

U87 tumor spheroids. U87 spheroids were cultured *in vitro* using liquid overlay system.[38] Agarose was dissolved in PBS (2%, w/v) at 80 ℃ for 30 min, and sterilized at Autoclaves Sterilizers. Each well of 96-cell culture plates was coated with 50 μl of agarose solution. And then U87 cells (5000 cells/well) were seeded into 96-well tissue culture plates. Subsequently, plates were gently agitated at set intervals on the first day, and U87 tumor spheroids were allowed to grow for 7 days.

Penetrating ability to U87 spheroids. To compare the penetration ability of BSeC and BSeC@MSNs-RGD, U87 spheroids were treated with the BSeC and BSeC@MSNs-RGD (20 μM) for 12 h and then scanned at the different layers from the top of the spheroid to the middle using a confocal laser scanning fluorescent microscope.

Inhibitory effect to U87 spheroids. To confirm the inhibitory effect of BSeC@MSNs-RGD, the size of U87 tumor spheroids treated with BSeC and BSeC@MSNs-RGD (20 μM) was used to evaluate the inhibitory effect under an invert microscope at day 0, 1, 2, 3, and 5. Briefly, the major (d_{max}) and minor (d_{min}) diameters of each spheroid were determined, and spheroid volume was calculated by using the following formula: $V= (\pi \times d_{max} \times d_{min}) / 6$. The volume change ratio of U87 tumor spheroid was calculated with the formula: $R= (V_{dayi} / V_{day0}) \times 100\%$. Each assay was repeated in triplicate, and sextuplicate determinations were set for each dose level.

Destruction of brain cancer vasculogenic mimicry (VM) channels. VA Matrigel-based tube formation assay was used to assess the activity of BSeC@MSNs-RGD against the VM channels of glioma U87 cells. The Matrigel and 48-well plates were precooled at 4 ℃ overnight. A volume of 100 μL growth factor-reduced Matrigel (BD Biosciences) was plated onto 48-well plates, and incubated for 30 min at 37 ℃. U87 cells (1×10^4 cells/ml) were resuspended with serum-free DMEM containing free BSeC and BSeC@MSNs-RGD and then loaded on the top of the Matrigel. After incubation at 37 ℃ for 10 h, each well was analyzed directly under a microscope (Advanced Microscopy Group, Carlsbad, CA, USA).

***In vivo Pharmacokinetic Assay*.** Sprague−Dawley (SD) mice (about 160−200 g) used in this study were obtained from the Medical Laboratory Animal Center of Guangdong provice. Six mice were randomly divided into two groups, and they were fasted overnight before the experiment. The dose of BSeC and BSeC@MSNs was 5.0 mg kg^{-1} of mouse body weight (n = 3, per group) through intravenous injection. The blood samples were obtained at different time points (0, 0.5, 2, 4, 8, 12, 24, 48, and 72 h). BSeC was extracted by dissolving blood samples in HCl (0.75 M)/isopropanol at −20 ℃ overnight and the amount of BSeC in the plasma was evaluated by ultraviolet absorption of BSeC. The plasma clearance (Cl) and the area under the blood concentration curve (AUC), which was the main pharmacokinetic parameters, were calculated using WinNonlin 3.3 software.

Biodistribution and Hematological Analysis. The mice were fed with BSeC and BSeC@MSNs-RGD at a dosage of 5.0 mg kg^{-1} of mouse body weight (n = 5) through intravenous administration and then sacrificed at 72 h, and the organs including heart, liver, spleen, lung, and kidney were obtained. Meanwhile, the blood sample (72 h) was used for hematology analysis at Guangzhou Overseas Chinese Hospital. The drug concentration of BSeC in each organ was determined by ultraviolet absorption, as described above.

