ADVANCED FUNCTIONAL MATERIALS

Supporting Information

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Self-Assembly of Therapeutic Peptide into Stimuli-Responsive Clustered Nanohybrids for Cancer-Targeted Therapy

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Supplementary Methods

General Remarks

$HAuCl_4 \cdot XH_2O$ was bought from Aladdin. All synthetic peptide sources were obtained from CS Bio company in Shanghai. If not clearly stated, all other chemicals were purchased from Sigma-Aldrich. Acetonitrile and water (HPLC grade) were purchased from Fisher Scientific Ltd. All products were used as received without further purification.

Synthesis of Therapeutic Peptides (BBI and BBI-SH)

All peptides were synthesized on appropriate resins on an CS bio 336X automated peptide synthesizer using the optimized HBTU activation/DIEA in situ neutralization protocol developed by an HBTU/HOBt protocol for Fmoc-chemistry SPPS.[55] After cleavage and deprotection in a reagent cocktail containing 88% TFA, 5% phenol, 5% H2O and 2%TIPS, crude products were precipitated with cold ether and purified to homogeneity by preparative C18 reversed-phase HPLC. The molecular masses were ascertained by electrospray ionization mass spectrometry (ESI-MS).

Physicochemical Characterization of Peptides

The purity and molecular weight of peptides were characterized by analytical HPLC and ESI-MS. Isothermal titration calorimetry (ITC) measurements were performed in a Microcal 2000 calorimeter (GE Healthcare) at 25°C in PBS, pH 7.4. Titrations were carried out by 20 stepwise injections, 2 μL at a time, of 200 μM BBI peptide in the syringe to 20 μM β-catenin protein in the cells. Data were analyzed using the Microcal Origin program. Data points at saturation were used to calculate a mean baseline value, which was then subtracted from each data point. As for

fluorescence polarization (FP) assay, succinimidyl ester-activated carboxyfluorescein (FAM-NHS) was conjugated to BBI via its N-terminal amino group in DMF, and the resultant product FAM-BBI was HPLC-purified and lyophilized. The BBI/β-catenin binding experiments were performed in Microfluor® 2, 96-well black plates (Thermo Fisher Scientific) and readings were taken by a Tecan Infinite M2000 fluorescence plate reader. Serially diluted β-catenin proteins were prepared in Tris-HCl buffered saline (10 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 7.0) and incubated with 100 nM FAM-BBI in a total volume of 150 μL per well. After a 2-h incubation at room temperature, fluorescence polarization was measured at λex =470 nm and λem =530 nm. Nonlinear regression analyses were performed to give rise to Kd values.

Fabrication of ^{Ctrl}pParticle and AuNp

^{Ctrl}pParticle was a PEG control for the pParticle. During the synthesis of ^{Ctrl}pParticle, BBI-SH was replaced by the same amount of NH2-PEG-SH (MW 2000Da), and other steps were same as the protocol of pParticle. AuNp was a peptide or PEG-free control for pParticle. As for the synthesis of this sample, an aqueous solution of tetrachloroauric acid (HAuCl4·XH2O, 10 mL, 1mM) were directly reduced by a total volume of 500 μL solution containing freshly prepared 0.1 M ascorbic acid (VC) and 0.1 M carnosic acid (CA).

Fabrication of pParticle without CA and Au-Cluster

For contrast bio-experiments, two negative controls (Au-Cluster and pParticle without CA) were synthesized in the present study. The synthetic procedure of pParticle without CA was roughly the same, but not added CA and changed the concentration of VC to 0.2 M. As for the Au-Cluster, the synthetic procedure was modified from that of pCluster. In details, the aqueous

solution of BBI-SH was changed to the ultrapure water, and VC&CA solution were changed to 0.2 M VC solution.

Characterization of pCluster and Its Intergradations

The morphology was observed by a TEM, HT7700, and operated at no more than100 kV. The hydrodynamic sizes (1 mg/mL in PBS, 1 mL) were measured by dynamic light scattering (DLS) using the Malvern Zetasizer Nano ZS system. For zeta potential measurement, the clustered nanoparticles (1 mg/mL, 1 mL) were incubated with PBS at different pH at 37 ℃ for 30 min and measured by DLS. Fourier transform infrared (FT-IR) spectroscopy was explore by Nicolet 6700, and the UV−vis absorption spectra was collected by Shimadzu 3000 spectrophotometer.

Preparation of Texas Red-Labelled pCluster or pParticle

Ten mg Texas Red (sulforhodamine 101 acid chloride) was first dissolved in 1 mL DMSO, and this solution were then added into 10 mL the pCluster (pParticle) solution which concentration is 1 mg/mL in PBS buffer. After 48 h stirring at 25℃ in the dark enviroronment, the mixed reaction solution was purified by dialysis against PBS with a dialysis tubing (MWCO =10 KDa). The product was obtained as an amaranth solid after lyophilization.

Cellular Uptake of pCluster

Cellular uptake of pCluster was detected using confocal laser scanning microscopy (CLSM, FV1200, Olympus) and flow cytometer (BD Biosciences, NJ). pClusters were first re-dispersed in culture medium at a concentration of 50 μg/mL. HCT116 and Hep3B cells were cultured in

corresponding growth medium for 24 h. The culture medium was then replaced with the growth medium containing Texas Red-labelled pCluster at pH7.4 or pH6.5, and cultured at 37°C for 6 h. Cell imaging or flow cytometry analysis were then carried out after washing the cells with PBS twice to remove the excess pClusters. For cell imaging, Hoechst33342 (Molecular probes) was used as the controls. To prevent cell falls from the bottom, we used paraformaldehyde to fix cells before observation. For confocal fluorescence microscopy imaging, the excitation was provided by the continuous wave laser at 405 nm (3.15 mW) and 543 nm (0.7 mW), respectively.

