

## Supporting Information

### Systemic delivery of siRNA by hyaluronan-functionalized calcium phosphate nanoparticles for tumor-targeted therapy

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#### 1. Synthesis of alendronate-hyaluronan graft polymers (AHA<sub>780k</sub> and AHA<sub>48k</sub>)

##### 1.1 Materials

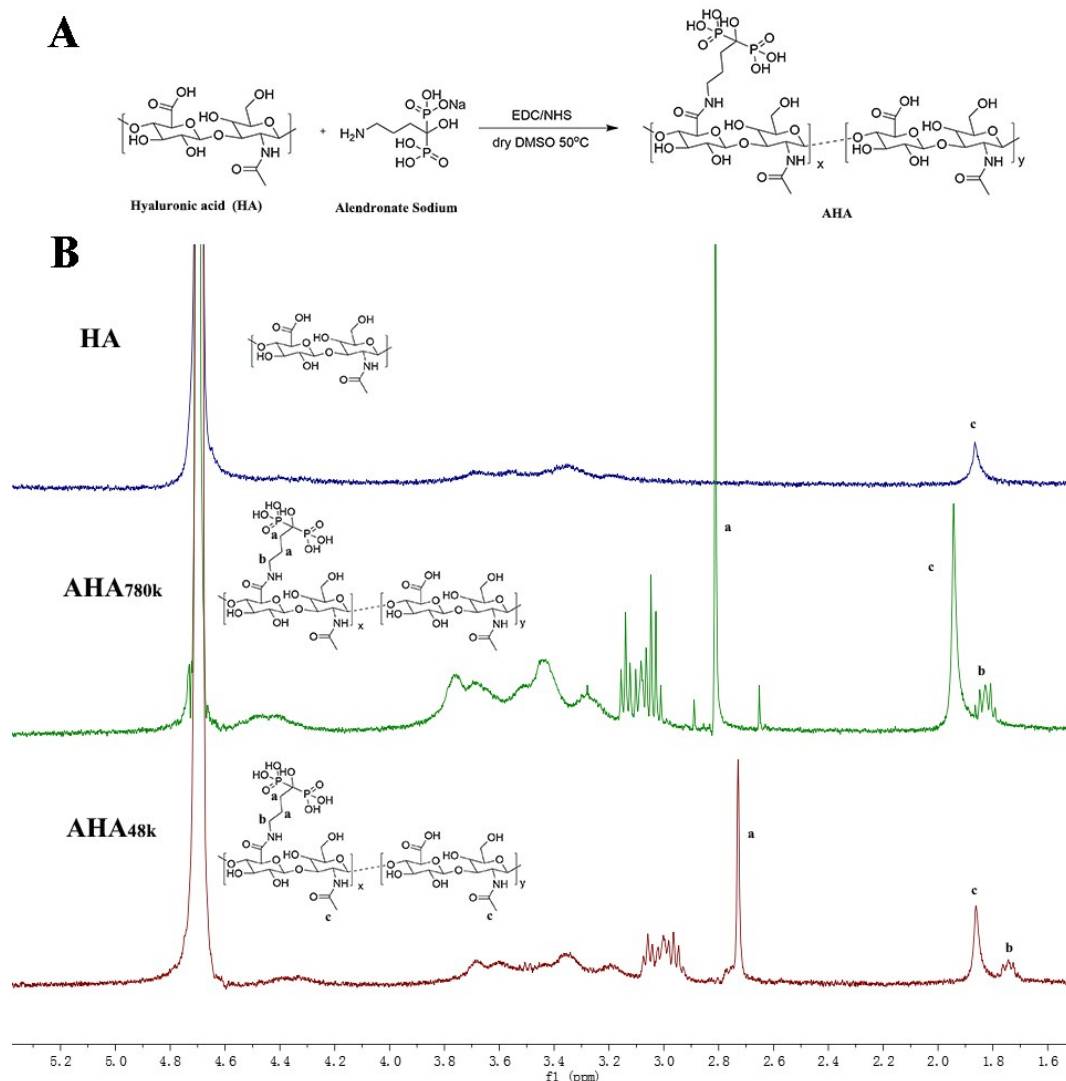
1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) was obtained from J&K Scientific Ltd. (Beijing, China). Hyaluronic acid (HA, MW=780 kDa and 48kDa) was purchased from HuaXi FuRuiDa Ltd. (Shandong, China). Dimethyl sulfoxide (DMSO) was purchased from Beijing Chemical Works. (Beijing, China).

