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Supporting Information

A modular approach for cytosolic protein delivery: metal ions-induced self-assembly of gold nanoclusters as a general platform

Lirui Wang[a], Chunlei Zhang[a,b], Tianliang Li[a], Meng Duan[a], Fangfang Xia[a], Xinhong Li[c], Cunfeng Song[a], Shaojun Pan[a], Bin Liu[a] , Daxiang Cui[a,b]**

[a]Institute of Nano Biomedicine and Engineering, Shanghai Engineering Research Centre for Intelligent Diagnosis and Treatment Instrument, Department of Instrument Science and Engineering, School of Electronic Information and Electrical Engineering, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai 200240, PR China

[b]National Center for Translational Medicine, Collaborative Innovational Center for System Biology, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai 200240, PR China

[c]Shanghai Key Lab of Veterinary Biotechnology, School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai, 200240, PR China

Materials and Experimental methods

Materials. Gold (III) chloride trihydrate ($HAuCl_4 \cdot 3H_2O$, 99%) were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Calcium chloride ($CaCl_2$), Cytochalasin-D (Cyto D), Genistein was purchased from Aladdin Reagent Co. Ltd. (Shanghai, China). Chlorpromazine and DMSO were purchased from Solarbio (Beijing, Shanghai). FITC were obtained from Sigma-Aldrich (St. Louis, MO). β-Gal and RNase A were obtained from J&K Scientific (Shanghai, China). BSA, LysoTracker Red DND-99 and Live/dead cell staining kit were purchased from Yeasen Corporation (Shanghai, China). Cell Counting Kit (CCK-8) was purchased from Dojindo Laboratories (Kumamot, China). PULSinTM was obtained from Polyplus Transfection (France). In situ β-Gal staining kit, β-Gal assay kit, BCA protein assay kit and Hoechst 33342 were purchased from Beyotime (Jiangsu, China). Human gastric cancer (MGC-803) cell line, lung cancer (A549) cell line and human embryonic kidney 293 (HEK293) cell line were available in the Cell Bank of Type Culture Collection of Chinese Academy of Sciences. All reagents used for cell culture were obtained from Gibco. Ultrapure water (18.2 MΩ cm, Millipore Co., USA) was used for the preparations of all solutions.

Synthesis of GNCs. The glutathione (GSH)-capped gold nanoclusters (GNCs) were synthesized according to the procedures previously reported by our group.[1-3] Briefly, freshly prepared $HAuCl_4$ (2 mL, 0.05 M) and GSH (2.5 mL, 0.1 M) were added to 90.5 mL of deionized water in a 100 ml conical flask. After vigorous stirring for 3 min, the mixture was added with 5 mL of icy cold TBAB solution and quickly stirred for further 5 min. Then the above mixture solution was controlled at 28 ℃ and maintained for half

of an hour without stirring. Subsequently, the pH of the solution was adjusted to 3.0 by adding 1M HCl. The Au (I)-thiolate complex precipitates were removed by centrifugation. After 12 h incubation in ice bath, the supernatant was added with NaCl (to about 20mM) and methyl alcohol (2Vol of aqueous solution).The solution becomes turbid and was centrifuged to remove free gold ions as well as free GSH. Finally, the precipitates were suspended in ultrapure water and dialyzed against water for 72h to obtain GNCs.

Synthesis of MHA@GNCs. MHA protected gold nanoclusters (MHA@GNCs) were synthesized following a previous reported method.[4] Typically, 1 mL of MHA(10 mM) and 0.25 mL of HAuCl4 (20 mM) were prepared freshly and quickly mixed with 3.35 mL of distilled water. Then 0.3 mL of NaOH (1M) was added to the above mixture to dissolve the Au (I)-MHA complexed. Subsequently, freshly prepared NaBH4 solution (112 mM, 0.1 mL) was added further. After 3 h, the MHA@GNCs were collected and purified by a semipermeable membrane of molecular weight cutoff (MWCO) 3500 Da.

Synthesis of FITC-labeled BSA(BSA-FITC). The synthesis of BSA-FITC was according to a previous study.[5] Briefly, BSA solution (dissolved in PBS) was mixed with FITC solution (dissolved in DMSO) at a BSA/FITC molecular ratio of 1:3. Then the mixed solution was stirred for 24 h at 25 ℃, following by further intensively dialysis against PBS and distilled water (MWCO 3500 Da). Finally, the product was collected and lyophilized to acquire yellow powders (BSA-FITC), and stored at -20 °C for further use. β-Gal-FITC were obtained by the similar procedures.

Preparation of supramolecular assemblies. Firstly, the above synthesized GNCs (2 mg/mL), the $CaCl_2$ (1 mM) dissolved in ultrapure water and proteins in specific concentration were well-prepared in the ultrapure water, respectively. The $CaCl_2$ solution and protein solution were freshly prepared and dissolved thoroughly before used. Taking the synthesis of Ca^{2+}-GNCs-BSA nanocomplex as an example, 0.1 mL of BSA solution (2.4 mg/mL) was added into 0.89 ml of aqueous solution containing GNCs (2 mg/mL, 0.09 mL), following by vigorous stirring. After 5 min, 0.02 mL of 1 mM $CaCl_2$ aqueous solution was added dropwise into the above mixed solution and the mixture solution was stirred for 3 h at 30°C. Finally, the centrifugation method at 10000 rpm for 10 min was used to purify the assembled nanocomplex. The precipitate was washed three times and finally dispersed in ultrapure water. Co-assembly of GNCs and BSA mediated by different ratios of Ca^{2+} as well as the coassembly of GNCs and other proteins by Ca^{2+} were performed in the similar steps.

BSA loading efficiency. The encapsulation efficiency (%EE) for BSA was determined according to the following Eqs.: %EE=((initial BSA – remaining BSA in supernatant)/initial BSA)*100.

Characterization. The morphology and size of GNCs and MHA@GNCs were evaluated on a field emission transmission electron microscopy (FE-TEM) (TAKOS, F200X, USA). The morphology of assemblies was estimated by 120 kV biological transmission electron microscopy (TEM). UV-Vis spectra were recorded with a Varian Cary 50 spectrophotometer (Varian Inc., USA). Photoluminescence (PL) spectra were determined by a Hitachi FL-4600 spectrofluorometer. The size distribution and zeta potential of nanoassemblies were detected by dynamic light scattering on a Nicomp 380

ZLS Zeta potential/Particle sizer (PSS Nicomp, USA). Circular dichroism (CD) measurements were carried out using a Jasco J-815 CD spectrometer (Jasco Corporation, Tokyo, Japan). FTIR spectra were measured using a Nicolet 6700 spectrometer (Thermo Fisher Corporation, USA). XPS spectrum were collected on a X-ray photoelectron spectrometer (Shimadzu, 1/AXIS UltraDLD, Japan).

Cell culture and intracellular protein delivery. MGC-803 cells, A549 cells and HEK293 cells were chosen as modal cells. Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), and 100 units/mL penicillin sulfate and streptomycin were used to culture these cell lines at 37 °C under a humidified 5% CO2 atmosphere. The cells were cultured in 24-well plates overnight till 80% confluence before protein delivery. Then the cells were incubated with the nanoassemblies for 4h in serum-free media. After incubation, the cell media was discarded and subsequently, the cells were washed with PBS for 3 times. Then the fluorescence intensity was quantitatively determined by flow cytometry (BD FACSCalibur, Mountain View, CA) and observed by a Confocal Quantitative Image Cytometer CQ1 (Yokogawa Electric Corporation, Tokyo, Japan).