Cell Culture and Cell Viability Assay

Human colon cancer cell line HCT116 was purchased from ATCC, and maintained in McCoy's 5A medium supplemented with 10% FBS. Human hepatoma cell line Hep3B, human lung adenocarcinoma cell line A549 and murine melanoma cell line F16B10 were also purchased by ATCC and maintained in DMEM with 10% FBS. Cells were maintained at 37°C in an atmosphere of 5% CO2. The in vitro cytotoxicity was measured using a standard MTT (Thermo Fisher scientific) assay in the above cell lines. Cells were plated in 96-well plates at a density of 2500 cells/well (100 μL). After 24 h, cells were treated with pCluster, pParticle, Au-Cluster and CA at the indicated concentrations and times, respectively.

Penetration of pCluster in the Isolated Tumor Mass by Fluorescence Microscope

MC38 tumor masses were isolated from c57 mice, and incubated in RPMI 1640 medium at pH 6.5 or 7.4. Texas red-labelled pCluster were added into the medium to reach a final concentration of 0.1mg/mL. After 4 h, tumor masses were washed with PBS following freezing

slice. The result was observed with a fluorescence microscope.

Western Blot Analysis

Cells were treated with pCluster (200 nM), pParticle (200 nM), Au-Cluster (same weight with pCluster) or CA (1 μM or 10 μM) for 24 h, respectively. Cells were then lysed in prechilled RIPA buffer containing protease inhibitors, and equal amounts of protein lysates were separated by 10% SDS-PAGE and transferred onto PVDF membranes (Roche Diagnostics, Mannheim, Germany). The membranes were subsequently incubated with the indicated primary antibodies at 4°C overnight. Antibodies against β-catenin and Cyclin D were purchased from Abcam. Antibody against β-actin was purchased from Sigma-Aldrich. This was followed by incubation with their respective HRP-conjugated secondary antibodies from Calbiochem, and immunoblotting signals were visualized using the Western Bright ECL detection system (Advansta, CA).

Cell Cycle Analysis

Cells were first serum starved for 12 h. After treatment with 200 nM of pCluster, pParticle and Au-Cluster or 1 μM CA for 24 h, cells were harvested, washed twice in PBS, and fixed in 70% ethanol on ice for at least 30 min. After that, cells were stained with propidium iodide (PI) solution (50 μg/ml PI, 50 μg/ml RNase A, 0.1% Triton-X, 0.1 mM EDTA). Cell cycle distributions were then analyzed by a flow cytometer (BD Biosciences, NJ).

Immunohistochemical (IHC) Staining

The tumor sections were incubated with the primary antibodies against β-catenin, Cyclin D,

Ki67, MMP9, CD133 or CD8 overnight at 4°C. The slides were stained by labeled streptavidin-biotin (LSAB) complex for 15-min incubation. Each slide was evaluated by randomly selected fields (×20) and documented for further statistical analysis.

The immunostaining intensity (I) standard is as follows: a numeric score ranging from 0 to 3 (0, no staining; 1, weak staining; 2, moderate staining; and 3, intense staining). The immunostaining area (A) standard is as follows: a numeric score ranging from 1 to 4 (1, positive area <10% ; 2, 10%<positive area <50%; 3, 50%<positive area <90%; and 4, positive area>90%). Using an Excel spreadsheet, the score was I × A.

Cell Migration and Invasion Assays

By transwell chambers purchased from Corning (8.0 μm pore size), we studied cell migration and invasion. For cell invasion assay, chambers were pre-coated with BD Bioscience Matrigel (1:10 dilution,). And then, cancer cells were seeded in the upper chamber with 200 μL of medium containing 1% FBS (at a density of 1×10^5 cells/mL). The lower chambers were cover with 10% FBS (900 μL) medium. After incubation for 24 h, migrating/invading cells were fixed by 4% PFA and stained with 0.1% crystal violet. And then the samples were photographed by microscope. We used ImageJ to analyze the results.