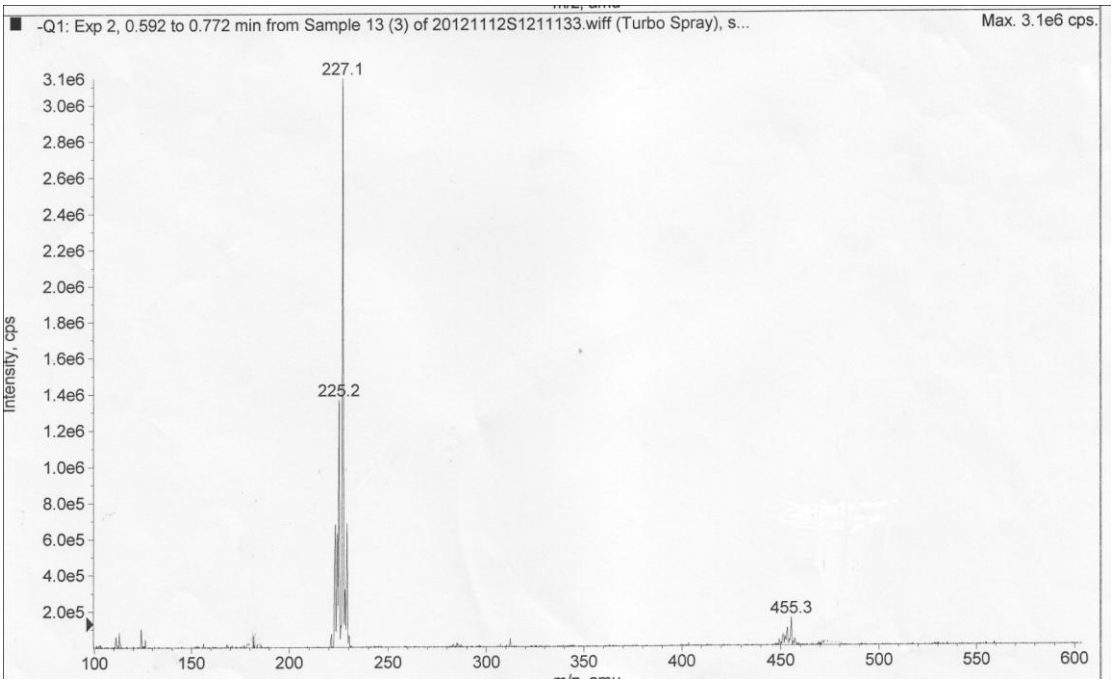

Figure S1. ESI-MS for BseC

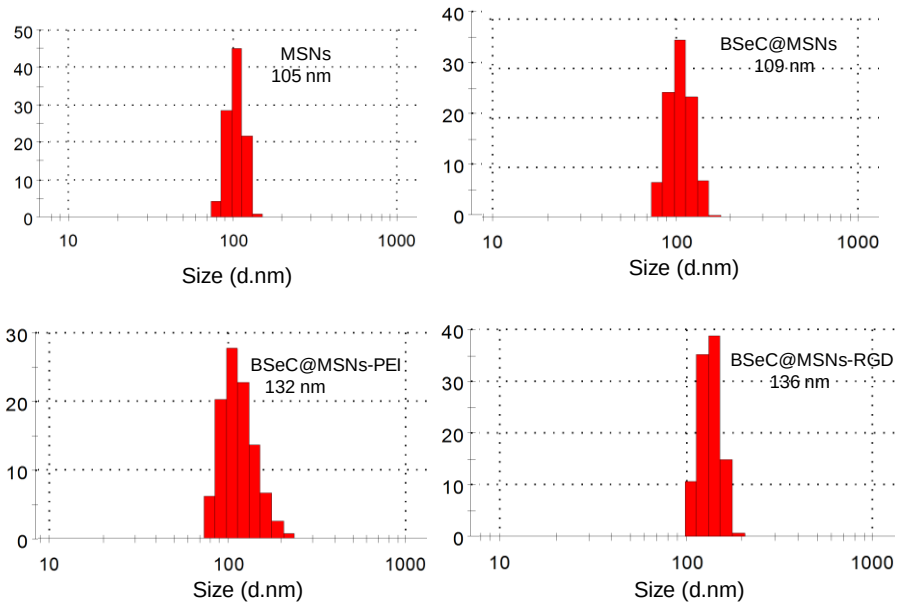

Figure S2. Particle size distribution of MSNs, BseC@MSNs, BSeC@MSNs-PEI and BSeC@MSNs-RGD

