

*Supporting Information for*

## **Lumazine synthase protein cage nanoparticles as modular delivery platforms for targeted drug delivery**

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### **Experimental**

#### **Preparation and purification of RGD4C or SP94-AaLS cages**

Cyclic RGD4C peptide or SP94 peptide with extra sequences (GGGCDCRGDCFCASGGG or SFSIIHTPILPL) was genetically inserted into the residues between 70 and 71 or added to the C-terminal end of AaLS with six consecutive histidines (His-tags) at the N-terminal end by an established PCR protocol using pET-30b and pET-Duet based plasmids, respectively. Introductions of desired peptide sequences were confirmed by DNA sequencing and molecular mass measurement of subunit of RGD4C or SP94 peptide inserted AaLS. The amplified DNAs were transformed into the competent *E. coli* strain BL21 (DE3) and the genetically modified AaLS were over-expressed. The cells were harvested by centrifugation and subsequently resuspended in lysis buffer (50 mM sodium phosphate, 100 mM NaCl, and pH 6.5). The suspension was treated with lysozyme (50 µg/ml) and incubated for 1 h at room temperature. The solution was centrifuged to remove cell debris and the resulting supernatant was heated at 65°C for 10 min. The further purification of the genetically modified AaLS was achieved by using an immobilized metal affinity chromatography (IMAC). AaLS was initially bound to the Ni-NTA column and the bound AaLS was eluted by a linear gradient from 5 to 100% of imidazole (1 M). To remove excess imidazole, ultrafiltration was performed and the purified RGD4C- or SP94-AaLS were further characterized by size exclusion chromatography, mass spectrometry, and transmission electron microscopy.

#### **Chemical conjugation of imaging or therapeutic molecules to AaLS**

To chemically conjugate fluorescent dyes, protein cages (RGD4C- or SP94-AaLS) were incubated with either 5 mol equivalents of fluorescein-5-maleimide (F5M) or 10 mol equivalents of 5-(and 6-) carboxy-fluorescein-succinimidyl ester (NHS-fluorescein) at room temperature with vigorous shaking overnight. Free dyes were removed by either extensive dialysis or spin column. The extent of the

conjugation was determined by subunit mass measurements using ESI-TOF mass spectrometry (Xevo G2 TOF, Waters).

To chemically attach Aldoxorubicin (AIDox), RGD4C-AaLS were incubated with 5 mol equivalents of AIDox (INNO-206, CytRx) at room temperature with vigorous shaking overnight. Unreacted prodrug was removed by spin column protocol as manufacturer described (BIO-RAD). In the case of bortezomib (BTZ), firstly SP94-AaLS were incubated with 5 mol equivalents of synthesized N-(3,4-dihydroxyphenethyl)-3-maleimido-propanamide (Catechol-Mal) at room temperature with vigorous shaking overnight. Unreacted Catechol-Mal was removed by extensive dialysis. The extent of the conjugation was determined by subunit mass measurements using ESI-TOF mass spectrometry. Then, the Catechol-conjugated SP94-AaLS (Catechol-SP94-AaLS) were incubated with 5 mol equivalents of BTZ at room temperature at pH 9.0 with vigorous shaking overnight. Free BTZ was removed by using spin column.

