Supporting Information

One-pot synthesis of fluorescent BSA-Ce/Au nanoclusters as ratiometric pH probes

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Experimental section

Chemicals and Materials. BSA, ovalbumin, sinapinic acid (SA), tris buffered saline (TBS, 10X, pH 7.4), and cerium(IV) sulfate ($Ce(SO_4)_2$) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Hydrogen tetrachloroaurate(III) trihydrate ($HAuCl_4$) was obtained from Alfa Aesar (Heysham, England). Phosphoric acid, and monobasic, dibasic, and tribasic sodium salts of phosphate were obtained from J.T. Baker (New Jeresey, USA). Antibiotic-antimycotic solution, fetal bovine serum (FBS), L-glutamine, and DMEM were obtained from Biowest (Lewes, UK). Alamar Blue reagent was obtained from BioSource International (Camarillo, USA). Ultrapure water (18.25 MΩ cm^{-1}) from a Milli-Q system (Millipore, Billerica, MA, USA) was used in all experiments.

One-pot Preparation of BSA-Ce/Au NCs. A solution (1.0 mL) containing BSA (0.76 mM; 50 mg mL^{-1}), $HAuCl_4$ (10 mM), $Ce(SO_4)_2$ (1.0 mM) and NaOH (100 mM) in a 1.5 mL eppendorf tube was incubated at 70°C for 20 min. The solution color remained yellow before and after heating, but gradually turned to orange during cooling. To purify BSA-Ce/Au NCs, the cooled solution was subjected to dialysis against phosphate buffer (5.0 mM, pH 7.4) through a 20 kDa molecular weight cutoff membrane (Float-A-Lyzer G2, Spectrum Laboratories, Rancho Dominiguez, CA, USA) for 36 hours. The solutions were further purified and concentrated to 2-fold through freeze-drying and re-dispersed in ultrapure water. As controls, the same preparation and purification processes were applied to preparations of BSA-Ce complexes and BSA-Au NCs from alkaline BSA solutions containing only Ce(IV) and Au(III), respectively.

Spectroscopic measurements. The absorbance spectra of the purified solutions containing BSA-Ce complexes, BSA-Au NCs, and BSA-Ce/Au NCs were recorded using a GBC Cintra 10e double-beam spectrometer (Victoria, Australia). The fluorescence excitation and emission spectra were recorded using a Cary Eclipse fluorescence spectrometer (Varian, CA, USA). The excitation wavelengths for BSA-Ce complexes, BSA-Au NCs, and BSA-Ce/Au NCs were all set at 325 nm, while their emission wavelengths were set at 410 nm, 650 nm, and both, respectively. Fluorescence lifetime measurements were carried out using a photon-counting system from Time-Harp 200 (PicoQuant GmbH, Berlin, Germany), using a 375 nm laser to excite the samples. We note that a laser at 325 nm was unavailable in the fluorescence

lifetime measurement system. X-ray photoelectron spectroscopy (XPS) spectra were obtained using a VG ESCA210 electron spectroscope (VG Scientific, West Sussex, UK) by placing freeze-dried samples onto silica substrates.

Thermogravimetric Analysis (TGA). TGA curves were obtained using a Du Pont 951 thermogravimetry analyzer with a heating rate of 10 °C per minute. 5.0 mg of freeze-dried solid samples were analyzed.

MALDI-MS measurements. Mass spectra were obtained in a linear-positive ion detection mode using an Autoflex MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). The purified solutions of BSA-Ce complexes, BSA-Au NCs, and BSA-Ce/Au NCs were all diluted 100 fold with ultrapure water, which (1.0 μL) was then mixed with 1.0 μL matrix solution (10 mg mL^{-1} sinapinic acid in 500:500:1 (v/v/v) acetonitrile/water/trifluoroacetic acid). The mixtures were cautiously pipetted onto a stainless-steel 96-well MALDI plate and dried at ambient temperature (25 °C) prior to MALDI-MS analysis. The samples were irradiated with a nitrogen laser (337-nm output) at 100 Hz. The desorbed ions were stabilized energetically during a delayed extraction period of 200 ns, and were then accelerated through the TOF chamber in a linear positive mode at +20 kV before entering the mass analyzer. The laser fluence was adjusted to 400 μJ, and each mass spectrum was obtained by averaging over 10,000 laser pulses.