Intracellular trafficking of the supramolecular assemblies. MGC-803 cells were incubated in 24-well plates overnight and then incubated with the nanocomplexes in serum-free media for 1, 2, 4 and 8h, respectively. The acidic compartments of the cells were stained by LysoTracker Red and the nuclei of the cells were stained with Hoechst 33342. The localizations of naocomplexes in MGC-803 cells were observed on a

Confocal Quantitative Image Cytometer CQ1 (Yokogawa Electric Corporation, Tokyo, Japan).

To verify the endocytosis pathways, the cells were incubated at 4 ℃ or pre-incubated with several endocytosis inhibitors including chlorpromazine (5 μM) for clathrin-mediated endocytosis, genistein (5 μM) and methy-beta-cyclodextrin (50 μM) for cavelolin-mediated endocytosis, Wortmannin (50 nM) and cytochalasin-D (2 μM) for macropinocytosis for 1h before the addition of nanocomplex. Untreated cells served as controls. The fluorescence intensity was analyzed by flow cytometry.

Detection of the β-Gal enzymatic activity. The intracellular β-Gal enzymatic activity was determined by an in situ β-Gal staining kit and a β-Gal assay kit according to the manufactures' instructions.[6, 7] Generally, MGC-803 cells were cultured with β-Gal nanocomplexes for 4 h as described above. Then the culture media was removed and the cells were washed with PBS for 3 times, fixed with 4% paraformaldehude for 15 min, and further washed with PBS for 3 times. Finally, the cells were incubated overnight at 37 °C with a working solution containing 5% 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). The cells were then washed with PBS and the intracellular blue products were observed by an optical microscope (Olympus, Japan).

The intracellular β-Gal activity after transfection were quantitatively measured by a β-Gal assay kit. Firsty, cells were incubated with 50 μL of cell lysates together with 50 μL of working solution containing the enzyme substrate. Then the plate was incubated at 37 °C for 30 min. After that, 150 μL stop reaction solution was added into the mixture solution of each well and the optical density of solutions at 420 nm was measured by

the microplate reader (Thermo Scientific, Germany). The optical density of free β-Gal solution at an equal enzyme content was detected and regarded as 100% relative β-Gal activity. Three repeats were conducted for each sample.

Cell viability. The cell viability of MGC-803 cells was tested by a well-established CCK8 assay. Briefly, cells were seeded in a 96-well plate with 10^4 cells for each well and cultured in media overnight. Then the medium were removed and fresh serum-free medium containing different concentrations of nanocomplexes were added. After incubation for 4 h, the fresh DMEM medium supplemented with 10% FBS was used to replace the above medium and culture the cells for further 20 h. After that, a standard CCk8 assay was performed. Five repeats were conducted for each sample.

Dead/live stainining. To visually observe the low cytoxicity of Ca^{2+}-GNCs-protein nanoparticles, the cells were washed with PBS and further stained with 2 μM calcein-AM (for live cells) and 1.5 μM PI (for dead cells) after 24 h incubation. Finally, the cells stainning with green or red fluorescence were observed by a Confocal Quantitative Image Cytometer CQ1 (Yokogawa Electric Corporation, Tokyo, Japan).

Fluorescent in Situ Hybridization to mRNA using an Oligonucleotide Probe. In situ hybridization method was performed referring to the previous literatures[8, 9] . Briefly, the digoxigenin-labelled oligonucleotide probe dT50 can hybridize to poly(A) tail of mRNA in cancer cells. Then the green fluorescence of FITC on digoxigenin was used as signal to monitor intracellular mRNA level.

BCA protein assay. Protein concentration was dertermined following the manufacture protocol of the BCA protein assay kit. Firstly, reagent A and B were fully mixed at the

volume ratio of 1:50 to obtain the BCA working solution. Secondly, the samples were added into a 96 microplate wells following by the addtion of 200 μL of working solution. Then the solutions were thoroughly mixed and put at room temperature. After 1 h incubation, the absorbance value of the samples at 562 nm were recorded on a Synergy™ 2 Multi-Detection Microplate Reader.

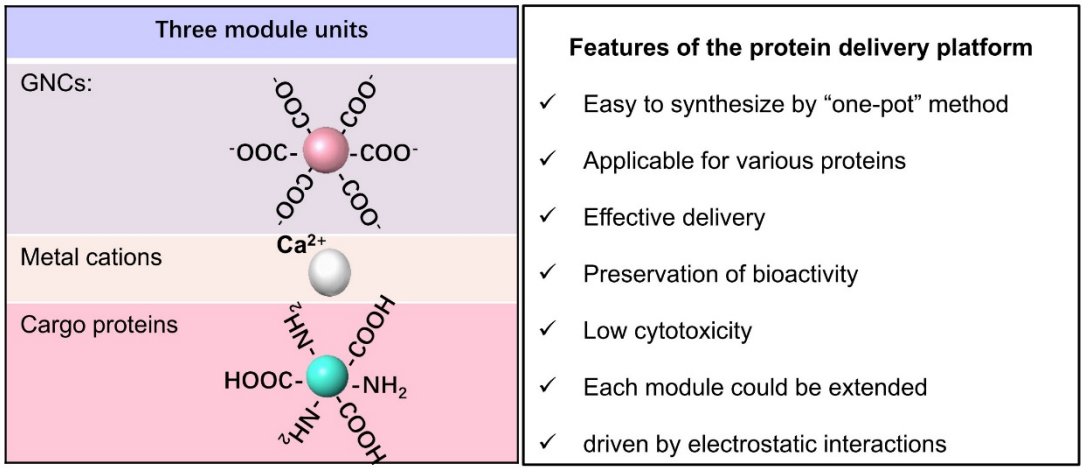

Fig. S1 Three modules of the designed protein delivery platform and its features.

Table S1 Zeta potential of GNCs.

	Zeta potential
GNCs	-18.56 ±1.98

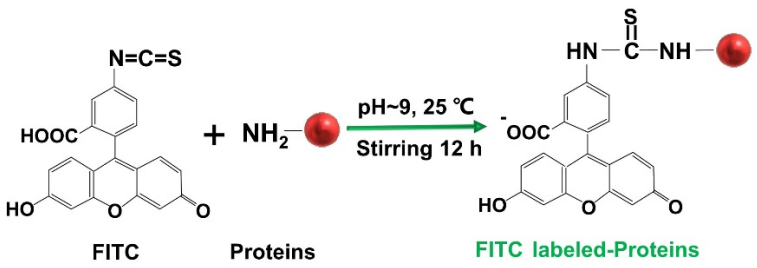

Fig. S2 The preparation of FITC-labeled BSA.

Table S2 The loading efficiency of BSA into Ca^{2+}-GNCs-BSA nanoparticles.