### **Cell culture and fluorescence microscopy**

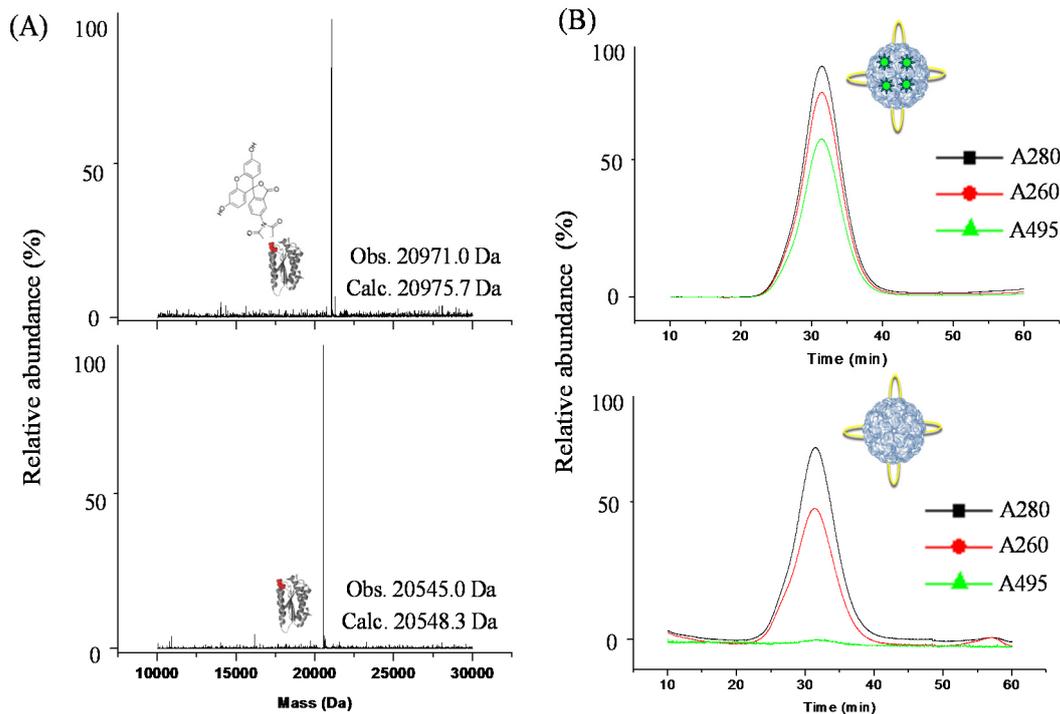
KB cells and HepG2 cells were obtained from the Korean cell line bank (KCLB). The KB cells were cultured in RPMI1640 medium with L-glutamine (300 mg/L), 10% fetal bovine serum (FBS, Invitrogen Corp., Carlsbad, CA), 25 mM HEPES and 25 mM NaHCO<sub>3</sub>. HepG2 cells were grown in RPMI 1640 medium with L-glutamine (300 mg/L) supplemented with 10% (v/v) FBS and antibiotics (100 µg/mL penicillin and 50 µg/mL streptomycin). They were maintained at 37°C under 5% CO<sub>2</sub>.

To prepare samples for the fluorescence microscopy, KB cells and HepG2 cells (1 10<sup>5</sup>/well) were grown in 12-well microscopy chambers (Ibidi GmbH, Martinsried, Germany). After the cells were settled for 24 h, they were washed with PBS containing 0.1% Tween 20, and fixed with 4% paraformaldehyde at 4°C for 20 min, and again washed with PBS. The cells were incubated with 6-diamidino-2-phenylindole (DAPI, Sigma). Fluorescence images were obtained using a FV1000 microscope (Olympus, UOBC).