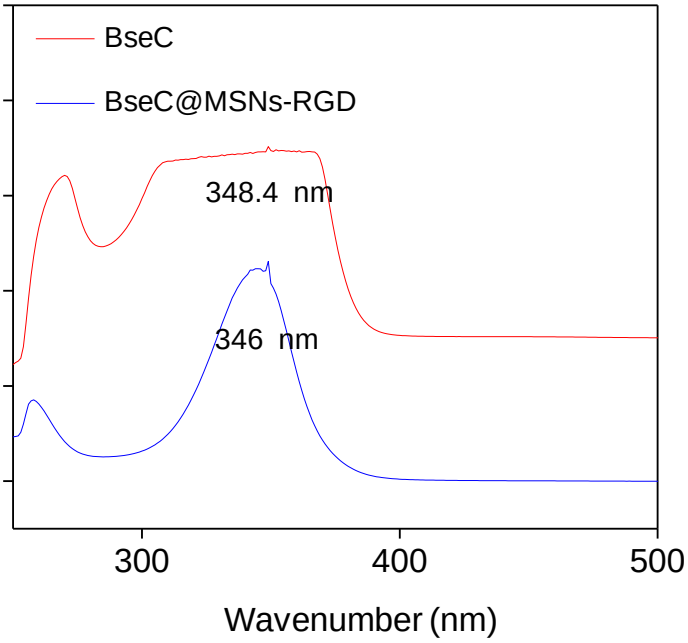

Figure S3. UV-Vis spectral analysis of BSeC and BseC@MSNs-RGD.

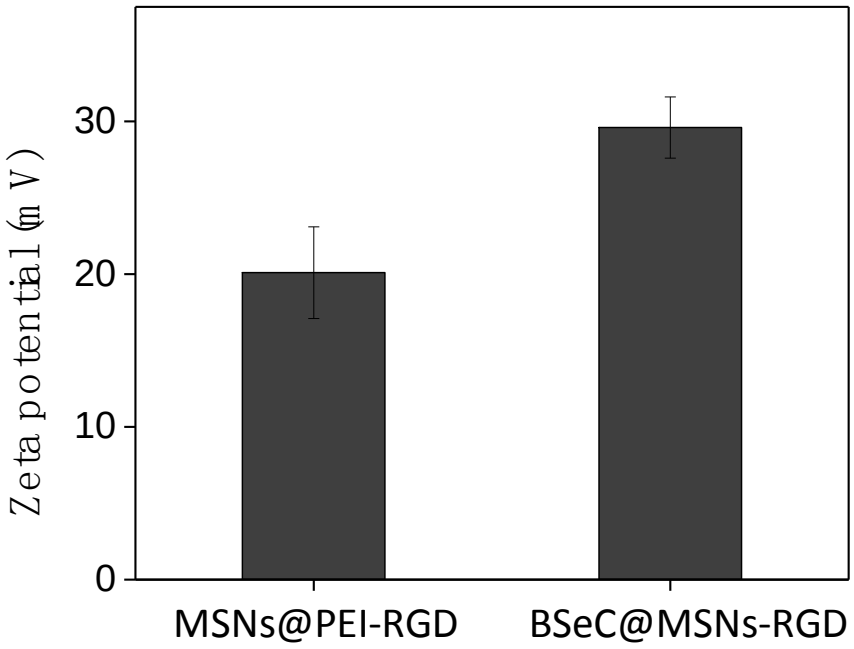

Figure S4. Zeta potential of MSN@PEI-RGD and BSeC@MSNs-RGD.