##### 1.2 Methods and Results

Two types of alendronate-hyaluronan graft polymers (AHA<sub>780k</sub> and AHA<sub>48k</sub>) with different molecular weight of hyaluronan (HA<sub>780k</sub> and HA<sub>48k</sub>) were synthesized. Briefly, HA (1 g, 2.5 mmol) was dissolved in 100 mL of degassed deionized water. EDC (959 mg, 3.75 mmol) and NHS (575 mg, 3.75 mmol) were slowly added in the HA solution. After 20 min stirring, alendronate sodium (813 mg, 2.5 mmol) was added in the mixture. The reaction solution was adjusted at pH 4~5 and kept stirring for 2 days at room temperature. After the end of reaction, the product was purified by dialysis (MWCO = 8000 Da) against distilled water and lyophilized. The structures of all products were confirmed by <sup>1</sup>H-NMR (AVANCE III, 400 MHz, Bruker, Billerica, MA) spectrometry.

The alendronate-hyaluronan graft polymer was successfully synthesized by conjugating the amine group of alendronic acid with carboxyl group of hyaluronic acid via EDC/NHS coupling technique (**Figure S1A**). The structures of AHA polymers were confirmed by <sup>1</sup>H NMR analysis. **Figure S1B** showed the <sup>1</sup>H-NMR spectra of HA, AHA<sub>780k</sub> and AHA<sub>48k</sub>. The proton peak of HA appeared between 1.8~2.0 ppm (single) and 3.0~4.0 ppm (multiple), where the single peak (c) was

representative CH<sub>3</sub>-group of acetyl section at 3'-sugar rings to calculate the substitution degree (SD). In AHA structure, the proton peak of the CH<sub>2</sub> closed to the HA appears at ~1.7 ppm (b) and other two CH<sub>2</sub> group of alendronate was presented at ~2.7 ppm (a), where the ratio of a/b was equaled to 2/1. Hence, the calculated SD was approximately 51.5% (AHA<sub>780k</sub>) and 33.3% (AHA<sub>48k</sub>) by the formula [(a)+(b)]/(c)\*100 %. These results confirmed the successful conjugation of alendronic acid with hyaluronic acid.



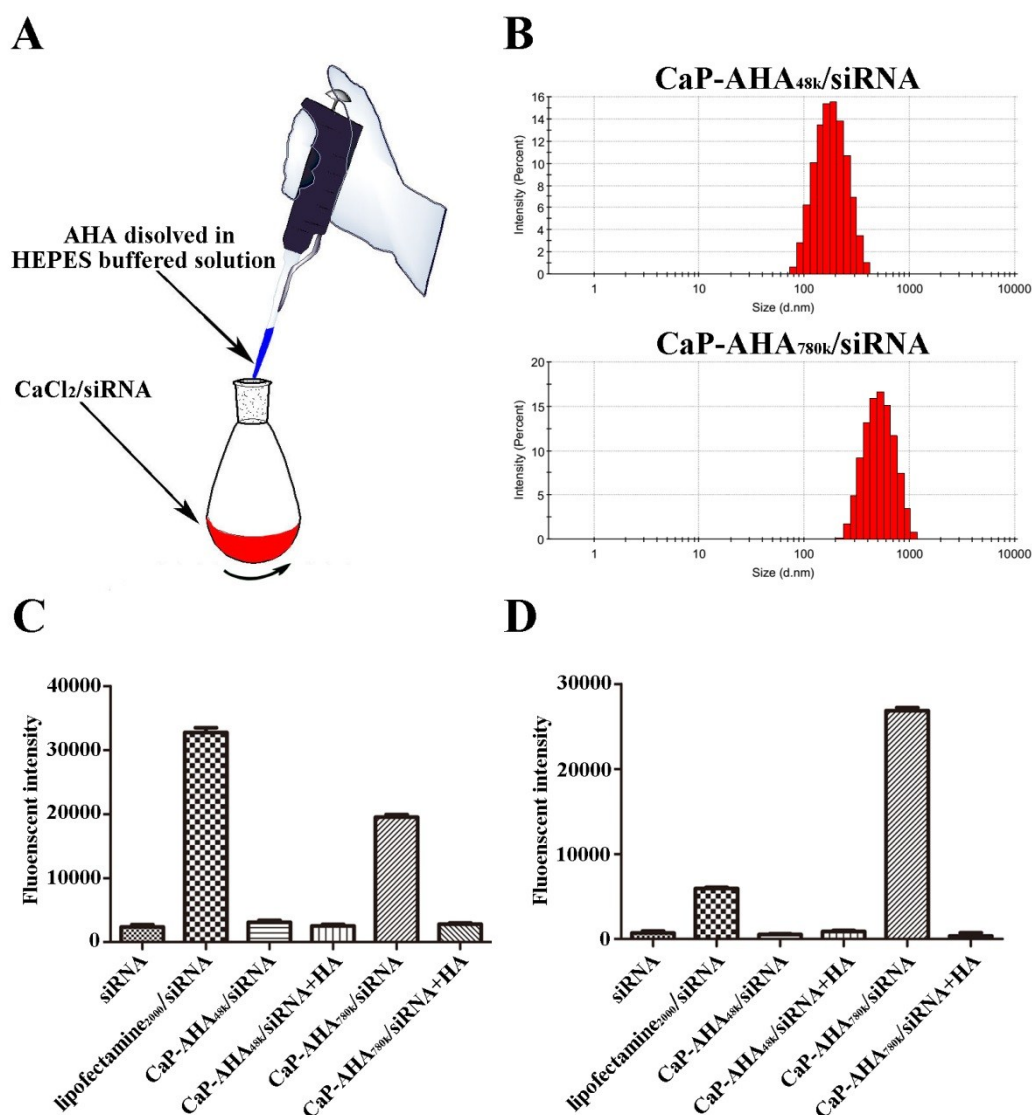
**Figure S1.** (A) Synthesis procedure of alendronate-hyaluronan graft polymers (AHA). (B) The <sup>1</sup>H-NMR spectrum of HA, AHA<sub>780k</sub> and AHA<sub>48k</sub>.

