

## Supporting Information

### Cell-Penetrating Hollow Spheres Based on Milk Protein

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#### Experimental Section:

**Materials:** Casein and rhodamine B isothiocyanate were bought from Sigma-Aldrich Company. Acrylic acid (AA; Nanjing Chemical Reagent Co.,Ltd.) was distilled under reduced pressure in nitrogen atmosphere before use. Potassium peroxydisulfate ( $K_2S_2O_8$ , Nanjing Chemical Reagent Co.,Ltd.) was recrystallized from deionized water before use. Cisplatin (CDDP) was supplied as a model drug from Shandong Boyuan Chemical Co.,Ltd.. All other reagents were of analytical grade and used without further purification. Human cervical cancer HeLa cells were obtained from China Pharmaceutical University (Nanjing, China). Cell culture reagents were all from Gibco.

**Preparation of Casein-PAA Nanoparticles:** The casein-PAA nanoparticles were prepared by polymerization of AA in casein aqueous solution in the presence of propionic acid. In a typical run, 0.08 g casein was added into 40 mL of 0.35 % (w/v) AA and 0.18 % (w/v) propionic acid aqueous solution and the temperature was raised to 90 °C. When the solution became homogeneous and clear, a predetermined amount of  $K_2S_2O_8$  was added under nitrogen atmosphere to initiate the polymerization of AA monomers. The reaction was allowed to proceed until opalescent suspension occurred. The resulting suspension was cooled at room temperature, filtered and dialyzed against water (pH = 3.0) for 24 h using a dialysis bag with a cutoff molecular weight of 12 kDa to remove residual molecules.

**Crosslinking of the Casein-PAA Nanoparticles:** The casein-PAA nanoparticles were crosslinked by glutaraldehyde with magnetic stirring. Next, the crosslinked casein-PAA nanoparticles were dialyzed against water (about pH = 3.0) for 48 h using a dialysis bag with a cutoff molecular weight of 12 kDa to remove glutaraldehyde and the other small molecules.

**Preparation of Casein Nanoparticles:** To get pure-protein spheres, the suspension of the crosslinked casein-PAA spheres was adjusted to pH 7 around and dialyzed against water (about pH = 7) using a dialysis bag with a cutoff molecular weight of 100 kDa to remove PAA.

**Preparation of Rhodamine-Labeled Casein Spheres:** Rhodamine B isothiocyanate was added into the suspension of full casein nanospheres and the dark reaction between the isothiocyanate groups and amido groups of the casein nanospheres took place under stirring for 24 h so that the rhodamine chemically bonded to the casein nanosphere. Next, the resulting suspension was dialyzed against water (about pH = 7.4) using a dialysis bag with a cutoff molecular weight of 12 kDa to remove free rhodamine. The last outer solution of the dialysis bag was measured by UV-Vis absorption spectroscopy to affirm that the free rhodamine had been ridden completely. Meanwhile, the rhodamine-labeled casein monomers were obtained in the same manner.

**Characterization of Size and Zeta Potential:** The average hydrodynamic diameter and size distribution of casein-based nanoparticles were measured by DLS using a Brookhaven BI9000AT system (Brookhaven Instruments Corporation, USA). The measurements were repeated twice and the results were the average of three runs. Zeta potential of the samples was obtained with Zetaplus (Brookhaven Instruments Corporation, USA). The measurements were repeated thrice and the results were the average of three runs.

**FT-IR Measurement:** The obtained dried samples were mixed with KBr powder and pressed to a plate for FT-IR measurement on a vacuum FT-IR Spectrometer (Bruker VERTEX80V, Germany).

**Morphological Characterization:** The morphologies of the spheres were investigated by TEM (JEOL TEM-100, Japan) and SEM (HITACHI S-4800, Japan), where an Au coating was used to enhance scattering contrast and electric conductivity. To study the interior structure of the spheres, a few granules of the lyophilized sample were embedded in epoxy resin and sections with about 70 nm thick were obtained.