Protein	Loading efficiency (%)
BSA	96.8±1.2

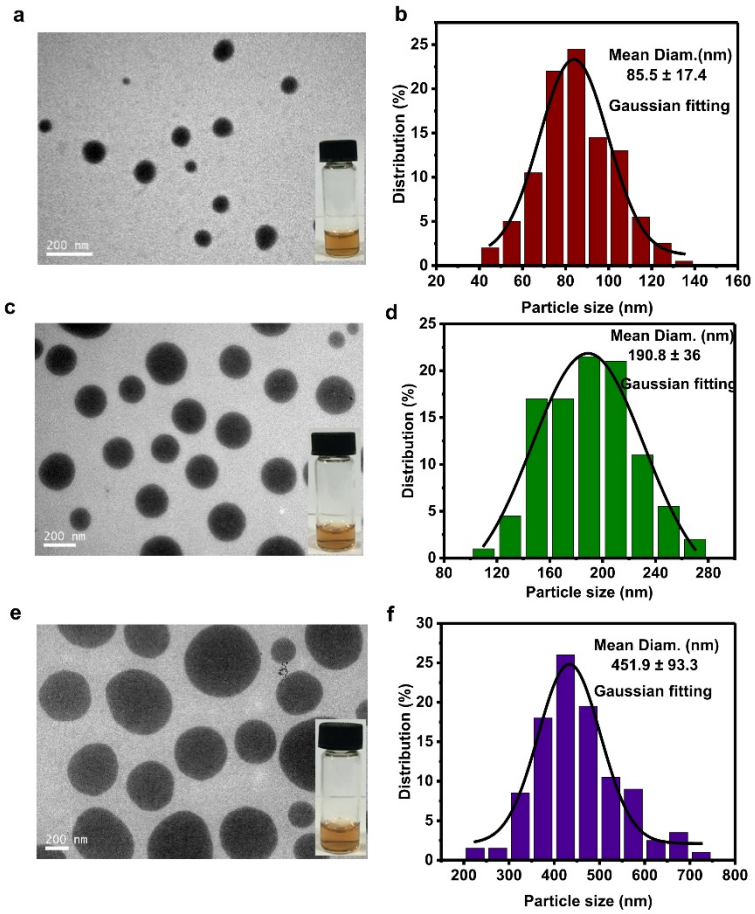

Fig. S3. TEM images of Ca^{2+}-GNCs-BSA nanoparticles assemblied with different Ca^{2+} ratios, with the digital photos of the aqueous solution inserted. Molar ratio of Ca^{2+}: GNCs : BSA is a) 8.25: 9 : 2, c) 13.75 : 9 : 2 and e)16.5 : 9 : 2. (b, d, f) The corresponding size distribution of nanoparticles in a, c and e.

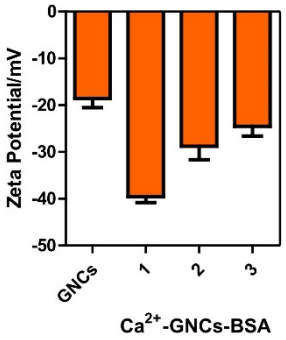

Fig. S4. Zeta potential of Ca^{2+}-GNCs-BSA aqueous solutions assembled with different Ca^{2+} ratios. Molar ratio of Ca^{2+}: GNCs : BSA is 8.25: 9 : 2 (1), 13.75 : 9 : 2 (2), and 16.5 : 9 : 2 (3). Mean ± SD, n=5.

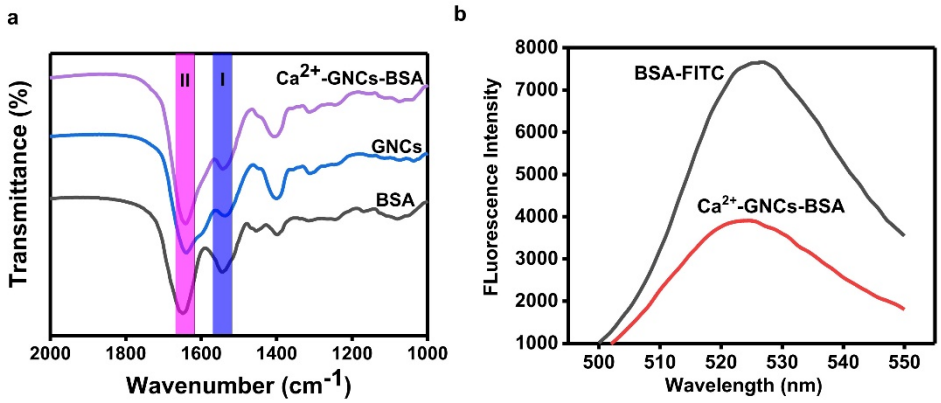

Fig. S5 a) FI-TR spectra analysis of Ca^{2+}-GNCs-BSA, GNCs and BSA, repectively. Bands in ~1640 – 1660 cm^{-1} and ~1550-1570 cm^{-1} stand for the amide I and amide II bands of the proteins. b) fluorescence intensity of Ca^{2+}-GNCs-BSA nanoparticles compared with BSA group.

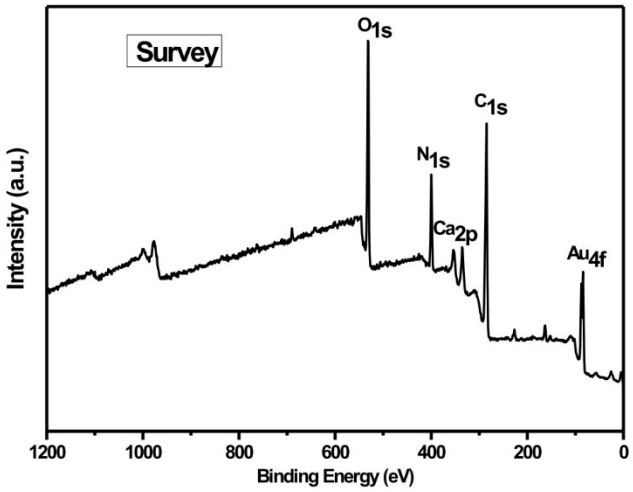

Fig. S6 XPS wide scans of Ca^{2+}-GNCs-BSA nanoparticles. Spectra of oxygen, O 1s, carbon, C 1s, nitrogen, N 1s, gold, Au 4f and calcium, Ca 2p were observed.

Table S3 XPS analysis of the content of each element in Ca^{2+}-GNCs-BSA nanoparticles.