## 2. Preparation and Characterization of CaP-AHA<sub>780k</sub>/siRNA NPs and CaP-AHA<sub>48k</sub>/siRNA NPs

The preparation process of CaP-AHA/siRNA NPs was illustrated as the cartoon in Figure S2A. Briefly, 40 μL of CaCl<sub>2</sub> solution (500 mM, pH 7.4) and 40 μL of siRNA solution (diluted in water) were mixed, and then 160 μL of HEPES buffered solution (HBS) (50 mM HEPES, 280 mM NaCl, 1.5 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4)

containing the predetermined amount of AHA (AHA<sub>780k</sub> or AHA<sub>48k</sub>, the percentage of phosphate groups from AHA in all phosphate groups of NPs: 90%) was subsequently added and incubated at 37°C for 1 hour. For the preparation of CaP/siRNA co-precipitation control, the identical procedure was conducted except that no AHA mixture was added in HEPES buffer solution to stabilize the particles. The hydrodynamic sizes and zeta-potentials of CaP-AHA<sub>780k</sub>/siRNA NPs and CaP-AHA<sub>48k</sub>/siRNA NPs were determined by dynamic light scattering (Malvern Zetasizer Nano ZS, Malvern, UK) at room temperature. Additionally, a comparative study on cellular uptake of CaP-AHA<sub>780k</sub>/siRNA NPs and CaP-AHA<sub>48k</sub>/siRNA NPs were conducted in A549 cells or 4T1 cells, which are of overexpressing CD44 receptor. Briefly, the cells were seeded  $2.5 \times 10^5$  per well in six-well plates. After 24 h proliferation, the NPs containing FAM-labeled siRNA (final concentration of 100 nM) were exposed to cells with or without the pre-treatment of HA and incubated for an additional 4 h at 37 °C. After incubation, the cells were harvested and washed three times with pre-cooled PBS solution. The intracellular fluorescence intensity of FAM-labeled siRNA was detected by the multiskan Mk3 microplate reader (Thermo scientific, US) at the wavelength of 488 nm.

As seen from **Figure S2B**, a significant larger average size ( $472.1 \pm 73.3$  nm) was found in CaP-AHA<sub>780k</sub>/siRNA NPs than that of CaP-AHA<sub>48k</sub>/siRNA NPs ( $158.8 \pm 1.8$  nm). The significant difference of average particle size is due to the increase of molecular weight of hyaluronan. However, the CaP-AHA<sub>780k</sub>/siRNA NPs had a significant higher level of cellular uptake than CaP-AHA<sub>48k</sub>/siRNA NPs either on A549 cells (**Figure S2C**) or 4T1cells (**Figure S2D**), which indicating that higher molecular weight hyaluronan could possess a better receptor-mediated endocytosis on CD44+ cells. By the conflicting results, the CaP-AHA<sub>x</sub>/siRNA NPs modified with a mixture of AHA<sub>780k</sub> and AHA<sub>48k</sub> should be further optimized.



**Figure S2.** (A) Schematic illustration of the preparation of CaP-AHA/siRNA NPs; (B) The intensity size of CaP-AHA/siRNA NPs based on different AHA(AHA<sub>780k</sub> and AHA<sub>48k</sub>); The cellular uptake assay of CaP-AHA/siRNA NPs on different cells: (C) A549 cells and (D) 4T1 cells. The final concentration of FAM-labeled siNC was 100 nM and the data were shown as mean  $\pm$  SD ( $n = 3$ ).