Supplementary Figures

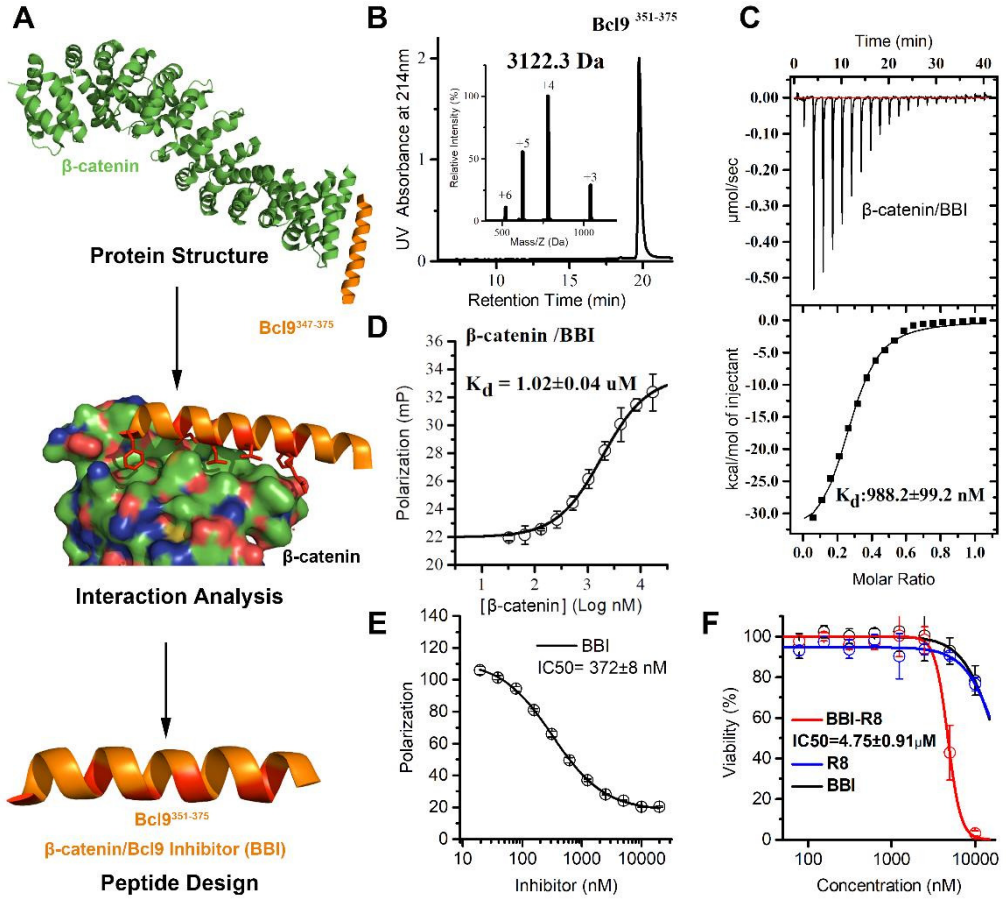

Figure S1. Design, synthesis and characterization of BBI. (**A**) Schematic diagram of BBI design. (**B**) Synthesized BBIs were purified by reverse phase HPLC and their molecular weights were determined by ESI-MS. The binding between BBI and β-catenin was quantified by isothermal titration calorimetry (**C**) and fluorescence polarization (**D**). Isothermal titration calorimetry (ITC) measurements were performed in a Microcal 2000 calorimeter (GE Healthcare) at 25°C in PBS, pH 7.4. Titration was carried out by 20 stepwise injections, 2 μL at a time, of 200 μM BBI peptide in the syringe to 20 μM β-catenin protein in the cells. Fluorescence polarization measurement was performed at room temperature on a Tecan Infinite M2000 plate reader. Briefly, FITC was covalently conjugated to the N-terminus of BBI. Serially diluted β-catenin proteins were prepared in PBS (pH 7.4) in 96-well plates and incubated for 30 min with 200 nM FITC-BBI in a total volume of 100 μL per well. The readings were then taken at λex = 490 nm, λem = 520 nm. (**E**) Competitive binding of BBI peptide to β-catenin as measured by fluorescence polarization techniques. Serially diluted BBI was prepared in PBS (pH 7.4), to which 200 nM FITC-$^{345\text{-}375}$BCL9 and 50 nM β-catenin were added in a total volume of 120 μL per well. After a 30-min incubation at room temperature, fluorescence polarization was measured at λex = 490 nm, λem = 520 nm. (**F**) Cell viability of Hep3B measured by MTT assay after treatment of BBI-R8, R8 and BBI, demonstrating that BBI has ability to inhibit the proliferation of Hep3B cells with hyperactivated Wnt signaling.

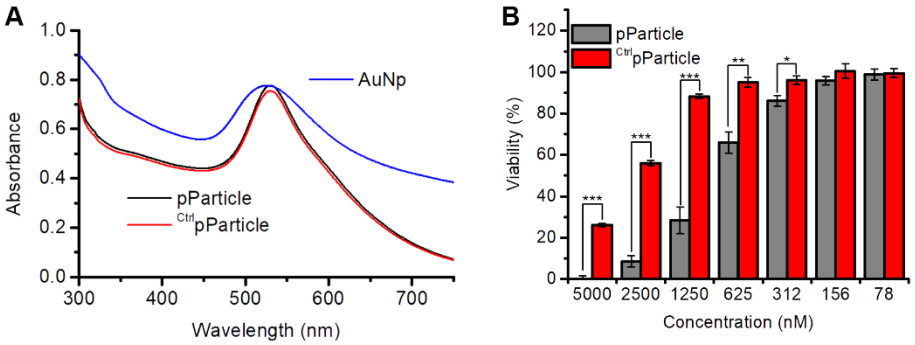

Figure S2. Ultraviolet–visible spectra and cytotoxicity toward Hep3B cells of pParticle. (**A**) pParticle, ^{Ctrl}pParticle and AuNp measured in PBS buffer at the concentration of 0.2 mg/ml. ^{Ctrl}pParticle was the control sample of pParticle which was synthesized by replacing BBI-SH to PEG-SH (MW 2000Da), and AuNp was a peptide-free gold nanoparticle synthesized by similar protocol as pParticle, which completely deleted the peptide during the synthesis. The wide plasma resonance peak of AuNp suggested the nonuniform particle size distribution, which indicated that our method of nanoparticle synthesis reduced from $[BBI\text{-}S\text{-}Au+]_n$ precursor can obtain better nanoparticle with sufficiently narrow size distribution. (**B**) Hep3B cell viability after incubation with different concentration of pParticle and ^{Ctrl}pParticle, measured by MTT assay (n =4, mean ± s.d.). Statistically significant differences were judged by t-test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