### **MTT assay**

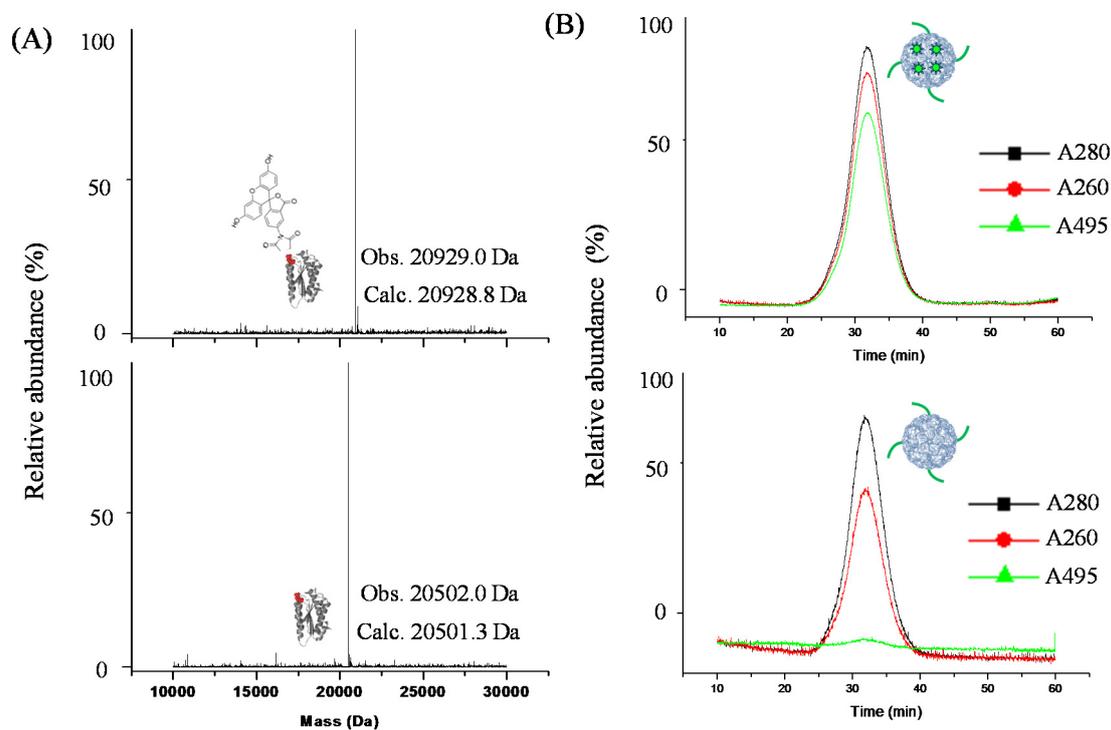
The cytotoxicity of AIDox-RGD4C-AaLS, BTZ-SP94-AaLS and free drugs was evaluated using the MTT cell viability assay with KB cells and HepG2 cells. The cells were seeded into 96-well plates and settled for 24 h. The cells were treated for 3 h with 100 µl of fresh medium containing different drug concentrations and then washed the cells with 200 µl of fresh medium and further incubated for 24 h at 37°C under 5% CO<sub>2</sub>. Subsequently, the cells were incubated in 200 µL of medium containing 0.5 mg/ml of MTT for 4 h and the medium was replaced by 200 µL of dimethylsulfoxide (DMSO) to dissolve the purple formazan crystals formed by viable cells. Absorbance was measured at 595 nm using a multiscanner (Infinite 200 PRO, TECAN). The cytotoxicity of RGD4C-AaLS and SP94-AaLS were also tested in parallel.

### Supporting Fig. S1



**Fig. S1.** Characterization of F5M-conjugated RGD4C-AaLS. (A) Molecular mass measurements of dissociated subunits of RGD4C-AaLS (bottom) and F5M-conjugated RGD4C-AaLS (top). Calculated and observed molecular masses were indicated. (B) Size exclusion elution profiles of RGD4C-AaLS (bottom) and F5M-conjugated RGD4C-AaLS (top). The samples were loaded onto a 10 x 300 mm superose 6 size exclusion column and eluted at a rate of 0.5 ml/min.