Cell culture. HeLa cells were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FBS, 1.0% antibiotic-antimycotic, 2.0 mM L-glutamine and 1.0% non-essential amino acids, in 5.0% CO_2 at 37 °C.

Cytotoxicity assay. Cell viability was determined using the Alamar Blue assay.[S1] HeLa cells were incubated in culture media at 37 °C for 24 hours, the cells were carefully rinsed with phosphate buffered saline (PBS), and the original culture media was replaced with freshly prepared media containing BSA-Ce/Au NCs of various concentrations (5.1– 51 mg mL^{-1} denoted by BSA concentration). The cells were then incubated for another 24 hours and were cautiously rinsed with PBS three times. The Alamar Blue reagent (diluted to 1X) in DMEM was then introduced. After 4 hours, the fluorescence of the reduced product of the dye, resorufin (7-hydroxy-3H-phenoxazin-3-one), in the cell solutions was recorded using a microplate fluorometer (Synergy 4 Multi-Mode Microplate Reader, Biotek Instruments, Vermont, USA) with an excitation wavelength of 545 nm and an emission wavelength of 590 nm. The cell

viability was calculated based on the measured fluorescence intensities, assuming that the control set of cells incubated in the medium containing dye alone has 100% viability.

Cellular uptake of BSA-Ce/Au NCs. HeLa cells were plated and cultured on 15 mm glass coverslips for two days. Pre-concentrated BSA-Ce/Au NCs were diluted with DMEM, both with final concentrations of 51 mg mL^{-1}. The as-prepared mixture was added to the cells, which were then incubated overnight in 5.0% CO_2 at 37 °C. To remove the excess BSA-Ce/Au NCs, the cell samples were washed three times with PBS.

Confocal fluorescence cell imaging. Before confocal measurements, the incubated cell samples were treated with TBS (pH 5.0, 7.4, or 8.5) for 5 minutes. TBS (pH 7.4) was adjusted with acetic acid and ammonium hydroxide to prepare solutions at pH 5.0 and 8.5, respectively. The cells were immediately fixed with 1.0% paraformaldehyde for 15 minutes, followed by three PBS rinsing steps. Each sample was separately mounted on a glass slide and sealed. A Zeiss LSM 710 fluorescence confocal microscope was used to obtain cell images. The fluorescence images of incubated HeLa cells in the emission wavelength regions of 410–500 nm (blue channel) and 600–700 nm (red channel) were recorded while the cells were irradiated with a laser at 405 nm. To analyze the images obtained, the free software ImageJ was employed to average the fluorescence intensities of the two channels.

Supplementary figures

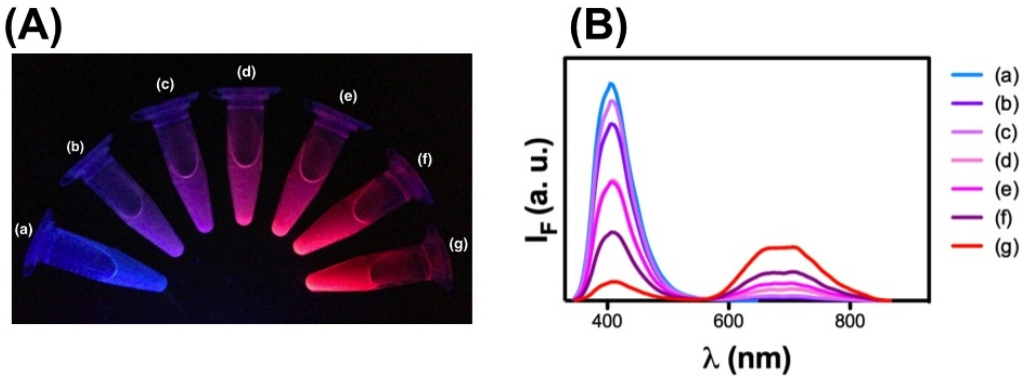

Fig. S1 (A) Photograph of BSA-Ce/Au NCs synthesized with different Ce/Au ratios, excited using a hand-hold UV lamp; (B) Fluorescence spectra of as-prepared BSA-Ce/Au NCs. Concentrations of $[Au^{3+}]$ (mM)/$[Ce^{4+}]$ (μM) in (a)-(g) are 0/1000, 0.5/950, 1.0/900, 2.0/800, 3.0/700, 5/0/500, 10/0, respectively.