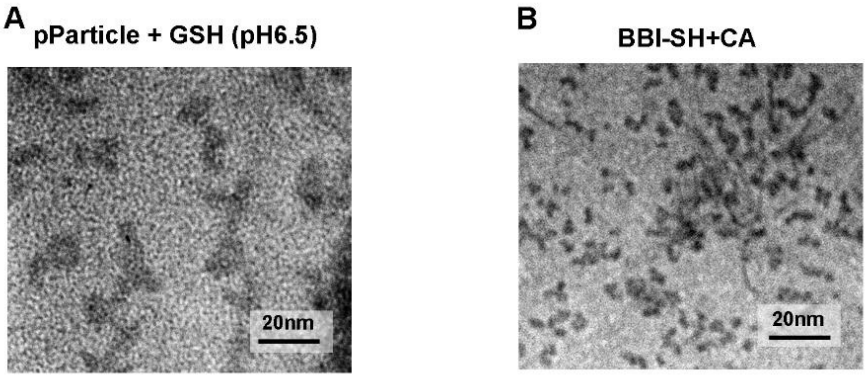

Figure S3. TEM image of the Redox-responsive pParticle. Transmission electron micrograph (TEM) of pParticle responding to reduced glutathione (**A**) and BBI-SH&CA (**B**).