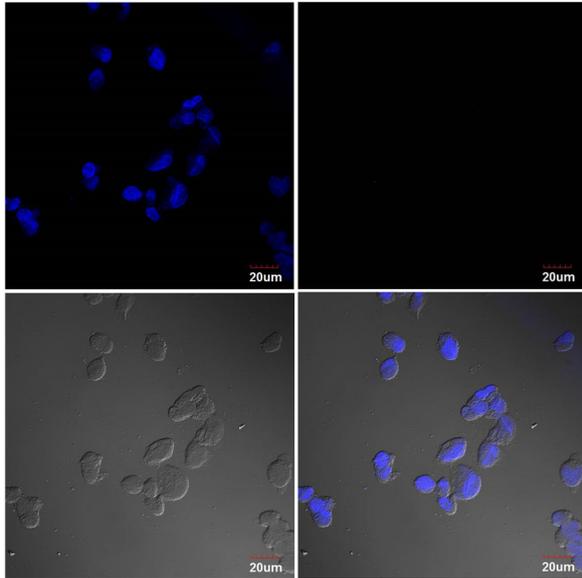
Supporting Fig. 2



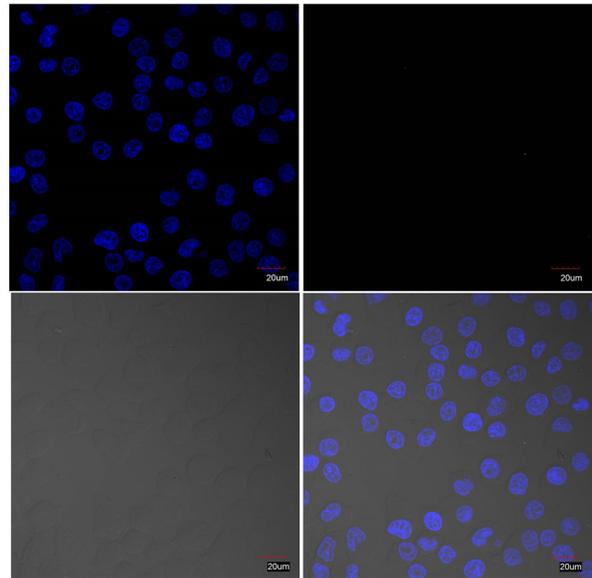
**Fig. S2** Characterization of F5M-conjugated SP94-AaLS. (A) Molecular mass measurements of dissociated subunits of SP94-AaLS (bottom) and F5M-conjugated SP94-AaLS (top). Calculated and observed molecular masses were indicated. (B) Size exclusion elution profiles of SP94-AaLS (bottom) and F5M-conjugated SP94-AaLS (top). The samples were loaded onto a 10 x 300 mm superose 6 size exclusion column and eluted at a rate of 0.5 ml/min.