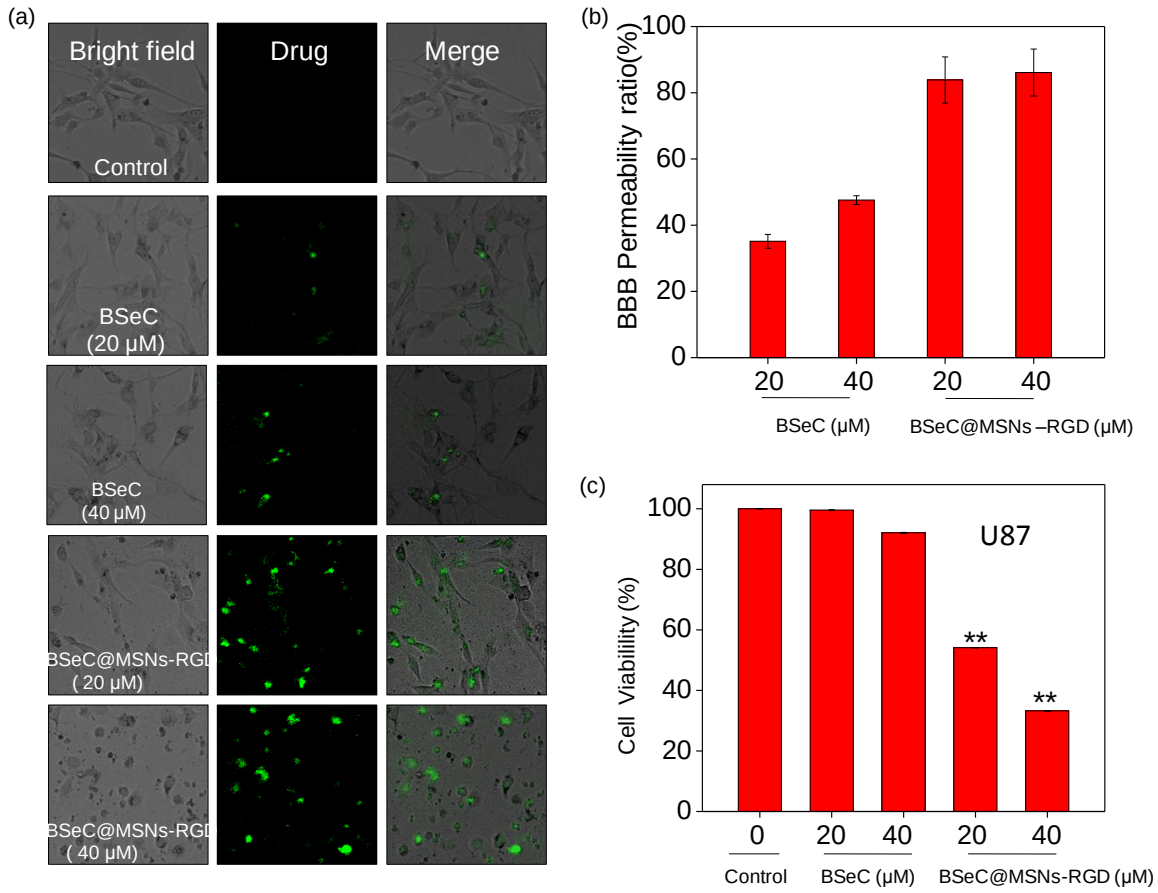

Figure S5. (a) The drug uptake on U87cells after penetrating for 72 h. (b) BBB permeability of BSeC and BSeC@MSNs-RGD from BBB. (c) Viability of U87 in the basolateral compartment after drug penetrating BBB for 72 h. Values expressed were means ±SD of triplicate. * $P < 0.05$ vs. control, ** $P < 0.01$ vs. control.

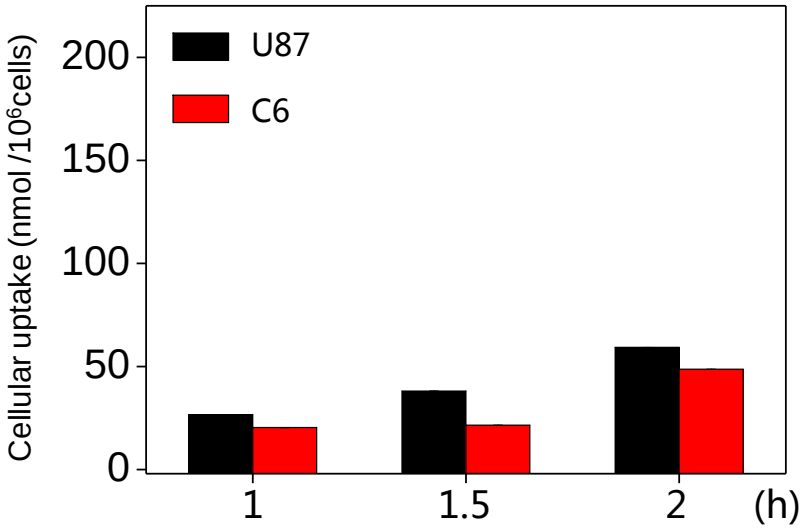

Figure S6. Quantitative analysis of cellular uptake of BSeC@MSNs-PEI in U87 and C6 cells. U87 cells and C6 cells were both treated with BSeC@MSNs-PEI (80 μM) for 1.0, 1.5 and 2.0 h, respectively. Values expressed were means ± SD of triplicate.