Element	Mass percentage	Atom percentage
C	51.34	67.24
O	18.89	18.57
N	11.07	12.43
Au	17.86	1.43
Ca	0.84	0.33
Total	100	

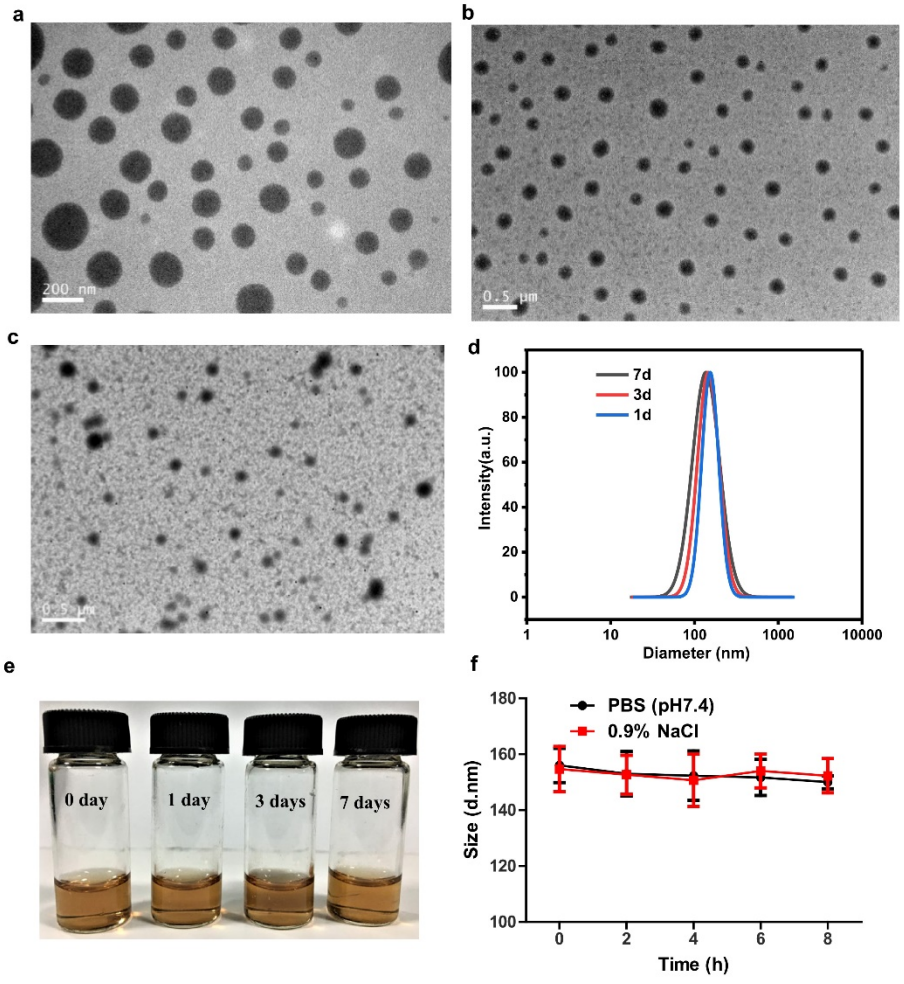

Fig. S7 . TEM images of of Ca^{2+}-GNCs-BSA nanoparticles in aqueous solution for a) 1 day, b) 3 days and c)7 days, respectively. Ca^{2+}: GNCs : BSA is 11: 9 : 2. d) DLS analysis of Ca^{2+}-GNCs-BSA nanoparticles in aqueous solution for 1, 3 and 7 days. e) Digital photos of Ca^{2+}-GNCs-BSA nanoparticles in aqueous solution for 0, 1, 3, and 7 days, respectively. f) Ca^{2+}-GNCs-BSA nanoparticles stability under physiological condition by DLS analysis.

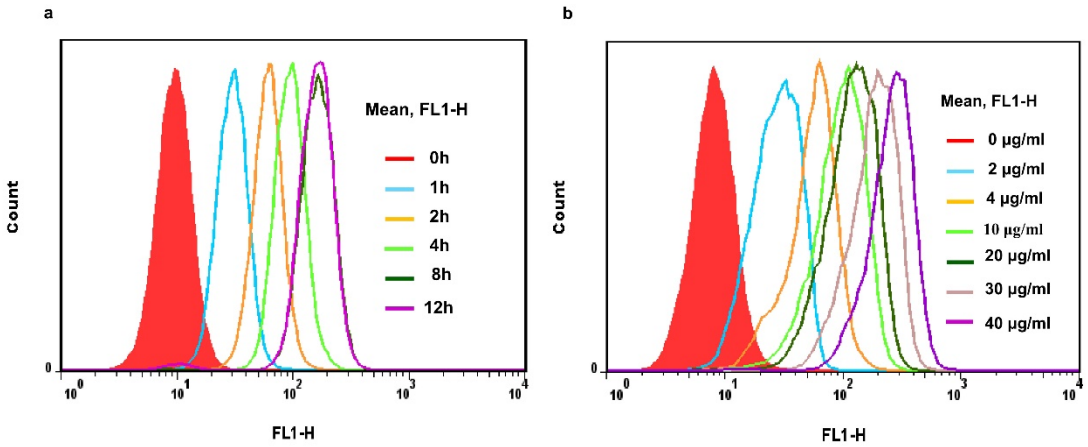

Fig. S8 . a) Flow cytometry analysis of the fluorescence intensity of MGC 803 cells treated with Ca^{2+}-GNCs-BSA complexes for 0 -12h. The concentrations of BSA in the samples were 10 μg/mL. b) Flow cytometry analysis of the fluorescence intensity of MGC 803 cells treated with different concentrations of Ca^{2+}-GNCs-BSA complexes for 4 h. The concentrations of BSA in the samples ranged from 0- 40 μg/mL.

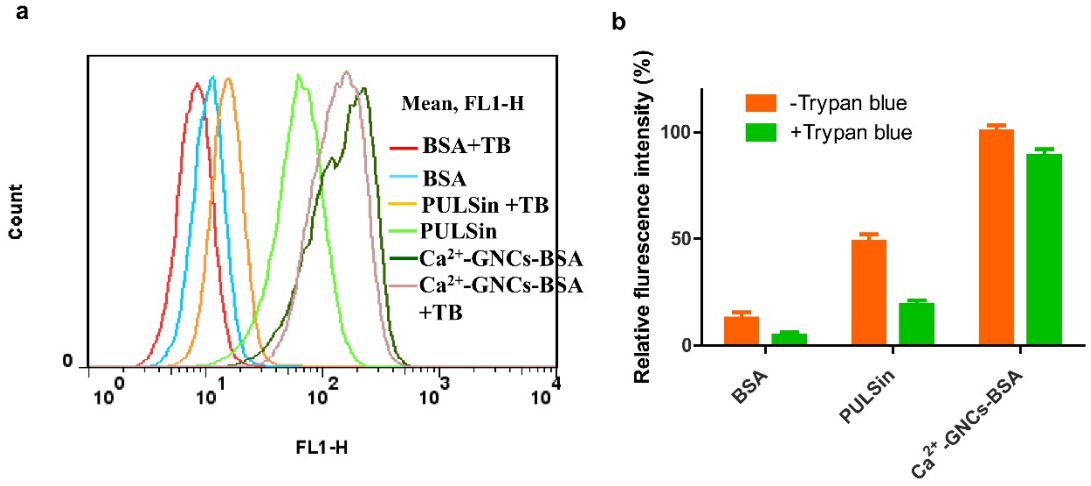

Fig. S9 . Fluorescence intensity of MGC-803 cells treated with BSA-FITC, PULSin + BSA or Ca^{2+}-GNCs-BSA-FITC complex for 4 h, before or after trypan blue quenching. The concentrations of BSA in the samples were 10 μg/mL. TB: Trypan blue.