**Supporting Fig. S3**

**(A)**

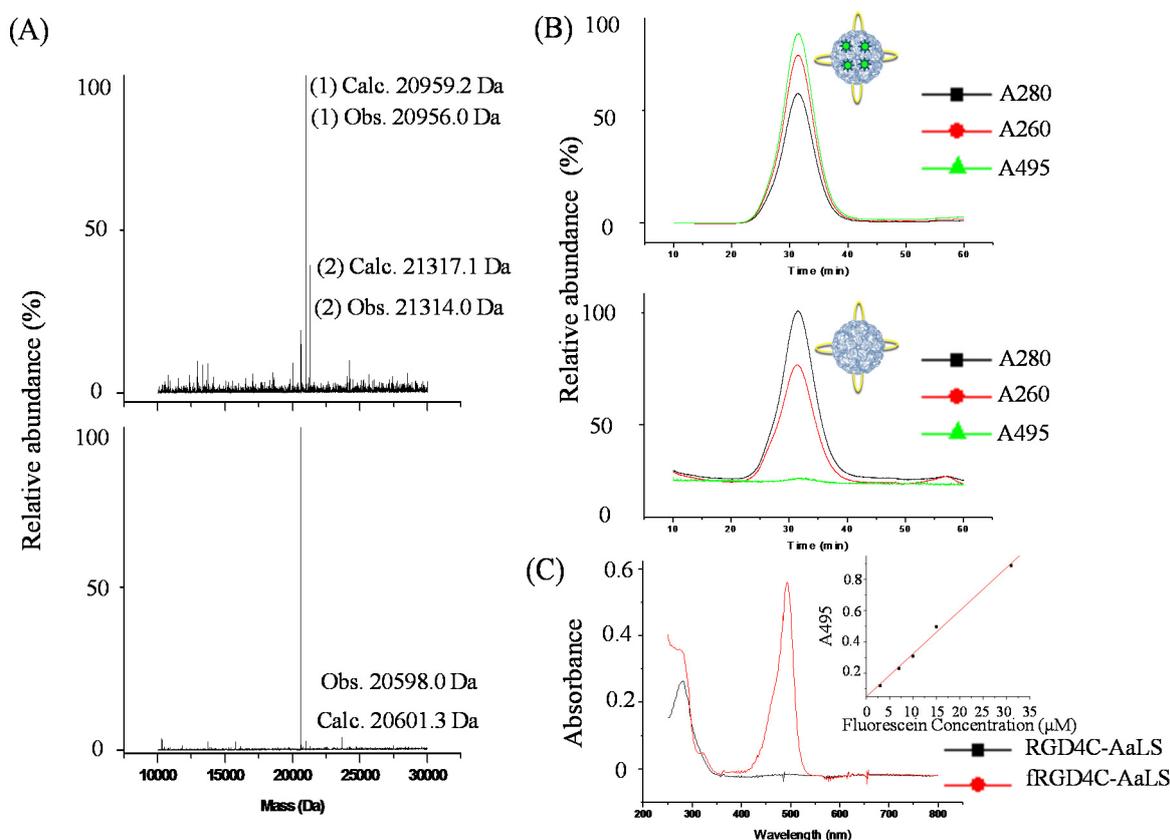


**(B)**



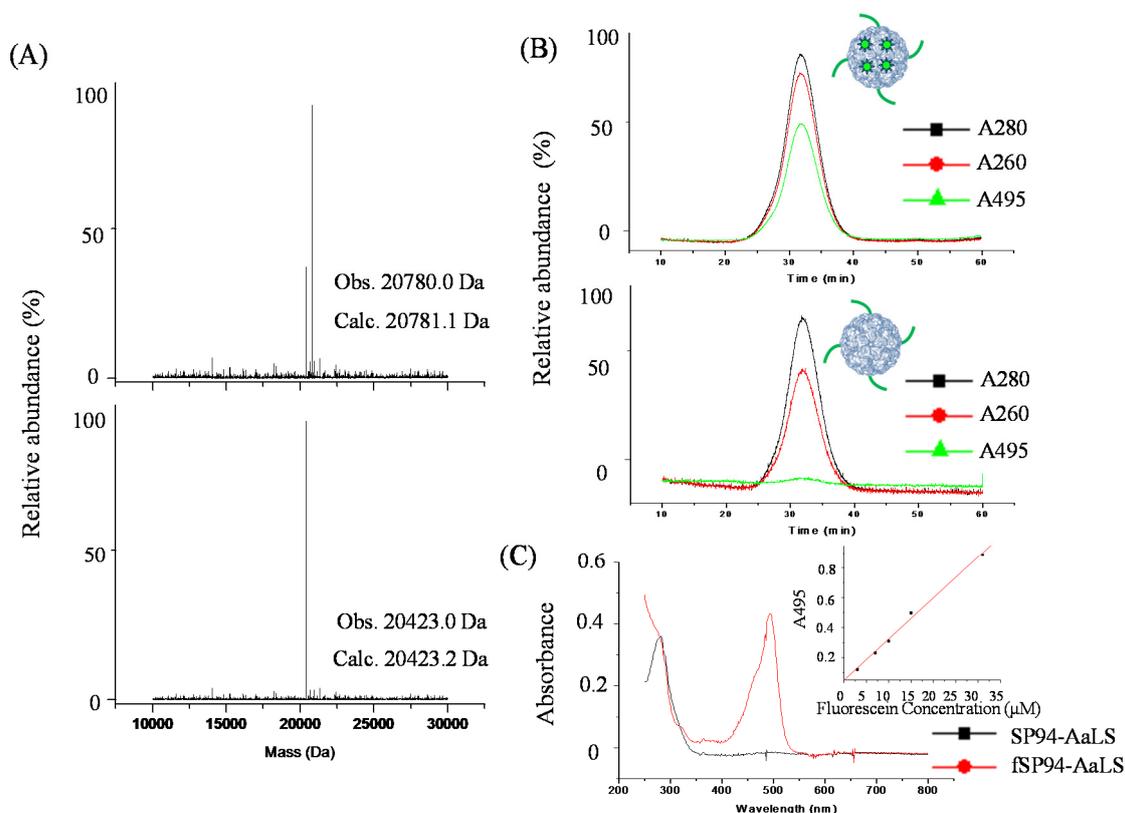
**Fig. S3** Fluorescent microscopic images of HepG2 cells treated with fRDG4C-AaLS (A) and KB cells treated with fSP94-AaLS (B). DAPI (top-left), fluorescein (top-right), DIC (bottom-left) and merged (bottom-right) images were presented. The nuclei were visualized as blue.