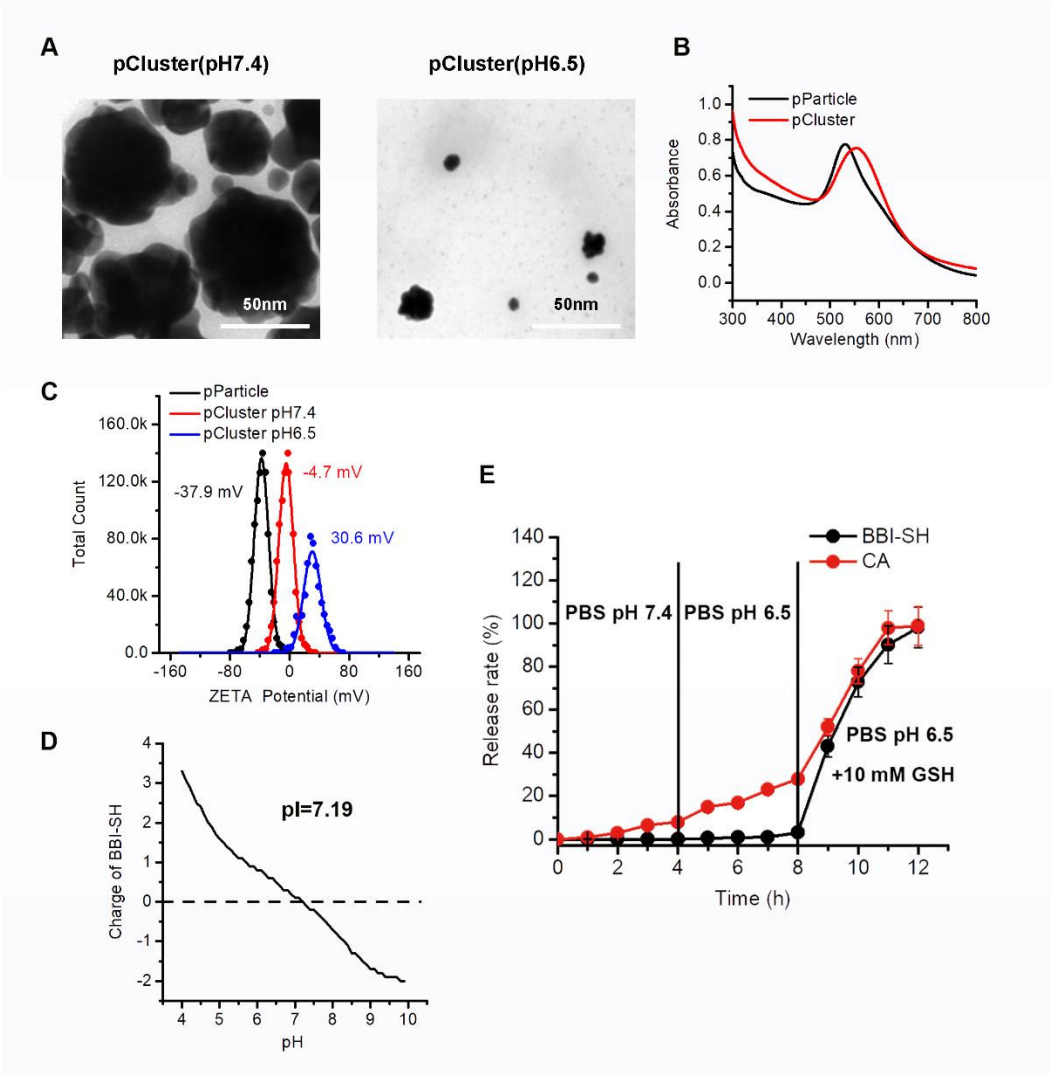

Figure S4. Characterization of pCluster. (**A**) Transmission electron micrograph (TEM) pCluster (pH 7.4) and pCluster (pH6.5). (**B**) Ultraviolet–visible spectra of pParticle and pCluster measured in PBS buffer at the concentration of 0.2 mg/ml). (**C**) The surface charge (Zeta potential) of pParticle (at pH 7.4), pCluster (at pH 6.5) and pCluster (at pH 7.4) measured in PBS buffer by DLS at 37 ºC after 30 min pH incubation. (**D**) The charges of N-terminal-acetylate BBI-SH from pH 4 to 10, which were also calculated by PROTEIN CALCULATOR. The pI was 7.19, and the charge at pH 7.4 and pH 6.5 were -0.2 and +0.7, respectively. (**E**) BBI-SH (black line) and CA (red line) release from pCluster under three different conditions, which include PBS at pH 7.4 to mimic a neutral environment, PBS at pH 6.8 to mimic a tumor extracellular environment, and PBS at pH 6.8 containing 10 mM GSH to mimic an intracellular redox environment. BBI-SH and CA release were quantified by HPLC.

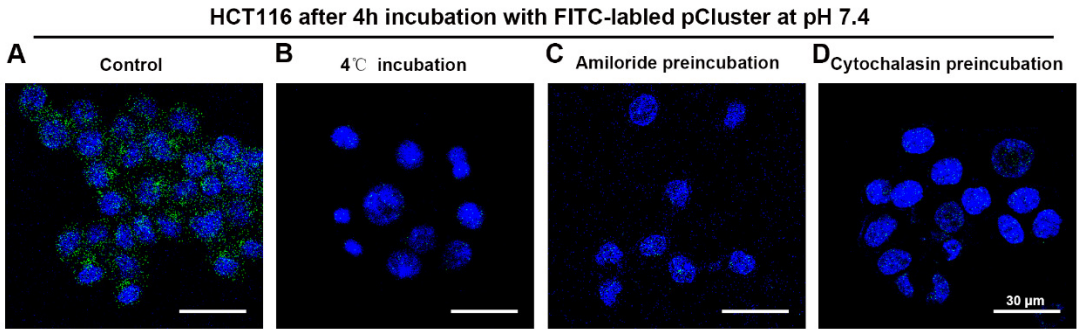

Figure S5. pH-responded pClusters internalize into cells via an ATP-dependent micropinocytosis pathway. Images of HCT116 cells in incubation with 20 μg/mL FITC-labeled pCluster at 37 ℃ (**A**) and 4℃ (**B**). Images of HCT116 cells in incubation with 20 μg/mL FITC-labeled pCluster at 37 ℃ after a 12-h pre-treatment with 3 mM Amiloride (**C**) and 2 μM Cytochalasin D (**D**).

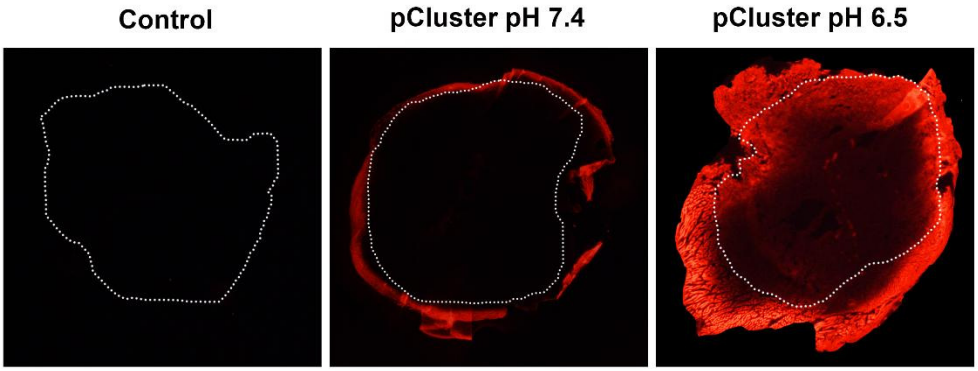

Figure S6. Ex vivo penetration of pCluster at different pH values. Tumor masses were isolated and incubated with FITC-labeled pCluster at pH 7.4 or 6.5 for 4 h. After washing, they were observed under fluorescence microscope. The area marked with white circle present the intratumoral region. Scale bar, 200 μm.