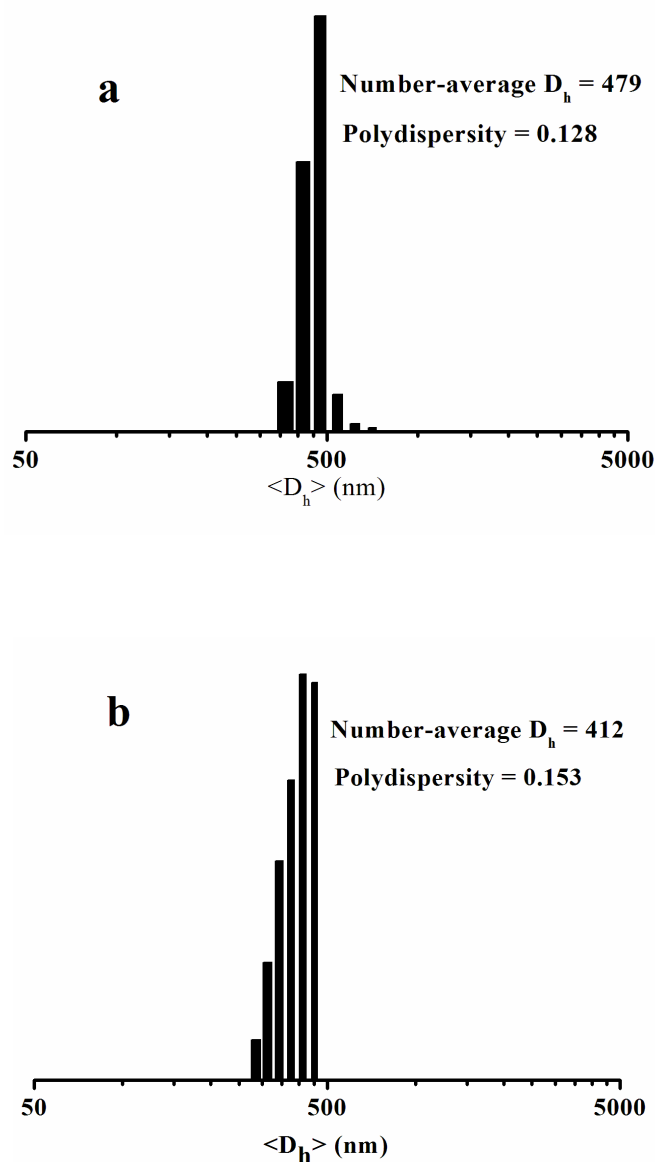
**Drug Loading and Release:** Drug loading was performed by adding CDDP into the casein nanospheres' suspension. Finally, CDDP-loaded casein nanospheres were separated from the aqueous phase by ultracentrifugation under 400000 g for 3 h. In vitro release of CDDP from casein nanospheres in phosphate buffered saline (pH = 7.4) at 37 °C was

evaluated by the dialysis method. The release medium was exchanged completely at defined time periods and CDDP concentration in the sampled medium was measured by an ICP-MS device (PerkinElmer ELAN9000, USA) with a calibration curve.

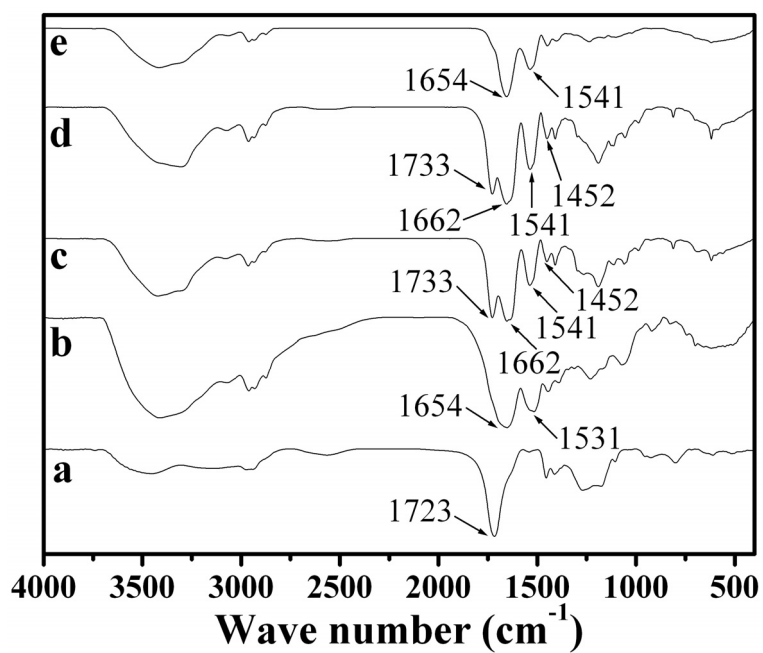
**In Vitro Cytotoxicity:** Cell viabilities of empty casein spheres, free CDDP and CDDP-loaded casein spheres on HeLa cell lines were determined by MTT assays. HeLa cells (8000 cells/well) were cultured in DMEM (Dulbecco's modified Eagle essential medium) supplemented with 10 % fetal bovine serum in a 96-well plate. Then, the cells were exposed to empty casein spheres, free CDDP and CDDP-loaded casein spheres for 48 h, respectively. After incubation, MTT solution was added to each well and cell viability was measured by the formed formazan absorbance at 570 nm.