## Supporting Fig. S4



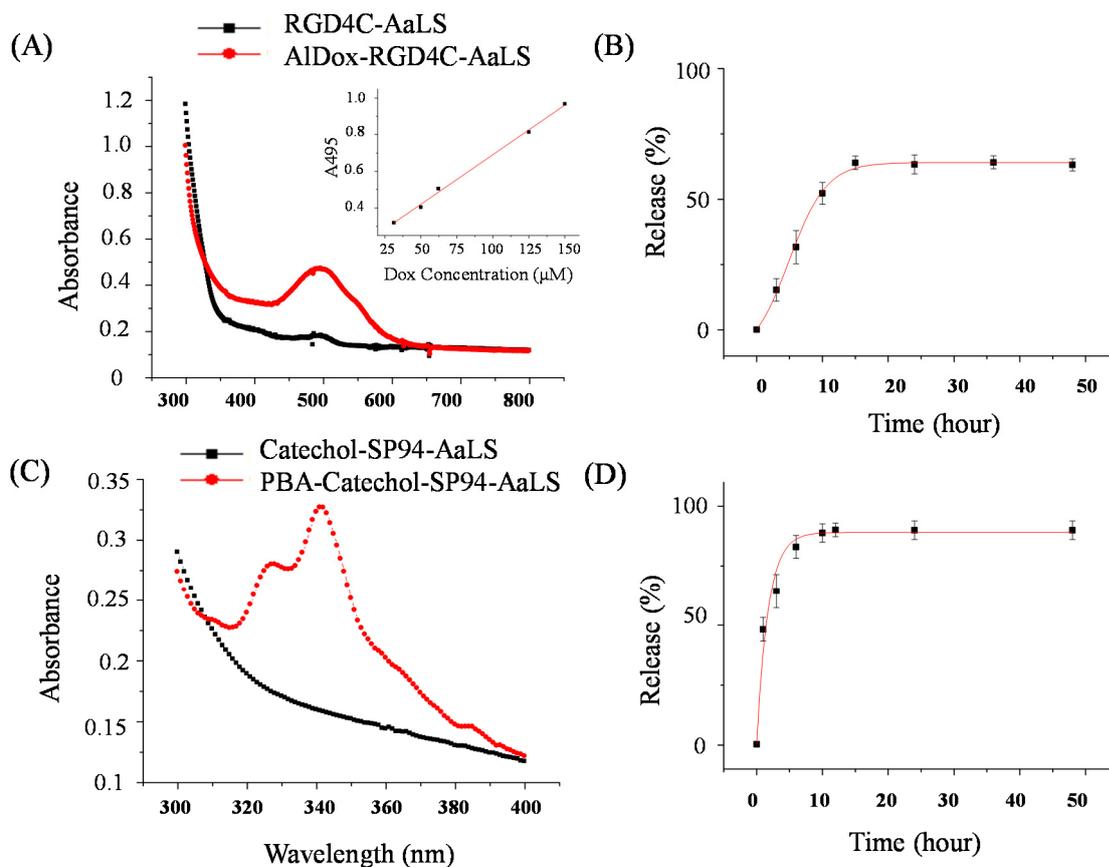
**Fig. S4** Characterization of fRGD4C-AaLS. (A) Molecular mass measurements of dissociated subunits of RGD4C-AaLS (bottom) and fRGD4C-AaLS (top). Calculated and observed molecular masses were indicated. RGD4C-AaLS were mainly labeled with one fluorescein molecule and approximately 30% of subunits were labeled with two fluorescein molecules. (B) Size exclusion elution profiles of RGD4C-AaLS (bottom) and fRGD4C-AaLS (top). The samples were loaded onto a 10 x 300 mm superose 6 size exclusion column and eluted at a rate of 0.5 ml/min. The SEC profiles of the fluorescein-conjugated RGD4C-AaLS (fRGD4C-AaLS) complexes were identical to those of RGD4C -AaLS, with the exception of the strong absorbance at 495 nm due to the fluorescein conjugation, indicating that the fluorescein labeling does not cause significant aggregation or disassembly of AaLS. (C) UV/Vis absorption spectra of RGD4C-AaLS (black) and fRGD4C-AaLS (red). A standard absorption curve of Aldox was plotted (inset). UV/Vis spectroscopic measurements showed that 80 fluoresceins were attached to RGD4C-AaLS.