### 3. Optimization of CaP-AHAx/siRNA nanoparticles

#### 3.1 Preparation and Characterization of CaP-AHAx/siRNA NPs

In order to obtain the optimal formulation, CaP-AHA<sub>10</sub>/siRNA NPs and CaP-AHA<sub>25</sub>/siRNA NPs were prepared by addition of different AHA mixture (AHA<sub>780k</sub>: AHA<sub>48k</sub> = 9:1 or 3:1(n/n), the percentage of phosphate groups from AHA in all phosphate groups of NPs: 90% and Ca<sup>2+</sup>: 500 mM) according to the preparation process described above. The hydrodynamic diameter and zeta potentials of the nanoparticles were determined at room temperature by dynamic light scattering (Malvern Zetasizer Nano ZS, Malvern, UK).

As seen in **Table S1**, compared to CaP/siRNA (>1  $\mu$ m), both CaP-

AHA<sub>10</sub>/siRNA and CaP-AHA<sub>25</sub>/siRNA could form the uniform NPs with mean size around 175 nm and 236 nm, respectively. Also, both CaP-AHA<sub>10</sub>/siRNA (~ -12.2 mV) and CaP-AHA<sub>25</sub>/siRNA (~ -13.1 mV) had a lower zeta potentials around -12 mV than CaP/siRNA (~ -3.54 mV). These above results showed that the negatively charged and hydrophilic HA acted as the outer layer of the nanoparticle could effectively prevent inter-particular aggregation and excessive crystal growth during the mineralization process.

**Table S1.** Characteristics of the three nanoparticles (n=3).

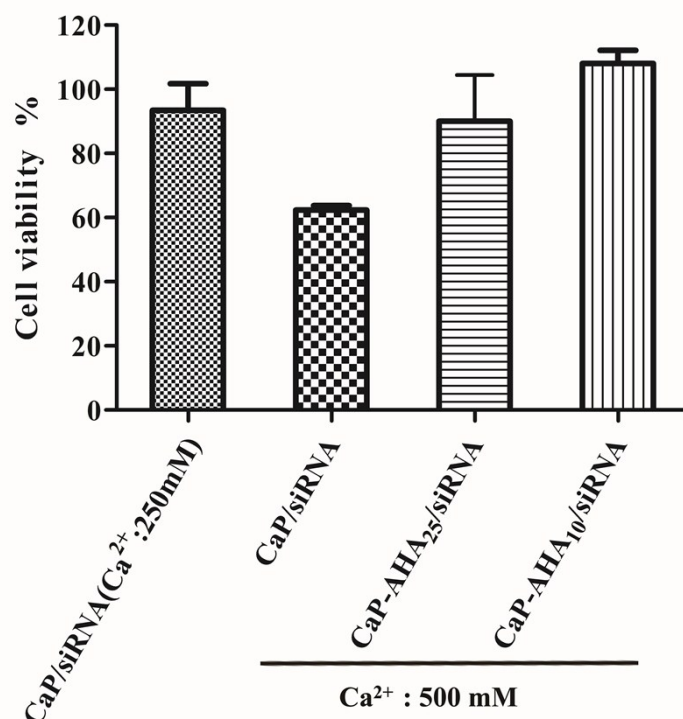
Preparations	Particle size (d, nm)	Polydispersity Index(PDI)	Zeta potential (mV)
CaP-AHA <sub>10</sub> /siRNA	174.9±8.3	0.249±0.105	-12.2±0.763
CaP-AHA <sub>25</sub> /siRNA	236.1±20.3	0.137±0.117	-13.1±1.51
CaP/siRNA	1186±170	0.646±0.116	-3.54±1.18

*\*The x in CaP-AHA<sub>x</sub>/siRNA represented the percentage of AHA<sub>48k</sub> in the AHA mixture used for preparation.*

### 3.2 Cytotoxicities of CaP-AHA<sub>10</sub>/siRNA NPs and CaP-AHA<sub>25</sub>/siRNA NPs

In vitro cytotoxicity of NPs on A549 cells was performed by MTT assay. Briefly, A549 cells were seeded in 96-well plates at a density of 10000 cells per well for 24h proliferation. The cells were treated with 200  $\mu$ L opti-MEM containing various NPs (the final concentration of siRNA 100 nM) for 6 hours incubation. After that, the medium was replaced by RPMI-1640 medium containing 10 % FBS and the cells were continuously incubated for 24 hours. Then, 20 $\mu$ L MTT (5 mg/ml) solution was dropped into each well and incubated for another 4 hours. After that, the culture medium was removed, and the formazan crystals were dissolved in 100 $\mu$ L DMSO, and the absorbance value were read by a microplate reader (Multiskan Mk3, Thermo scientific, US) at the wavelength of 490 nm. Cell viability (%) = [OD<sub>490</sub> (sample)/OD<sub>490</sub> (control)]  $\times$  100%.