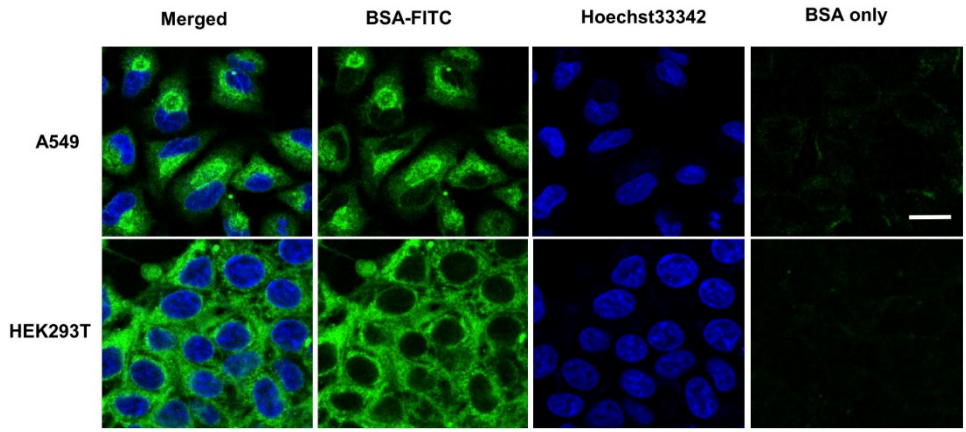

Fig. S10 . Fluorescence images of A549 cells and HEK 293T cells treated with BSA-FITC or Ca^{2+}-GNCs-BSA-FITC complex for 4 h, respectively. The concentrations of BSA in the samples were 10 μg/mL.The nucleus was staining with Hoechst 33342. Scale bar: 20 μm.

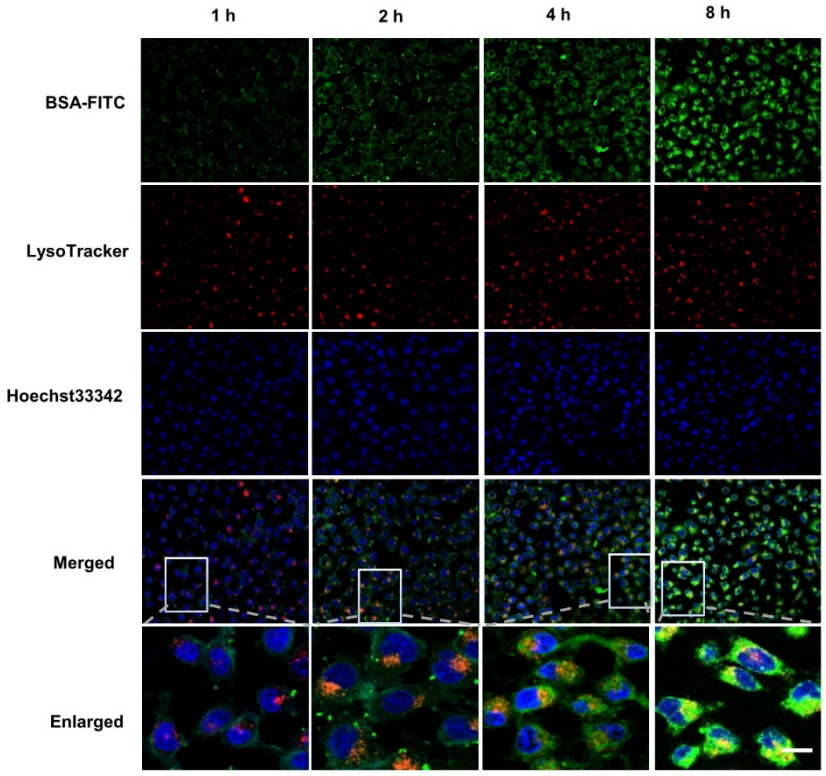

Fig. S11 Confocal images of living cells treated with Ca^{2+}-GNCs-BSA complexes for 1 - 8h. The concentrations of BSA in the samples were 10 μg/mL. Scale bar: 20 μm.

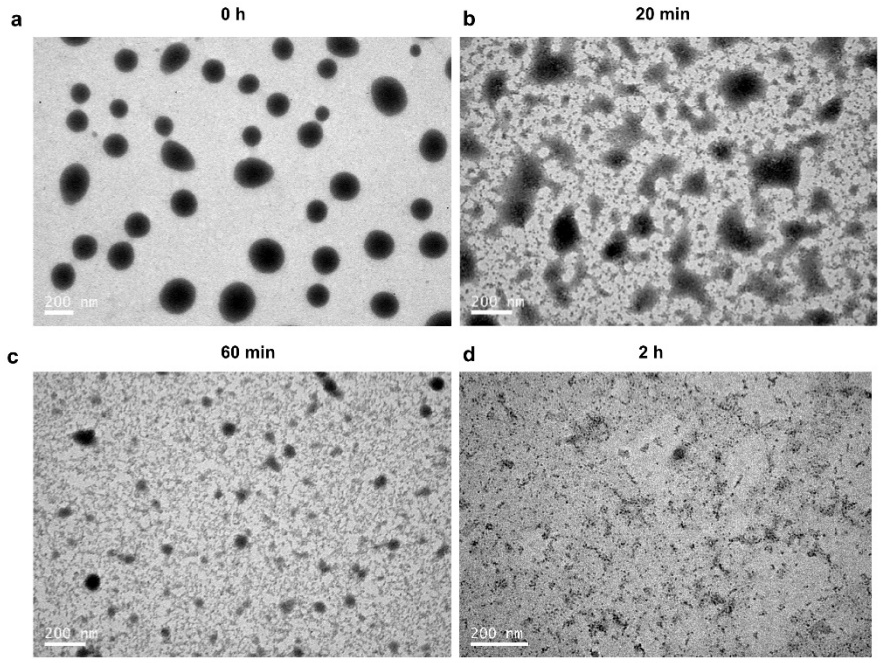

Fig. S12 . TEM images of Ca^{2+}-GNCs-BSA nanoparticles dispersed in acidic solution (pH ~5) for different time. a) 0 h, b) 20 min, c) 60 min and d) 2 h.