## Supporting Fig. S5



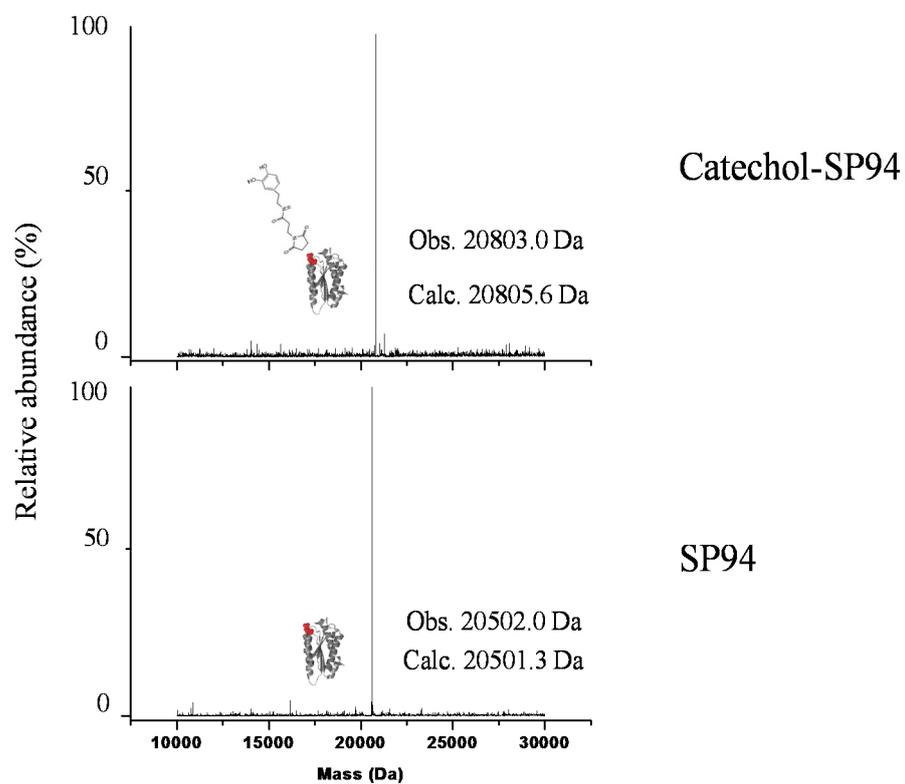
**Fig. S5** Characterization of fSP94-AaLS. (A) Molecular mass measurements of dissociated subunits of SP94-AaLS (bottom) and fSP94-AaLS (top). Calculated and observed molecular masses were indicated. Approximately 70% of the SP94-AaLS subunits were labeled with only one fluorescein molecule and the other subunits were not labeled. (B) Size exclusion elution profiles of SP94-AaLS (bottom) and fSP94-AaLS (top). The samples were loaded onto a 10 x 300 mm superose 6 size exclusion column and eluted at a rate of 0.5 ml/min. The SEC profiles of the fluorescein-conjugated SP94-AaLS (fSP94-AaLS) complexes were identical to those of SP94-AaLS, with the exception of the strong absorbance at 495 nm due to the fluorescein conjugation, indicating that the fluorescein labeling does not cause significant aggregation or disassembly of AaLS. (C) UV/Vis absorption spectra of SP94-AaLS (black) and fSP94-AaLS (red). A standard absorption curve of AIDox was plotted (inset). UV/Vis spectroscopic measurements showed that 44 fluoresceins were attached to SP94-AaLS.