**Figure S3** showed that the cytotoxicities of CaP/siRNA NPs, CaP-AHA<sub>10</sub>/siRNA NPs and CaP-AHA<sub>25</sub>/siRNA NPs on A549 cells. Compared to CaP/siRNA NPs (Ca<sup>2+</sup>: 250 mM), CaP/siRNA NPs (Ca<sup>2+</sup>: 500 mM) had a stronger cytotoxicity on A549 cells, which probably due to the changes of osmotic pressure induced by the increased Ca<sup>2+</sup> concentration released from the unstable CaP/siRNA NPs. Moreover, CaP-AHA<sub>10</sub>/siRNA NPs had lower cytotoxicity than CaP-AHA<sub>25</sub>/siRNA NPs, which indicated that higher proportion of AHA<sub>780k</sub> would benefit to cell viability because of its longer chain length and larger viscosity.

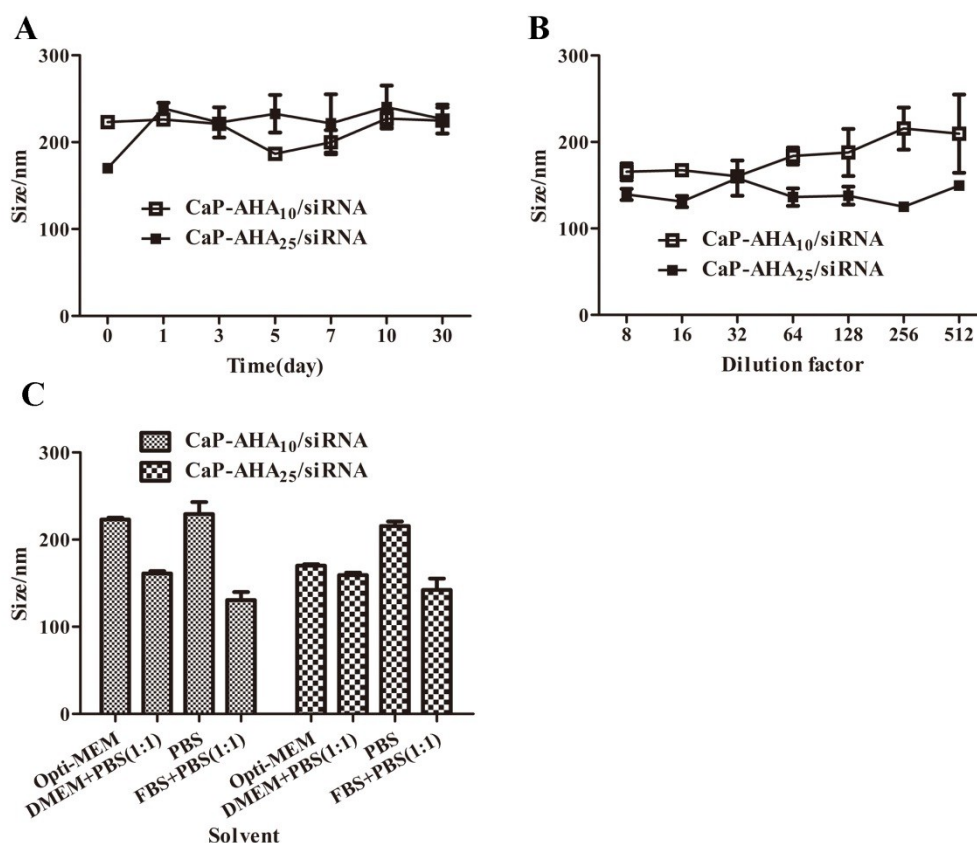


**Figure S3.** In vitro cytotoxicities of various NPs on A549 cells. The final siRNA concentration was 100 nM and all the data were given as the mean  $\pm$  SD (n=6).