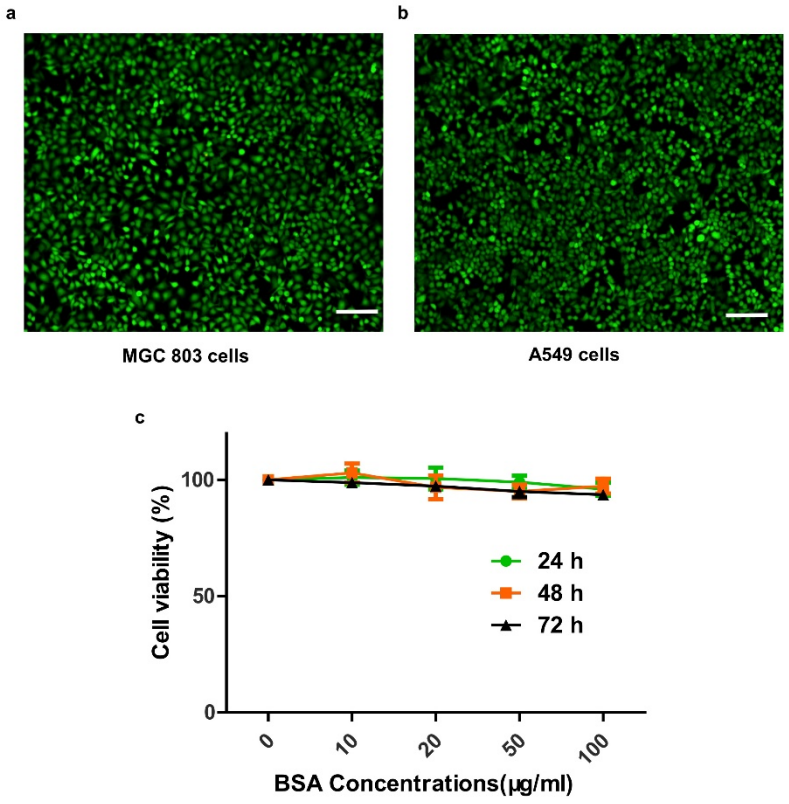

Fig. S13 Dead/live staining for detection the cell viability of Ca^{2+}-GNCs-BSA nanoparticles in a) MGC-803 cell line and b) A549 cell line, respectively. The concentrations of BSA in the samples were 20 μg/mL. Scale bar: 200 μm. c) Cell viability experiment at different BSA concentrations for 24 h, 48 h and 72 h.

Table S4 Properties of proteins in this study.

Proteins	Molecular weight	Isoelectric point
BSA	66.446 kDa	4.7
β-Gal	430 kDa	5.0
RNase A	13.7 kDa	9.6

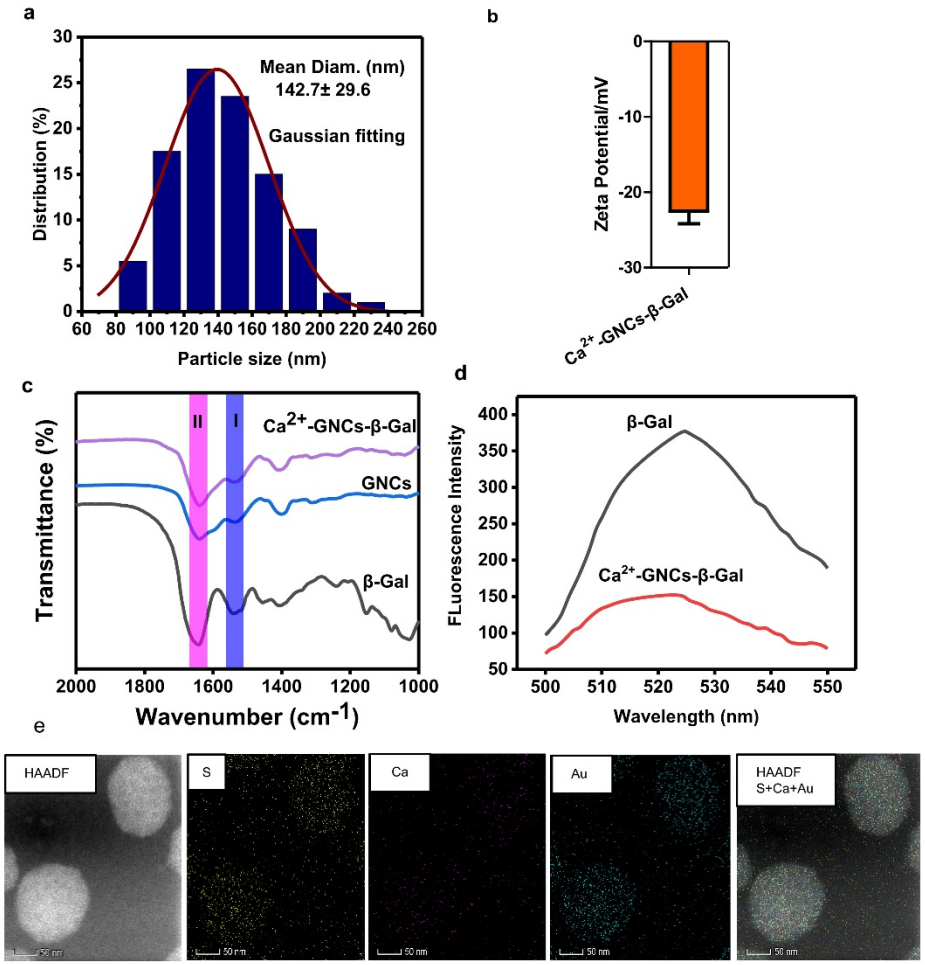

Fig. S14 a) The size distribution of Ca^{2+}-GNCs-β-Gal. b) Zeta potential of aqueous of Ca^{2+}-GNCs-β-Gal nanocomplexes. c) FI-TR spectra analysis f and Ca^{2+}-GNCs-β-Gal, GNCs and β-Gal, repectively. Bands from ~1640 to1660 cm^{-1} and from ~1550 to 1570 cm^{-1} stand for the amide I and amide II bands of the proteins. d) Fluorescence intensity of Ca^{2+}-GNCs-β-Gal nanoparticles compared with free β-Gal. e) The elemental mapping of Ca^{2+}-GNCs-β-Gal nanoparticles.

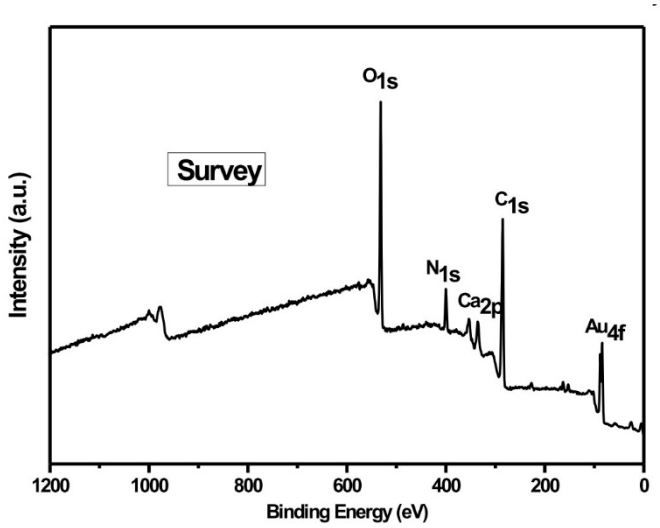

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Fig. S15 XPS wide scans of Ca^{2+}-GNCs-β-Gal. Spectra of oxygen, O 1s, carbon, C 1s, nitrogen, N 1s, gold, Au 4f and calcium, Ca 2p were observed.

Table S5 XPS analysis of the content of each element in Ca^{2+}-GNCs-β-Gal nanoparticles.