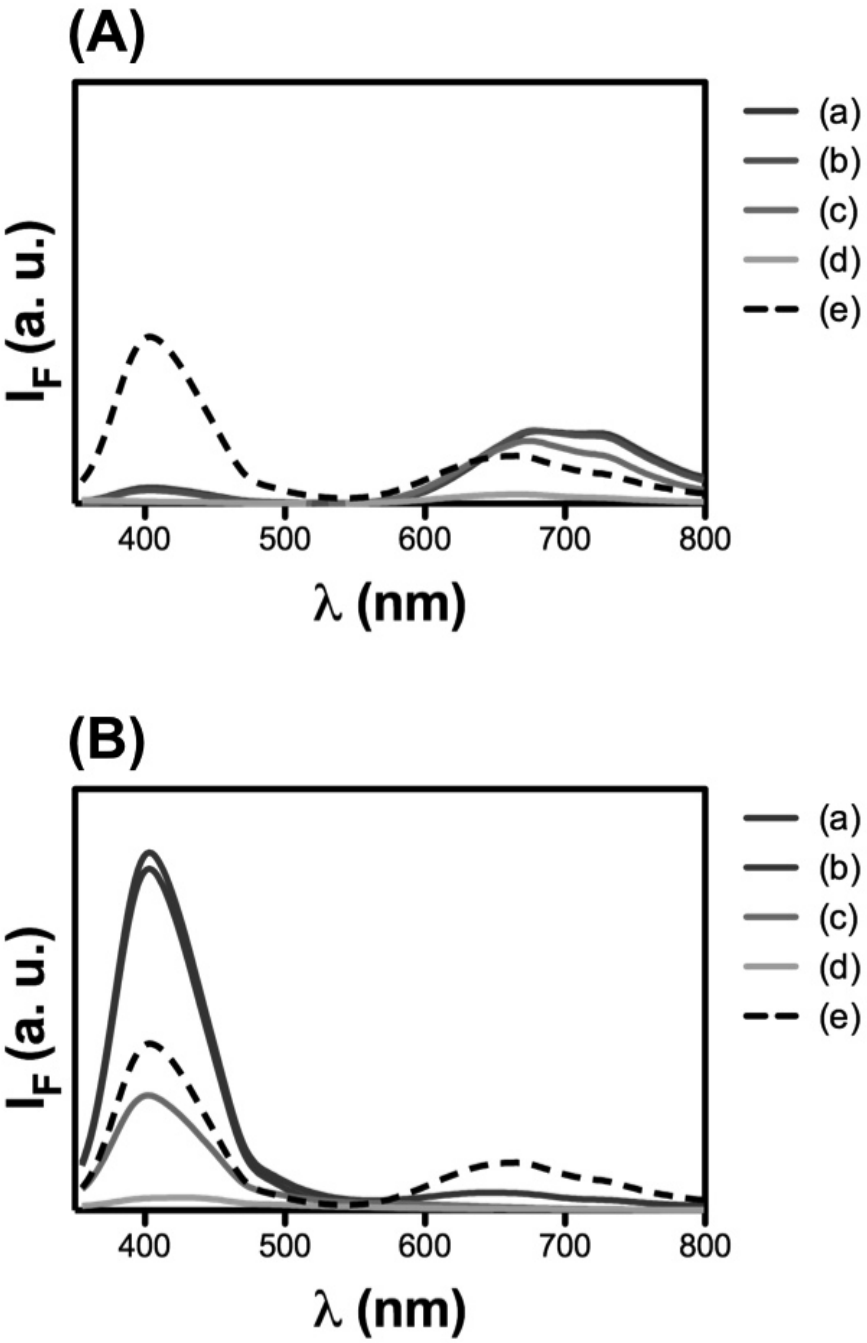

Fig. S2 Fluorescence spectra of NCs prepared from (A) BSA-Au NCs and Ce(IV), and (B) BSA-Ce complexes and Au(III) through a two-steps synthetic route. (A) Concentrations of Ce(IV) ions are (a) 0, (b) 0.5 mM, (c) 1.0 mM, (d) 3.0 mM. (B) Concentrations of Au(III) are (a) 0, (b) 5.0 mM, (c) 7.5 mM, (d) 10 mM. BSA-Ce/Au NCs prepared from one-pot synthesis were used as controls in (e) of (A) and (B). The solutions were heated at 70 °C for 20 minutes after adding the metal ions. White precipitants were observed in (c) and (d) of (A) and (d) of (B).