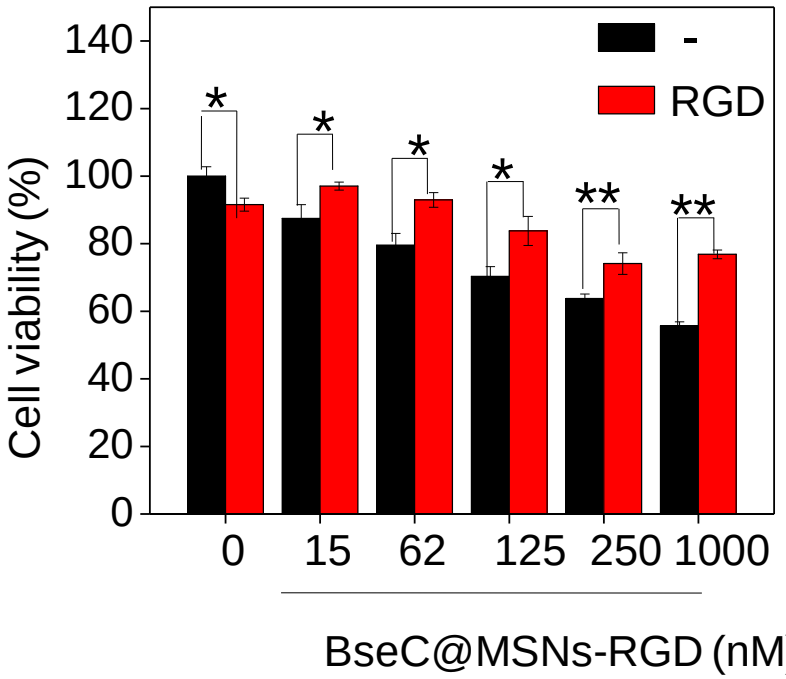

Figure S7. Cell viability was test by MTT assay for 24 h to determine the effects with the pretreatment of RGD (0.25 μg/ml)**.** The cell was incubated with BSeC@MSNs-RGD at different concentration simultaneously. Values expressed were means ±SD of triplicate. * $P < 0.05$ vs.control, ** $P < 0.01$ vs. control.

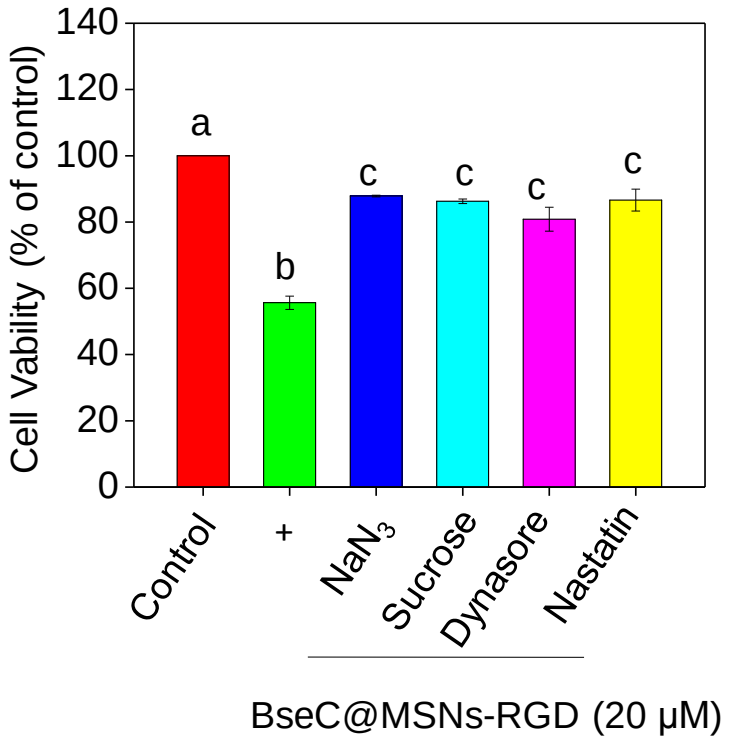

Figure S8. Cell viability was test by MTT assay for 24 h to determine the effects with the pretreatment of endocytosis inhibitors **.**The cell was incubated with BSeC@MSNs-RGD(1 μM) simultaneously. Values expressed were means ±SD of triplicate. * P < 0.05 vs.control, ** P < 0.01 vs. control. Bars with different characters (a, b and c) are statistically different at * *P* < 0.05 level.