**Confocal Laser Scanning Microscopy:** To analyze the cellular uptake of the crosslinked casein spheres by CLSM, HeLa cells were seeded at a density of  $1 \times 10^6$  cells/well in a 6-well plate containing a cover glass and allowed to adhere for 24 h in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C. The cells were equilibrated at 4 or 37 °C for 30 min prior to replacement of the medium with fresh temperature-equilibrated complete medium containing rhodamine-labeled casein spheres. After incubation for 3 h, the cover glass containing adherent HeLa cells was taken out, washed thrice with fresh temperature-equilibrated medium and fixed through inversely putting the cover glass onto the glass slide. Cells were observed by a CLSM device (Zeiss LSM 710, Germany) at an excitation wavelength of 543 nm.

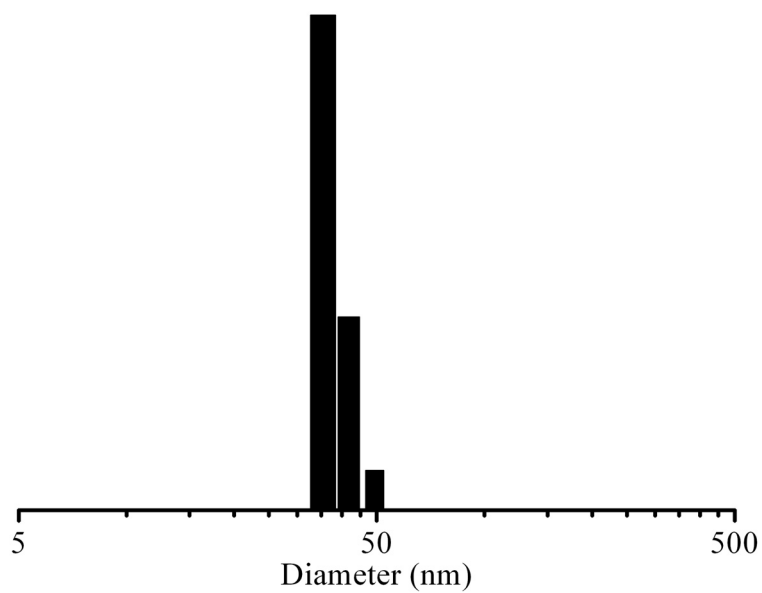
**Flow Cytometry:** The cells to be analyzed by flow cytometry were cultured and treated as described above except no cover glass in the well. Following 3 h incubation, the cells were washed thrice with fresh ice-cold PBS, incubated with 0.25% trypsin and then placed as a suspension into centrifuge tubes. After centrifugation, the resulting cells were washed twice in ice-cold PBS, resuspended in 500 µL of ice-cold PBS, and then immediately quantified by flow cytometry (Becton Dickinson FACSCalibur, USA). To provide statistical significance, 10,000 events were collected for each cell sample. In case of adding endocytosis inhibitors, HeLa cells were preincubated for 30 min in the presence of inhibitors (namely, sodium azide (0.05% wt), nocodazole (10 µM), filipin (10 µM), nystatin (10 µM) and cytochalasin D (3 µM)), the cells were then incubated with casein spheres in the presence of inhibitors for 3 h at 37°C.