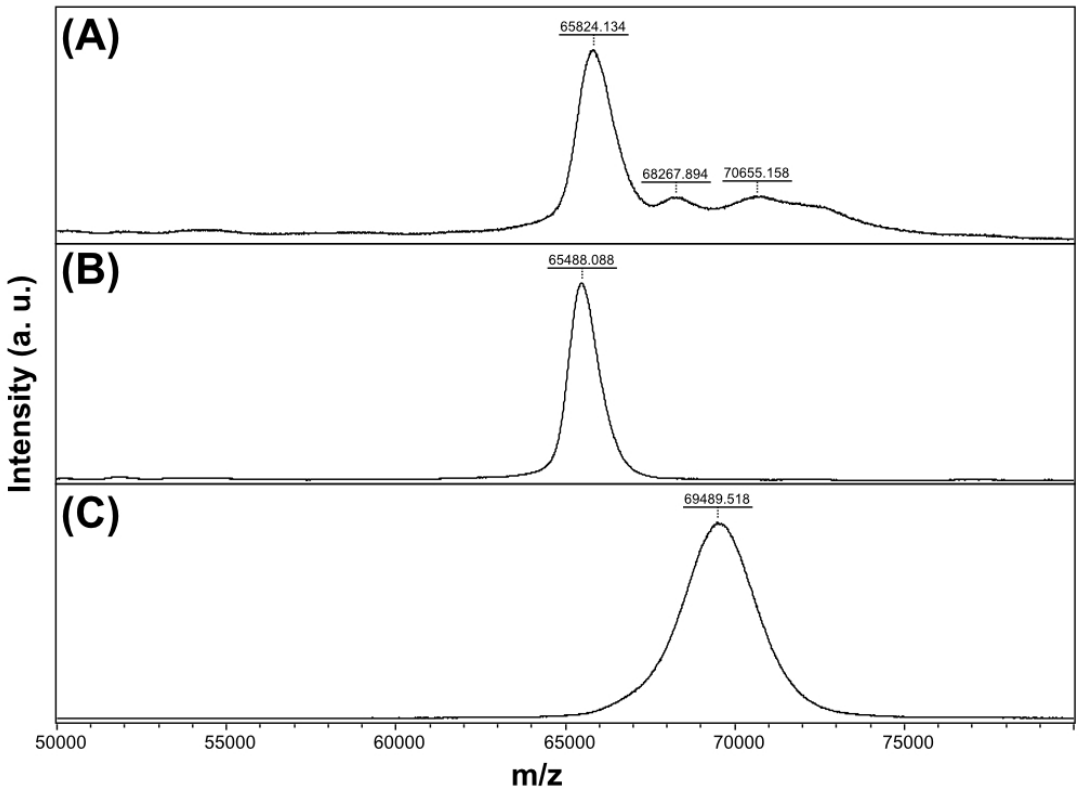

Fig. S3 MALDI-TOF mass spectra of (A) BSA-Au NCs, (B) BSA-Ce complexes, (C) BSA-Ce/Au NCs. The samples were all diluted ten times with DI water prior to measurements.