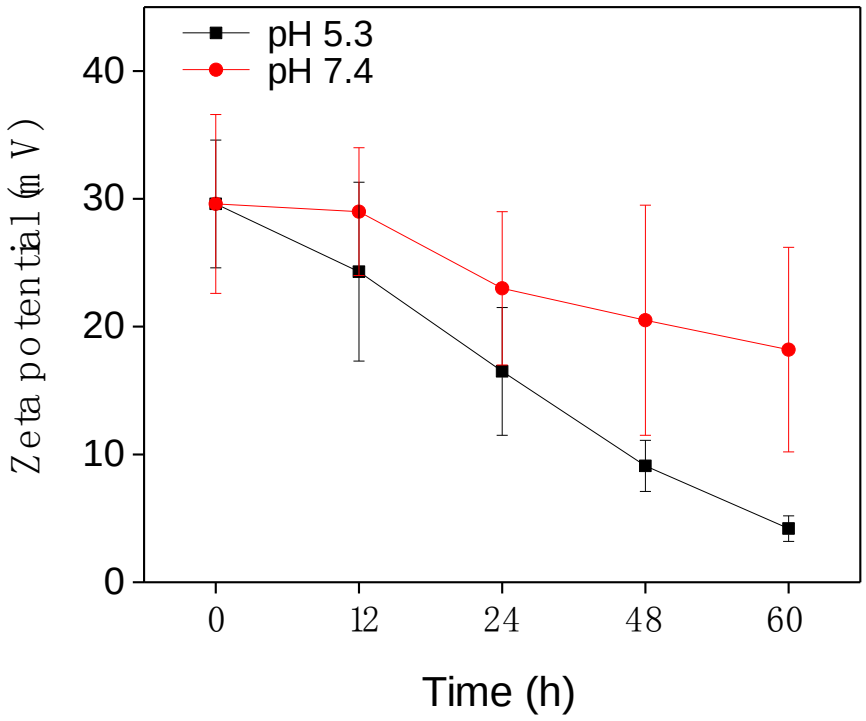

Figure S9. Characterization of polymer dissociation from BSeC@MSNs-RGD by measuring the change in Zeta potential in PBS (pH 7.4) and PBS (pH 5.3).

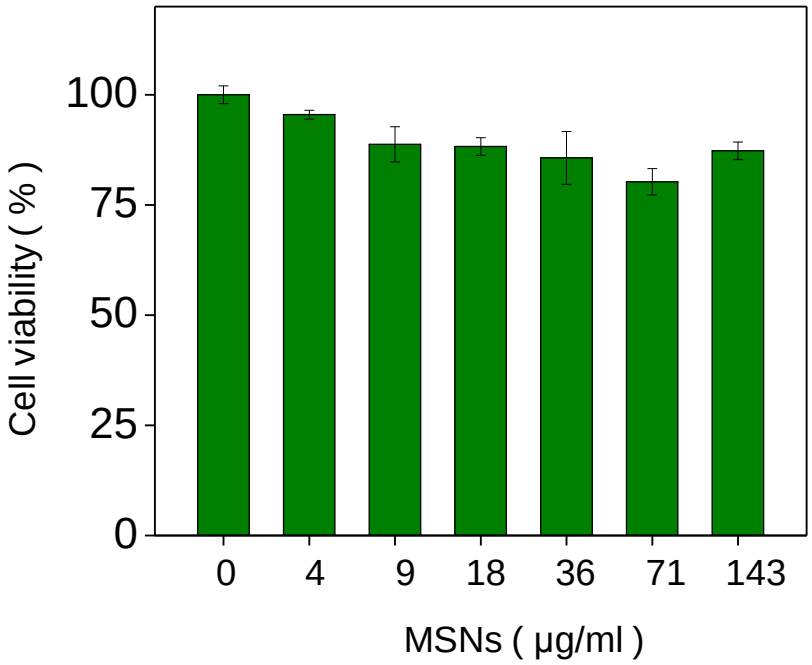

Figure S10. The viability of LO2 cells exposed to MSNs for 72 h.