### 3.3 Physical stability of CaP-AHA<sub>10</sub>/siRNA NPs and CaP-AHA<sub>25</sub>/siRNA NPs

The physical stability of CaP-AHA<sub>10</sub>/siRNA NPs and CaP-AHA<sub>25</sub>/siRNA NPs (Ca<sup>2+</sup>:500 mM, P%: 90%) was investigated as followed. Firstly, for the stabilities in different media, the NPs were diluted at the ratio of 1:7(v/v) with opti-MEM, PBS (K<sub>2</sub>HPO<sub>4</sub> 1.06 mM, NaCl 155 mM, Na<sub>2</sub>HPO<sub>4</sub> 3 mM, pH 7.4), mixture of PBS and FBS (1:1, v/v), DMEM medium containing 10% FBS, 100 U/ml penicillin and 100  $\mu$ g/mL streptomycin). After incubated at 37 °C for 6 h, the particle size was monitored by dynamic light scattering (DLS). Secondly, for the stability of dilution, the NPs were diluted with opti-MEM medium at a series of ratios (1:7, 1:15, 1:31, 1:63, 1:127, 1:255 and 1:511(v/v)) and incubated at 37 °C for 6 h, and then the particle size was monitored by DLS. Thirdly, for long-term stability of storage, the CaP-AHA/siRNA NPs suspensions was stored in Opti-MEM medium at room temperature, and record the change of size at predetermined intervals (0, 1, 3, 5, 7, 10 and 30 days) by DLS analysis.

**Figure S4A** showed that both CaP-AHA<sub>10</sub>/siRNA NPs and CaP-AHA<sub>25</sub>/siRNA NPs had a good stability within 30 days storage at room temperature. As seen in **Figure S4B**, with the increase of dilution factor, there was no significant difference between CaP-AHA<sub>10</sub>/siRNA NPs and CaP-AHA<sub>25</sub>/siRNA NPs, and they still kept the stable particle sizes in the range of 150~250 nm, which suggested that these two NPs had a good anti-dilution capability (more than 500 times). Moreover, **Figure S4C** showed these two NPs had a stable nanoscale size in different media (opti-MEM, DMEM+PBS(v/v=1/1), PBS, FBS+PBS(v/v=1/1)) because of their stable



**Figure S4.** The stability assay of CaP-AHA<sub>x</sub>/siRNA nanoparticles. (A) The long-term size stability of two nanoparticles within 30 days storage period; (B) The changes of particle size with different ratios of dilution in opti-MEM; (C) The changes of particle size after suspended in different disperse medium. All the data were given as the mean  $\pm$  SD (n=3).

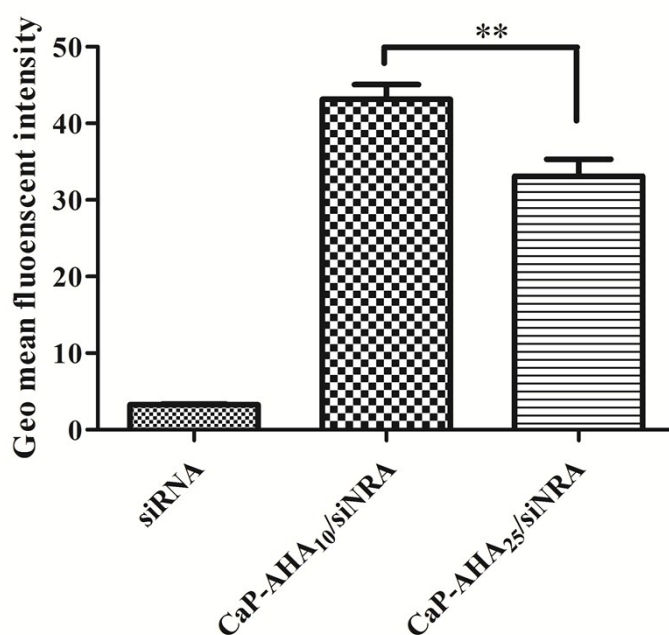
### 3.4 Cellular uptake of CaP-AHA<sub>10</sub>/siRNA NPs and CaP-AHA<sub>25</sub>/siRNA NPs

The cellular uptake of CaP-AHA<sub>10</sub>/siRNA NPs and CaP-AHA<sub>25</sub>/siRNA NPs (Ca<sup>2+</sup>:500 mM, P%: 90%) was investigated as followed. A549 cells were seeded  $2.5 \times 10^5$  per well in six-well plates. After 24 h proliferation, the NPs containing FAM-labeled siRNA at the final concentration of 100 nM were exposed to cells and incubated for an additional 6 h at 37 °C. After incubation, the cells were harvested and washed three times with pre-cooled PBS solution, and intracellular fluorescence intensities were detected by a FACS Calibur flow cytometry (Becton Dickinson, San Jose, CA, USA) immediately.

As shown in **Figure S5**, higher intracellular fluorescence intensities of FAM-siRNA were found in CaP-AHA<sub>10</sub>/siRNA NPs than that of CaP-AHA<sub>25</sub>/siRNA NPs. This difference is probably resulted from significant receptor-mediated endocytosis induced by higher proportion of AHA<sub>780k</sub> in the CaP-AHA<sub>10</sub>/siRNA NPs.