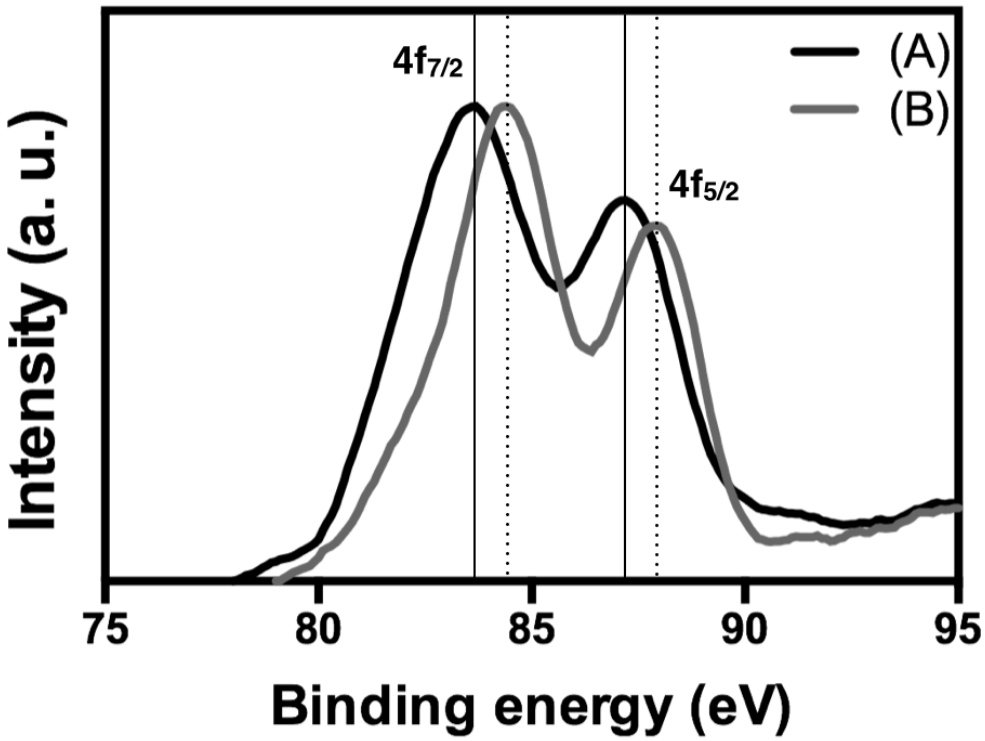

Fig. S4 XPS spectra of the Au components of (A) BSA-Au NCs and (B) BSA-Ce/Au NCs. The characteristic Au $4f_{7/2}$ and $4f_{5/2}$ peaks were observed for both, but shifts to higher binding energies for the BSA-Ce/Au NCs.

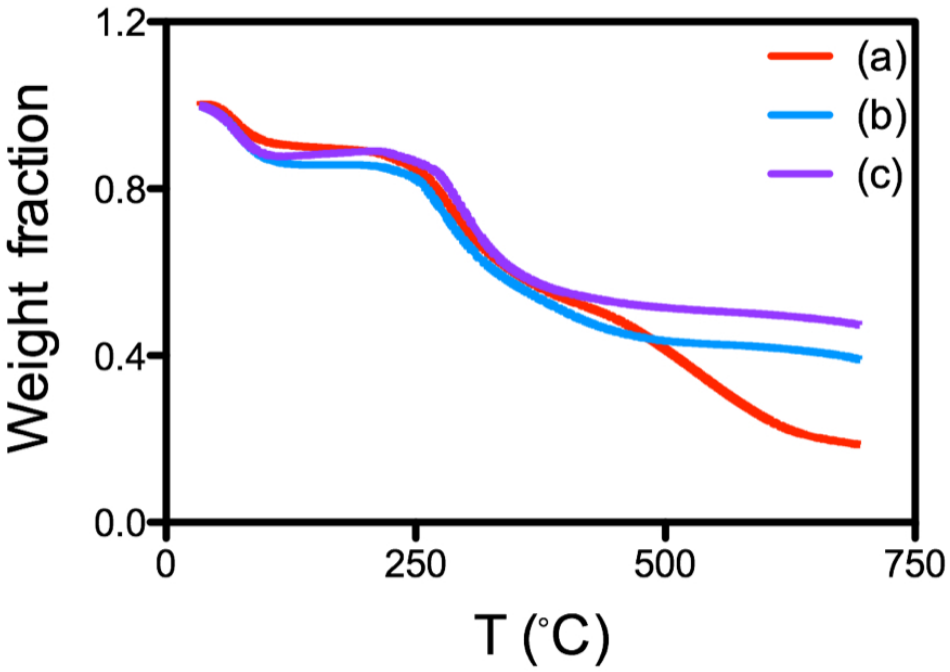

Fig. S5 Thermogravimetric analysis of (a) BSA-Au NCs, (b) BSA-Ce complexes, and (c) BSA-Ce/Au NCs from 30 to 700°C. The first drop around 100 °C responds to water loss. The second drop starting at around 280 °C is due to the loss of organic compound/peptides. The loss flattening after 300 °C is characteristic for cerium-containing compounds.