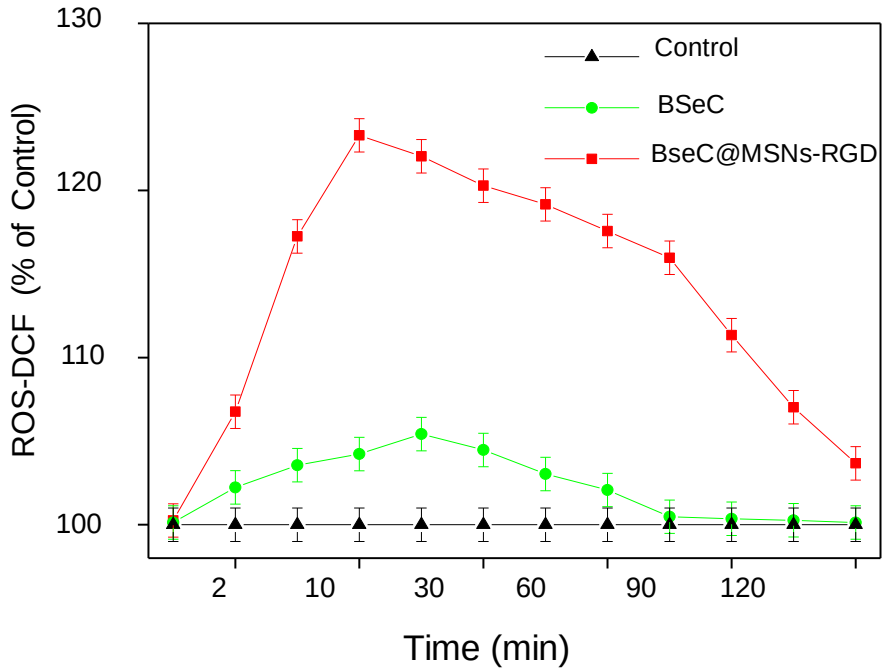

Figure S11. Overproduction of ROS in U87 cells exposed to BSeC and BSeC@MSNs-RGD (5 μM). Values expressed were means ±SD of triplicate.

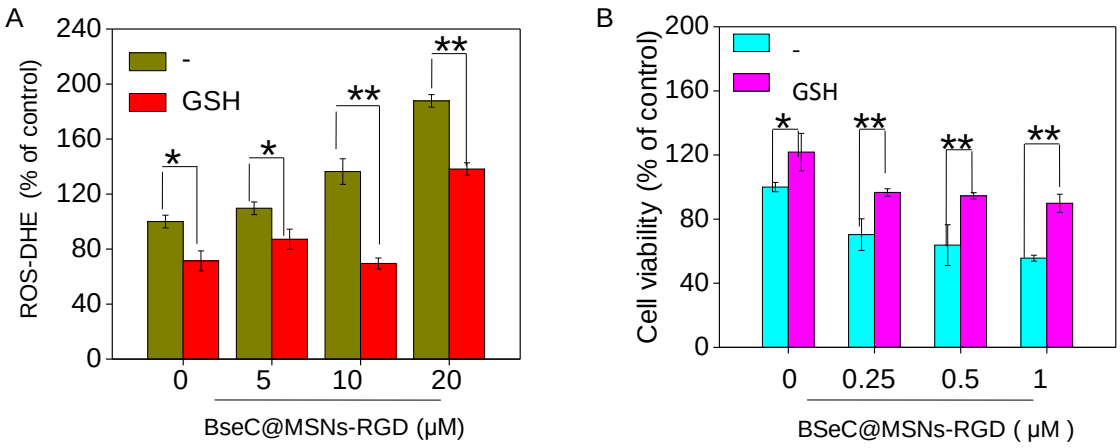

Figure S12. Effects of GSH (1 mM) on intracellular ROS generation and cell growth inhibition induced by BSeC@MSNs-RGD (20 μM). Cells were first treated with GSH for 2 h and then incubated to BSeC@MSNs-RGD. Values expressed were means ±SD of triplicate. * $P < 0.05$ vs. control, ** $P < 0.01$ vs. control.