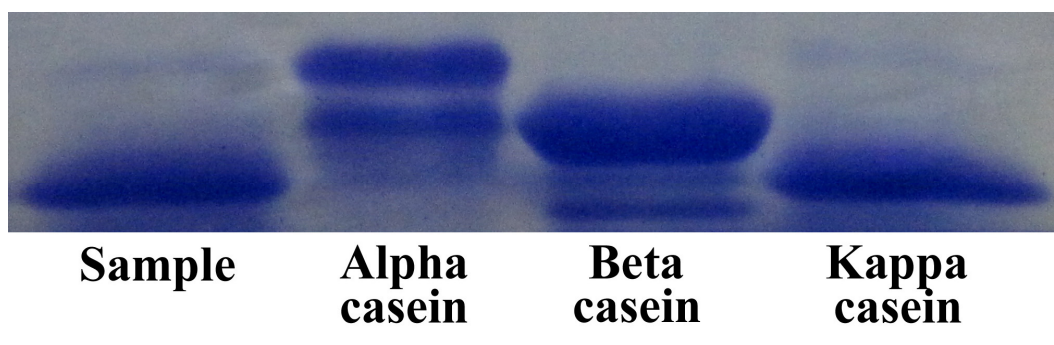
**Figure S1.** The size distribution of hollow full casein nanospheres at pH = 7.4 stored for 0 day (a) and 98 days (b). Compared with the size at pH = 3, the diameter of casein spheres at physiologic environment increased due to the stronger electrostatic repulsion generated by the ionization of the carboxyl groups in the particles with increasing pH. The stability of casein spheres at physiologic environment is obvious with 3-month store.



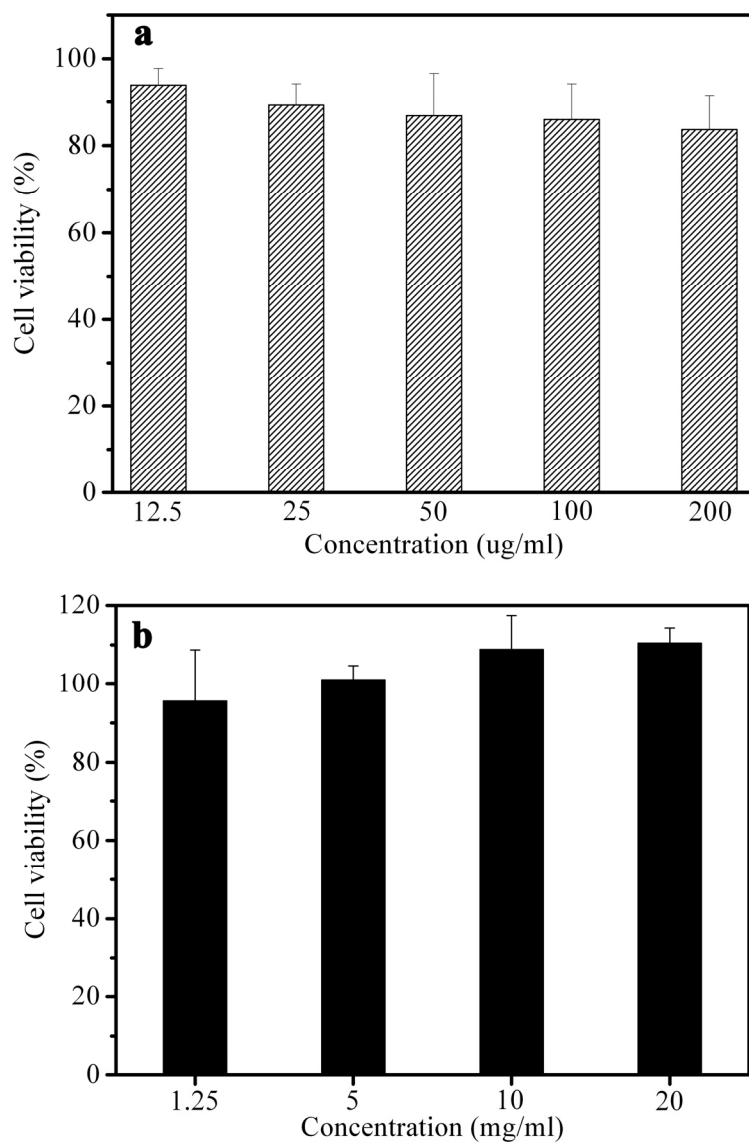
**Figure S2.** FT-IR spectra of (a) PAA, (b) casein, (c) casein-PAA spheres, (d) GA-crosslinked casein-PAA spheres, (e) GA-crosslinked casein spheres.



**Figure S3** The size distribution of casein micelles measured by DLS in the system before polymerization of AA monomer.



**Figure S4** SDS-PAGE pattern of free casein molecules separated by ultracentrifugation (MWCO: 100kDa) from the system before polymerization of AA monomers.



**Figure S5.** In vitro cytotoxicity of empty casein spheres in the concentration range from 12.5 ug/mL to 200 ug/mL (a) and 1.25 mg/mL to 20 mg/mL (b).