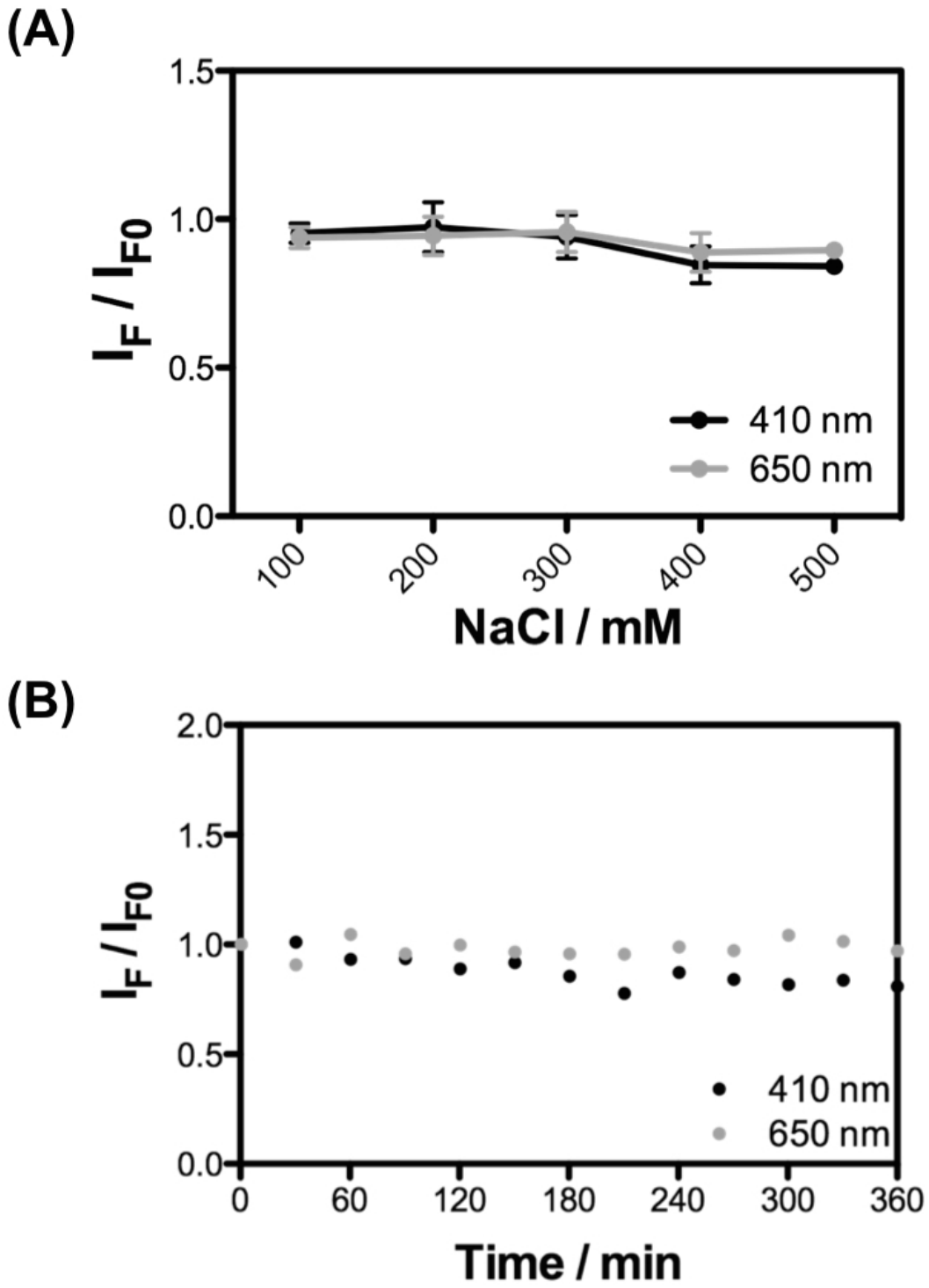

Fig. S6 (A) Phosphate buffer (pH 7.0, 20 mM) containing various NaCl (100-500 mM). (B) Photostabilities of BSA-Ce/Au NCs at 410 nm and 650 nm under continuous irradiation with 325 nm UV light for 6 hours. I_{410} and I_{650} represent the fluorescence intensities of BSA-Ce/Au NCs when excited at 325 nm, respectively.