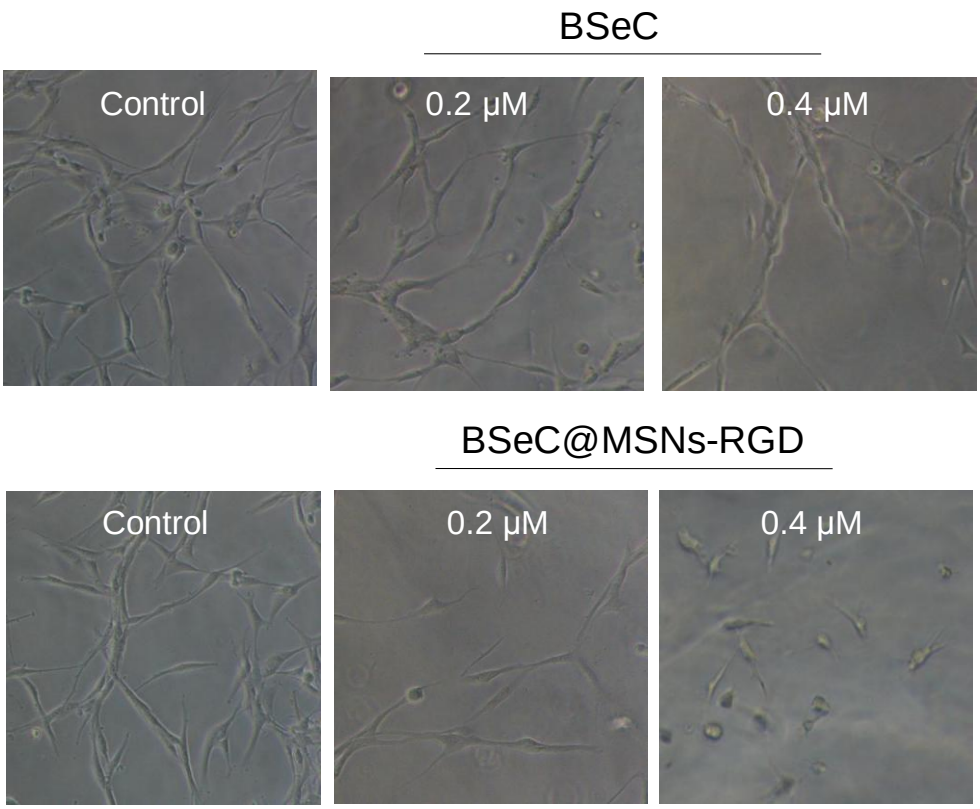

Figure S13. Destruction of brain cancer U87 VM channels after treatment with BSeC and BSeC@MSNs-RGD for 10 h.

Table S1. Loading capacity of BSeC@MSNs-RGD for BSeC and RGD

Samples	Loading capacity for BSeC (μg/mg MSNs)	Loading capacity for RGD (μg/mg MSNs)
BSeC@MSNs-RGD	120	1.8

Table S2. Cytotoxic effects of BSeC and BSeC@MSNs-RGD on brain cancer cells and normal cells after 72-h incubation.

Samples	IC_{50} (μM)			
	U87	C6	L02	CHEM-5
BSeC	> 800	> 800	> 800	> 800
BSeC@MSNs-RGD	0.13 ±0.02	2.30 ±0.07	3.82 ±0.04	5.85 ±0.05

Table S3. Pharmacokinetic parameters of BSeC and BSeC@MSNs-RGD after iv injection at an equivalent dose of 5.0 mg BSeC per kg of mouse body weight (n=3 per group).

Parameters	BSeC	BSeC@MSNs
$t_{1/2\beta}$ (h)	9.8	30.6
$AUC_{0\text{-}72\ h}$ (μg/L·h)	300.9	10567.8
C_{max} (μg/L)	89.2	485.2
Cl (L/h)	3.3	0.09