Element	Mass percentage	Atom percentage
C	50.29	66.87
O	23.58	23.54
N	6.66	7.60
Au	18.15	1.47
Ca	1.32	0.52
Total	100	

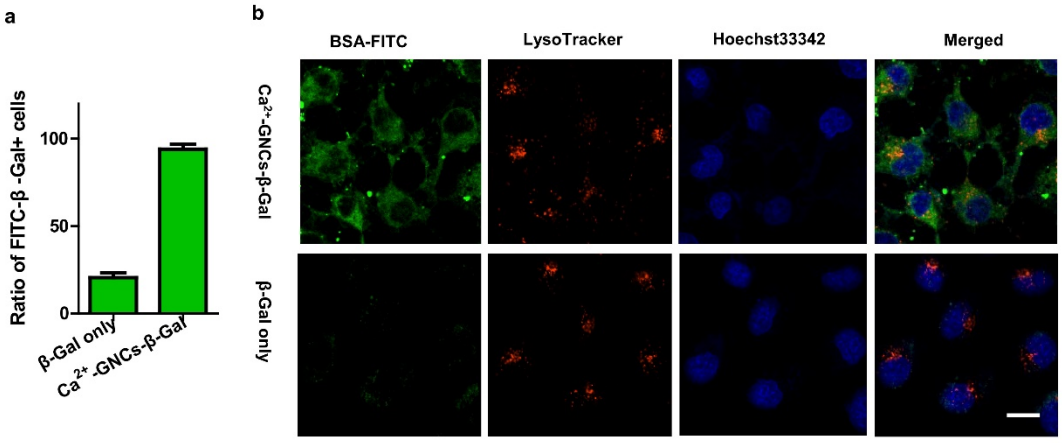

Fig. S16. a) Ration of FITC-β-Gal^{+} MGC-803 cells treated by Ca^{2+}-GNCs-β-Gal nanocomplex and β-Gal, respectively. b) Confocal images of MGC-803 cells treated by Ca^{2+}-GNCs-β-Gal nanocomplex and β-Gal, respectively. The endolysosomes were stained with Lysotracker red and the nuclei were stained with Hoechst33342. The dose of β-Gal in the sample was 20 μg/mL. Scale bar: 20 μm.

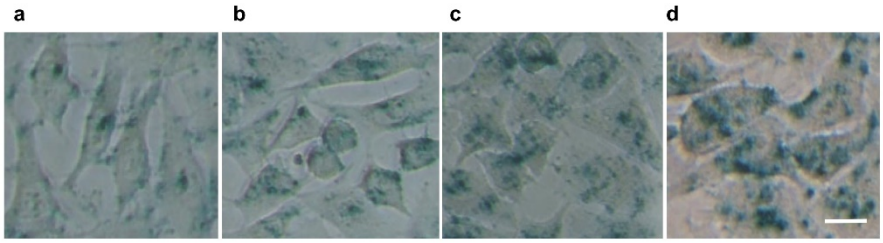

Fig. S17 X-Gal staining of MGC-803 cells treated with Ca^{2+}-GNCs-β-Gal nanocomplexes for 4 h. The concentrations of β-Gal in a-d group were 5, 8, 10, 12 μg/mL, respectively.

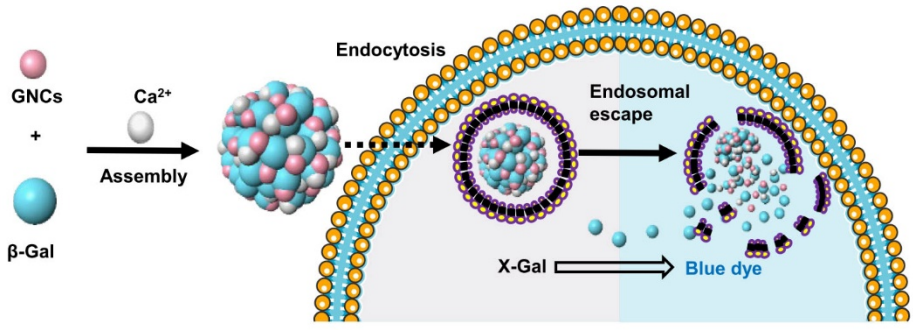

Fig. S18 A scheme showing the cellular uptake of Ca^{2+}-GNCs-β-Gal nanocomplex, the release of β-Gal, and β-Gal hydrolyses X-gal into a blue pigment, which can be used to determine the enzymatic activity of β-Gal.

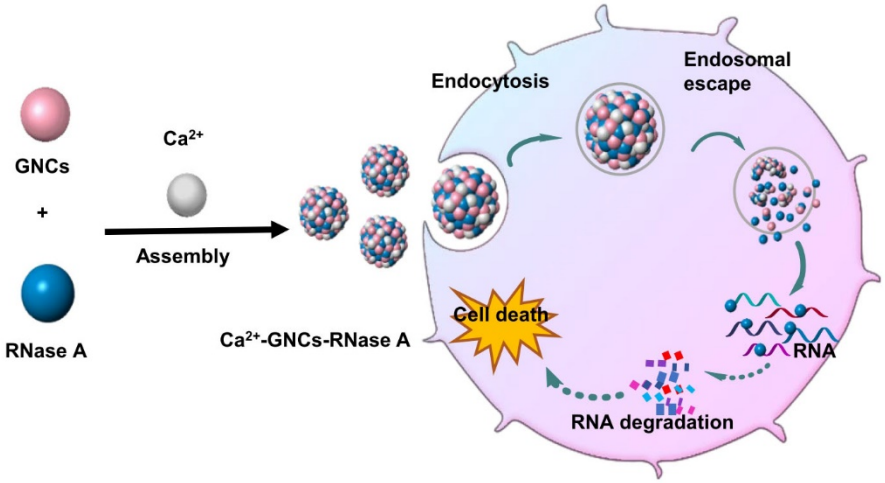

Fig. S19 Schematic illustration of Ca^{2+}-induced co-assembly of GNCs and RNase A into monodisperse nanoassemblies and proposed mechanism of the nanoassemblies in cytosolic protein delivery.

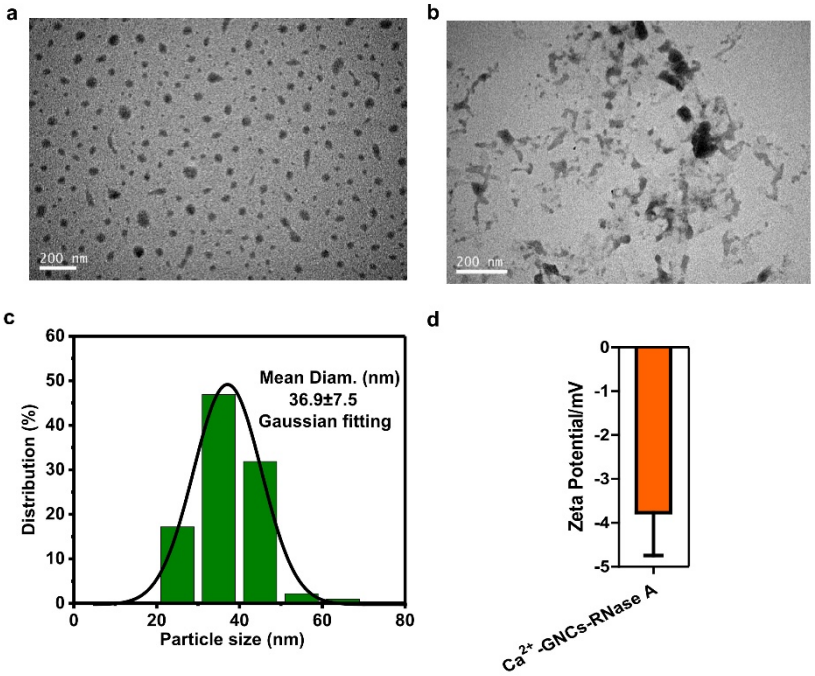

Fig. S20 TEM images of a) Ca^{2+}-GNCs-RNase A complexes and b) GNCs-RNase A. The monodisperse nanoparticles were not found in the absence of Ca^{2+}, suggesting the essential coordination role of Ca^{2+} in the synthesis of Ca^{2+}-GNCs-RNase A complexes. c) Size distribution of Ca^{2+}-GNCs-RNase A nanoparticles and d) the zeta potential of their aqueous solution.