Above all the experimental results, we believe that the CaP-AHA<sub>10</sub>/siRNA NPs would be the optimal core-shell nanoparticles for the following experiments.





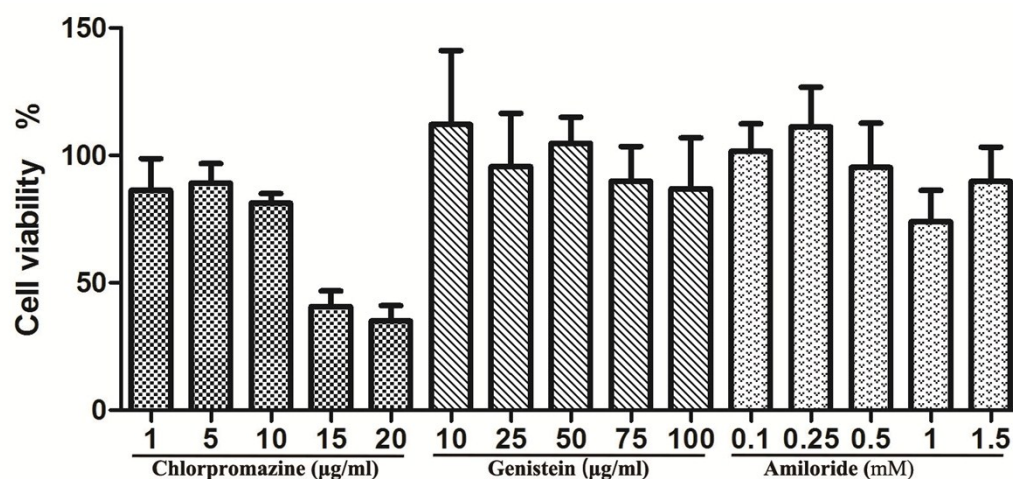
**Figure S5.** The cellular uptake intensity of CaP-AHA<sub>x</sub>/siRNA nanoparticles (FAM-labeled siRNA: 100 nM) on A549 cells were detected by flow cytometry after 6 h incubation. \*\* $p < 0.01$  and all the data were given as the mean  $\pm$  SD ( $n=3$ ).

#### 4. Cytotoxicities of various inhibitors in A549 cells

The cytotoxicities of various inhibitors on A549 cells were performed by MTT assay. Briefly, A549 cells were seeded in 96-well plates at a density of 5000 cells per well. After 24 h proliferation, cells were treated with 200  $\mu$ L OPTI-MEM containing serial concentrations of inhibitors (sample) or 5% glucose (control). After 6h incubation, the OPTI-MEM was removed carefully and another 200  $\mu$ L RMI1640 medium was added into each well for 24 hours incubation. Then, 20 $\mu$ l MTT (5mg/ml) solution was dropped into each well and incubated for another 4 hours. After that, the culture medium was removed, and the formazan crystals were dissolved in 100 $\mu$ l DMSO, and the absorbance value were read by a microplate reader (Multiskan Mk3, Thermo scientific, US) at the wavelength of 540 nm. The cell viability (%) was calculated according to the following formula: Cell viability (%) =  $[\text{OD}_{540}(\text{sample})/\text{OD}_{540}(\text{control})] \times 100\%$ .

**Figure S6** showed the cytotoxicities of various inhibitors on A549 cells. For genistein, there was no significant cytotoxicity on A549 cells within concentration range of 10~100  $\mu$ g/mL. For amiloride, no significant cytotoxicity was also found within the concentration range of 0.1~1.5 mM. However, a significant dose-dependent cytotoxicity was found in the concentration range of chlorpromazine (1~20  $\mu$ g/mL) when incubated with A549 cells. Therefore, in order to ensure 80% cell viability during the studies on the internalization mechanisms of CaP-AHA/siRNA nanoparticles, the final concentrations of amiloride (0.25 mM), chlorpromazine (10  $\mu$ g/mL) and genistein (50  $\mu$ g/mL) were selected in this study.