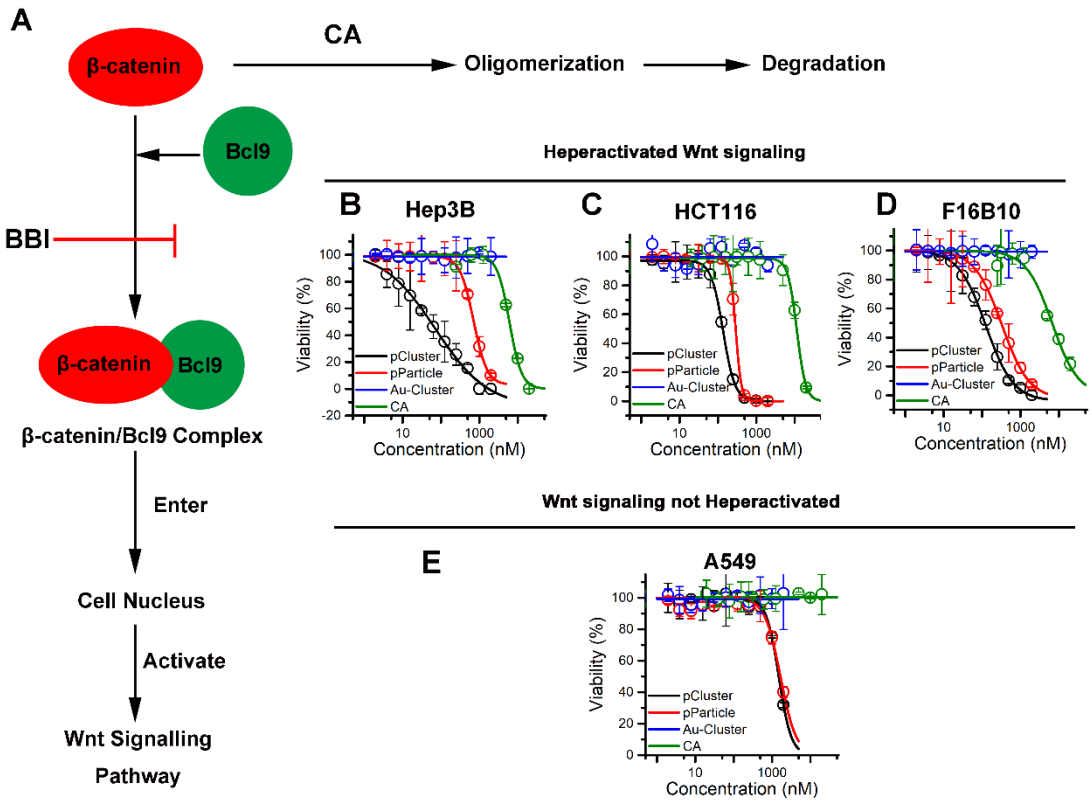

Figure S7. pCluster inhibits tumor growth in vitro by targeting Wnt signaling. (**A**) Mechanism diagram underlying synergistic inhibition of Wnt pathway by BBI and CA. BBI inhibits Wnt pathway by blocking the interaction between Bcl9 and β-catenin. CA inhibits Wnt pathway by inducing the degradation of cytoplasmic β-catenin. (**B**-**E**) Dose–response curves of different nanoparticles formulations against Hep3B, HCT116, F16B10 and A549 cells after 48 h incubation, which were measured by MTT assay (n =4, mean ± s.d.).

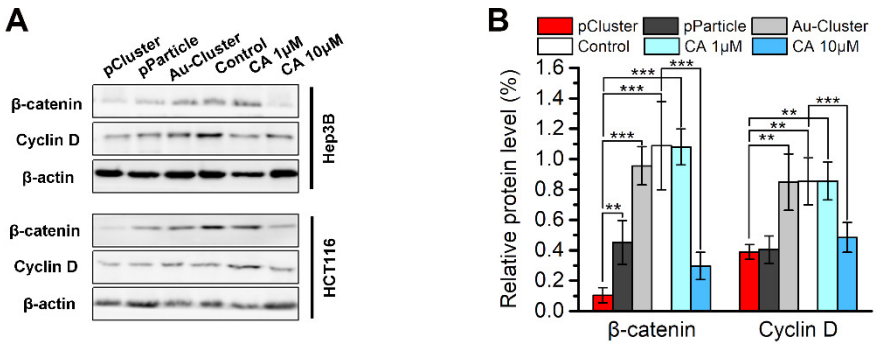

Figure S8. Western blot analysis was performed to monitor the changes in protein expression of Cyclin D and total β-catenin in Hep3B and HCT116 cells with the indicated treatments. β-actin was used as a loading control, and the western blot is representative of three independently preformed experiments (**A**). Shown in **B** is quantitative analysis of the expression of the indicated proteins by densitometry. p values were calculated by t-test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

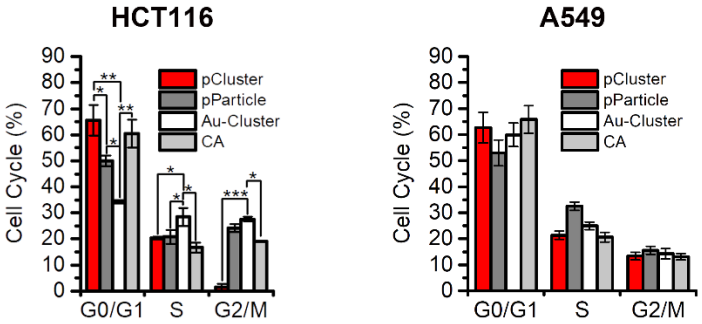

Figure S9. Cell cycle of HCT116 and A375 cells after indicated treatment. Cell cycle distributions were analyzed by flow cytometry in HCT116 and A549 cells with the indicated treatments for 24 h (n =3, mean ± s.d.). p values were calculated by t-test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