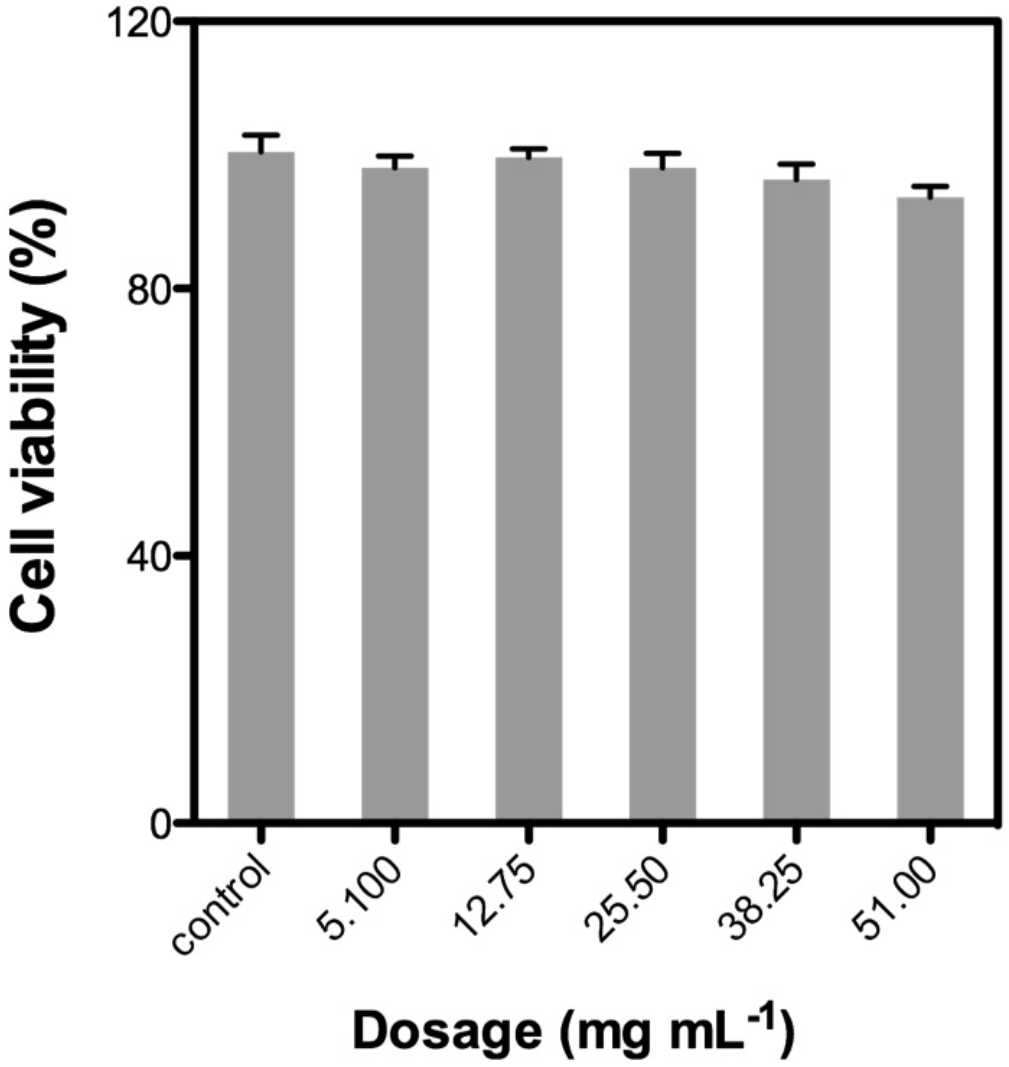

Fig. S7 Cell viability of HeLa cells treated with solutions of BSA-Ce/Au NCs at various concentrations for 24 hours.

Supplementary reference

S1. N. G. S. Al-Nasiry, M. Hanssens, C. Luyten and R. Pijnenborg, *Human Reproduction,* 2007, **22**, 1304-1309.

Acknowledgements

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