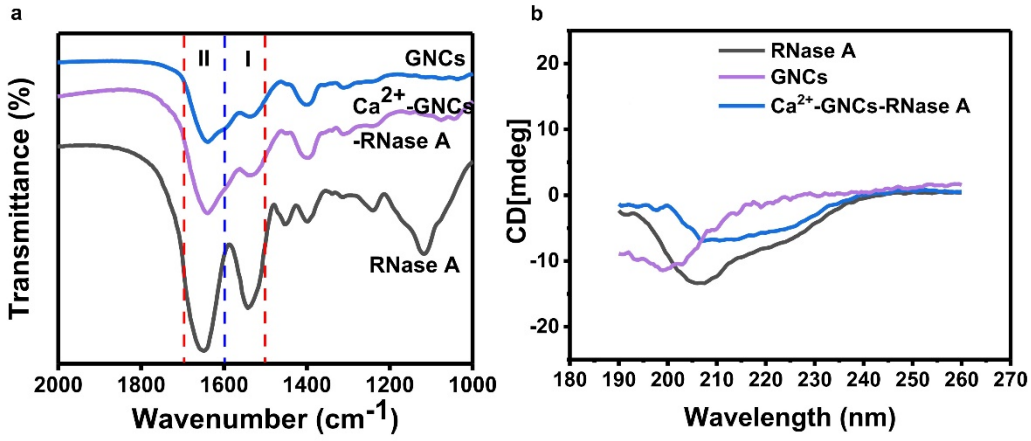

Fig. S21 a) FI-TR spectra analysis and b) CD spectra of RNase A, GNCs, and Ca^{2+}-GNCs-RNase A, respectively.

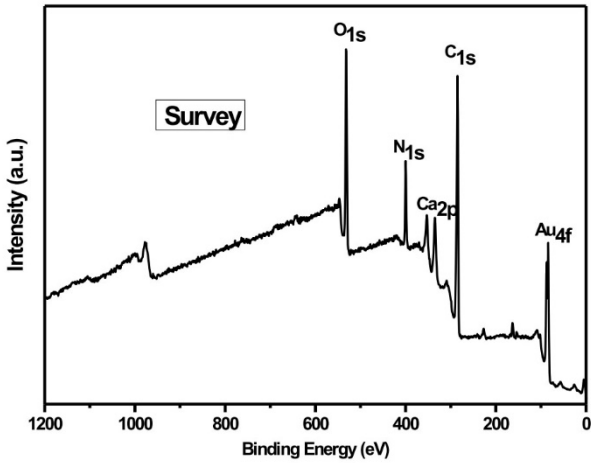

Fig. S22 XPS wide scans of Ca^{2+}-GNCs-RNase A. Spectra of oxygen, O 1s, carbon, C 1s, nitrogen, N 1s, gold, Au 4f and calcium, Ca 2p were observed.

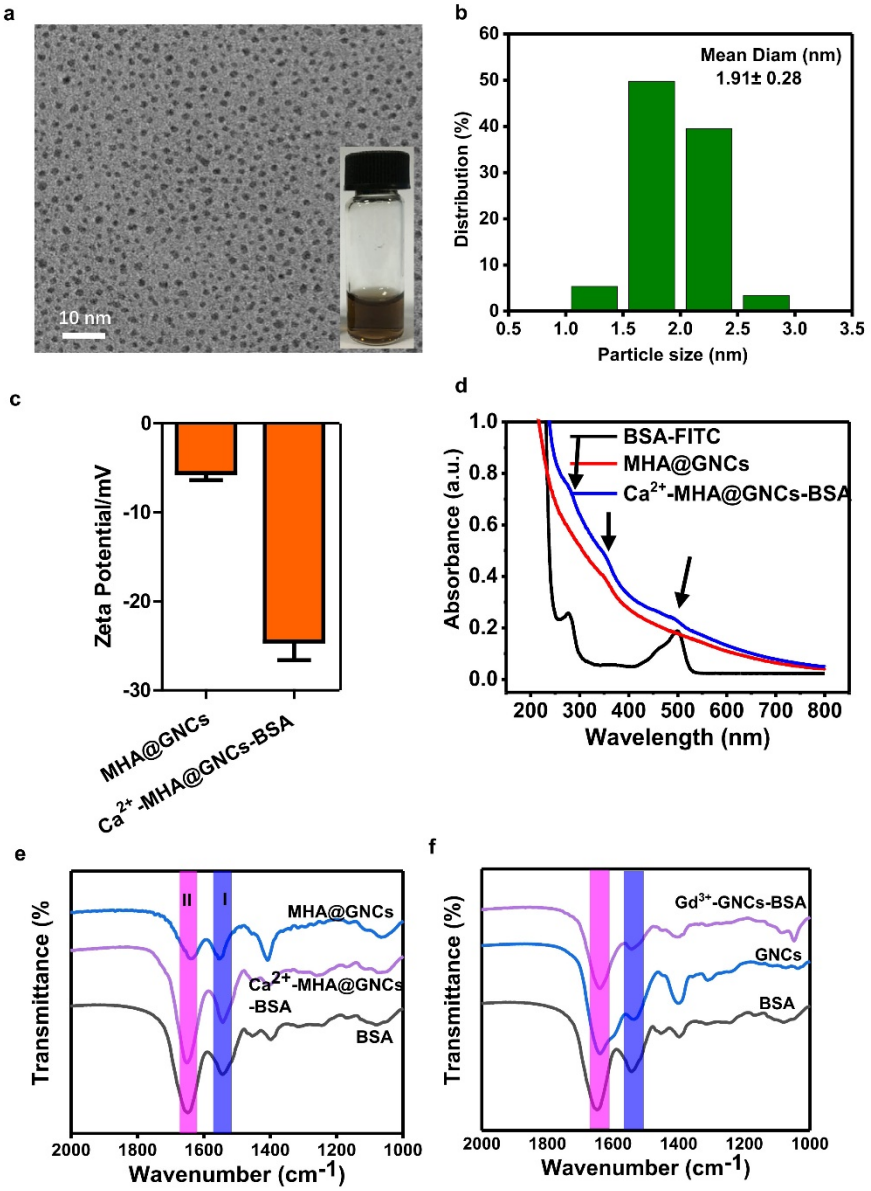

Fig. S23 a) TEM image of MHA@GNCs and b) the core size distribution of MHA@GNCs; c) Zeta potential of MHA@GNCs and Ca^{2+}-MHA@GNCs-BSA; d) UV-Vis analysis of MHA@GNCs and Ca^{2+}-MHA@GNCs-BSA; e) and f) FITR spectra analysis of Ca^{2+}-MHA@GNCs-BSA and Gd^{3+}-GNCs-RNase A using GNCs and BSA, respectively.

References

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