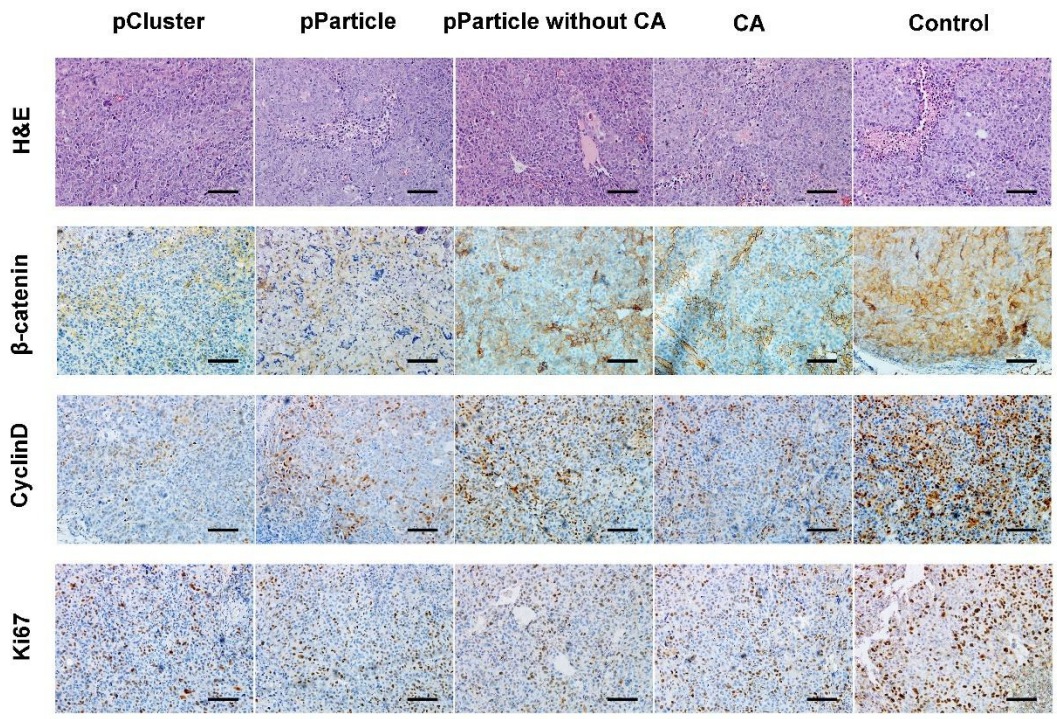

Figure S10. H&E and IHC-stained xenograft tumor sections. Representative H&E-stained tumor sections and IHC staining of β-catenin, Cyclin D and Ki67 proteins in the same tumors from mice with the indicated treatments. (×200) (scale bar: 50 μm)

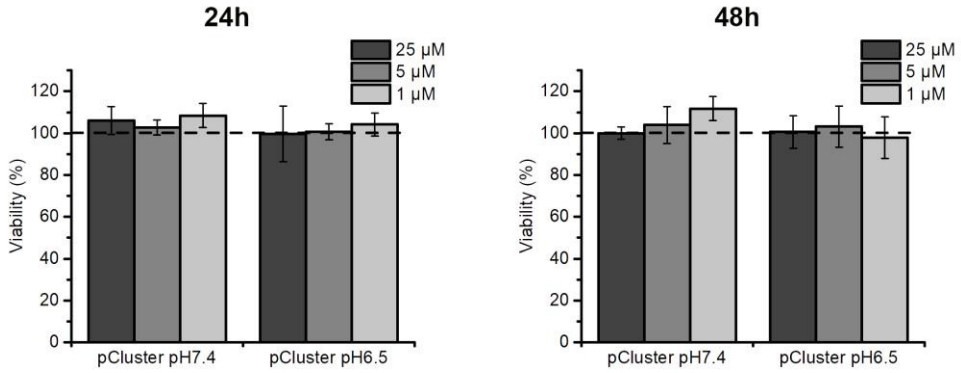

Figure S11. The effect of pCluster on the viability of PBMCs. The PBMCs used here were separated the blood from C57 mice. Cells were firstly incubated with the indicated concentrations of pCluster at pH7.4 or 6.5 for 24 or 48 h, and cell viability was then measured by the MTT assay.

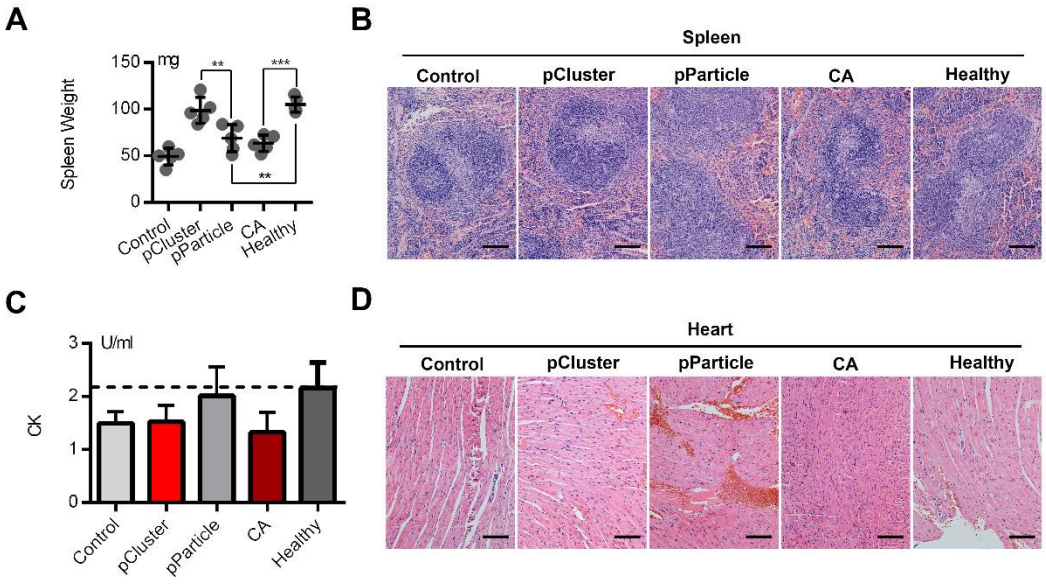

Figure S12. Toxicity evaluation of the spleen and Heart in mice with different treatments. (**A**) Spleen weight of mice with the indicated treatments. (**B**) Representative H&E staining of spleen tissues in mice with the indicated treatments (×200). (scale bar: 50 μm). (**C**) Serum creatine kinase (CK) activity of mice with the indicated treatments. Clinically, CK is assayed in blood tests as a marker of damage of CK-rich tissues such as in myocardial infarction (heart attack). (**D**) representative H&E staining of heart sections in mice with the indicated treatments (×200). (scale bar: 50 μm). p values were calculated by t-test (**, $p < 0.01$ or unmarked $p > 0.05$).