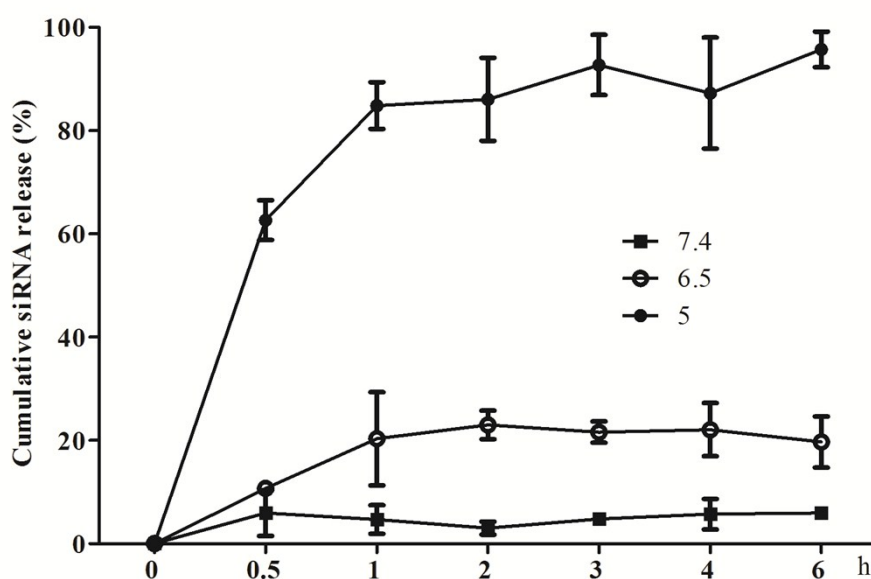


**Figure S6.** The cytotoxicities of various inhibitors on A549 cells. The data were shown as mean  $\pm$  SD (n=6).

### 5. *In vitro* release of siRNA

The releases of siRNA from CaP-AHA<sub>10</sub>/siRNA nanoparticles were investigated. The CaP-AHA<sub>10</sub>/siRNA NPs were suspended in the release media with different pH values (PBS at pH 7.4; 10 mM citric acid buffer at pH 5.0 and 6.5). After incubation for a predetermined time period, The samples were collected and centrifuged at 12000 g for 30 min, and the released siRNA in supernatant was analyzed by monitoring its absorbance at 260 nm using a spectrophotometer (NanoDrop, Thermo-Fisher Scientific Inc., Wilmington, DE).

As shown in **Figure S7**, only 5% of entrapped siRNA was released from CaP-AHA<sub>10</sub>/siRNA NPs after 6 h incubation at pH 7.4, however, 20% and 85% of siRNA was released within 2 h incubation at pH 6.5 and 5.0, respectively. These results suggested that the prepared CaP-AHA<sub>10</sub>/siRNA nanoparticles would exhibit good pH-responsive releases of siRNA in the conditions of early endosomal (pH 6.5) and lysosomal pH (pH 5).

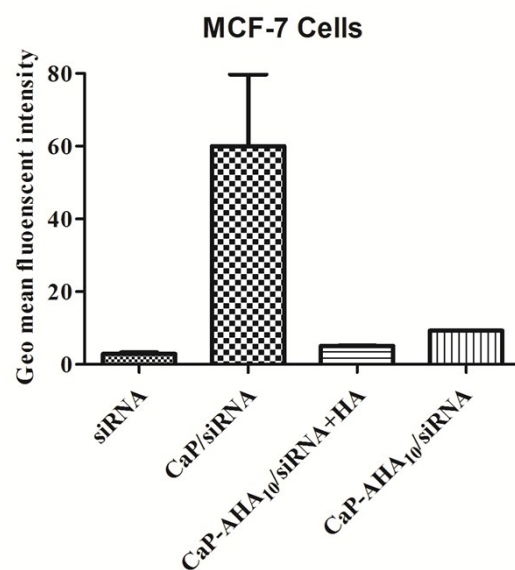


**Figure S7.** The Cumulative releases of siRNA from CaP-AHA<sub>10</sub>/siRNA NPs at different pH values. The data were shown as mean  $\pm$  SD (n=6).

#### 6. Cellular uptake of CaP-AHA<sub>10</sub>/siRNA NPs in MCF-7 cells

The cellular uptake of CaP-AHA<sub>10</sub>/siRNA NPs was further investigated in MCF-7 cells. The cells were seeded  $2.5 \times 10^5$  per well in six-well plates. After 24 h proliferation, the NPs containing FAM-labeled siRNA at the final concentration of 100 nM were exposed to cells and incubated for an additional 6 h at 37°C. After incubation, the cells were harvested and washed three times with pre-cooled PBS solution, and intracellular fluorescence intensities were detected by a FACS Calibur flow cytometry (Becton Dickinson, San Jose, CA, USA) immediately.

As shown in **Figure S8**, the CaP-AHA<sub>10</sub>/siRNA NPs showed a low cellular uptake similar as the naked siRNA and the CaP-AHA<sub>10</sub>/siRNA NPs pretreated with free HA on MCF-7 cells. Meanwhile, CaP/siRNA NPs displayed higher intracellular fluorescence intensities of FAM-siRNA than CaP-AHA<sub>10</sub>/siRNA NPs.



**Figure S8.** The intracellular fluorescence intensities detected by flow cytometry after 6 h incubation of different nanoparticles (FAM-labeled siRNA: 100 nM).