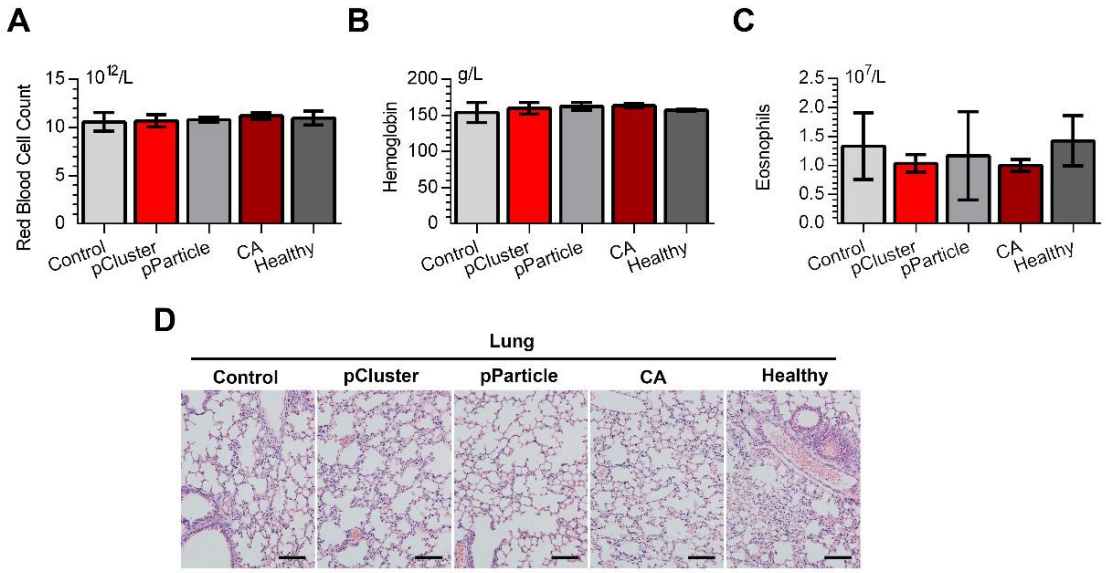

Figure S13. Toxicity evaluation of blood indexes and Lung in mice. The count of red blood cells (**A**), hemoglobin (**B**) and eosnophils (**C**) in mice with the indicated treatments. p values were calculated by t-test (unmarked p >0.05). (**D**) Representative H&E staining of lung tissues in mice with the indicated treatments (×200). (scale bar: 50 μm)

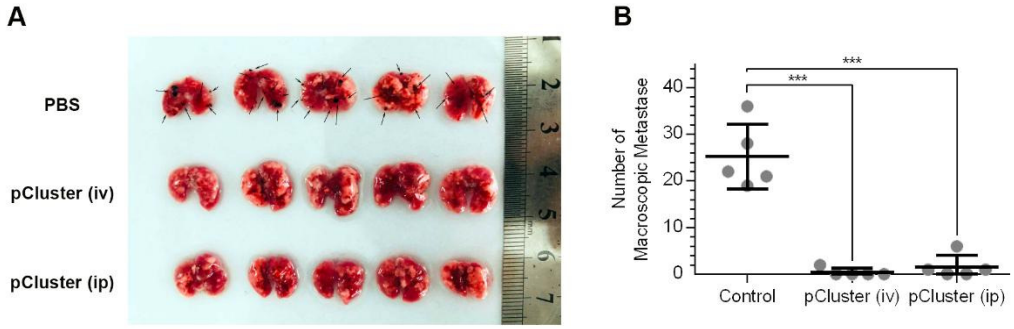

Figure S14. Comparison of therapy efficacy between i.v and i.p. administration of pCluster in the Tail vein metastasis model. (**A**) The photographs of lungs taken 10 days after injection of 5×10^4 cells/mL B16F10 cells. Macroscopic analysis of the lungs confirmed that pCluster decreased the lung colonization by B16F10 cells. (**B**) The number of superficial macroscopic metastases in the lungs (n =5). p values were calculated by t-test (***, $p < 0.001$).

Supplementary Table

Table S1. Acid responsiveness of the pCluster synthesized by different ration of CA and BBI

Molar ratio (CA:BBI)	pH 7.4		pH 6.5	
	Size[1] (nm)	Charge[2] (MV)	Size[1] (nm)	Charge[2] (MV)
0.5:1	20.9	10.2	6.8	36.3
1:1	40.2	4.8	8.9	31.2
2:1	**89.3**	**-4.7**	**10.8**	**30.6**
4:1	210	-8.6	96.9	3.68
8:1	620	-11.2	287	-2.3

[1] Size is the hydrate particle size measured by dynamic light scattering.

[2] Charge is the Zeta potential of the pCluster measured by electrophoretic light scattering.