Supporting Fig. S6



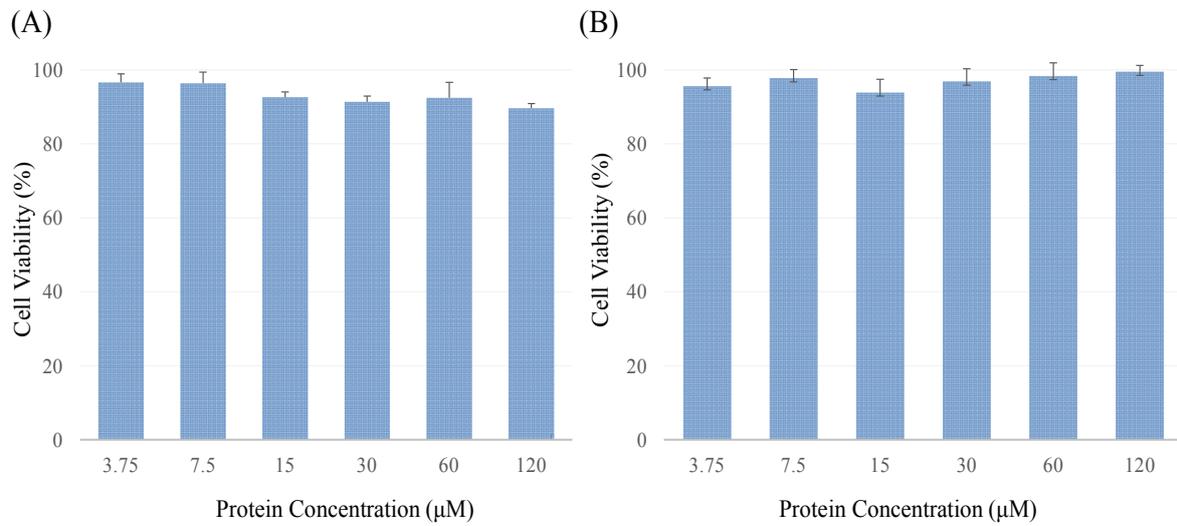
**Fig. S6** *In vitro* binding and releasing assay of drug molecules. The amounts of AIDox per protein subunit were quantify by measuring the absorbances of AIDox-conjugated RGD4C-AaLS (AIDox-RGD4C-AaLS) at 495 nm and comparing the absorbance to that of a standard curve of free AIDox. (A) UV/Vis absorption spectra of AIDox-RGD4C-AaLS (red, circles) and RGD4C-AaLS only (black, squares) at pH 9.0. A standard absorption curve of AIDox was plotted (inset). (B) Time-dependent release profile of Dox from AIDox-RGD4C-AaLS at pH 5.5. (C) UV/Vis absorption spectra of PBA treated Catechol-SP94-AaLS cage (red, circles) and Catechol-SP94-AaLS only (black, squares) at pH 9.0. (D) Time-dependent release profile of PBA from PBA-Catechol-SP94-AaLS at pH 5.5.

Supporting Fig. S7



**Fig. S7** Molecular mass measurements of dissociated subunits of SP94-AaLS (bottom) and Catechol-SP94-AaLS (top). Calculated and observed molecular masses were indicated.

**Supporting Fig. S8**



**Fig. S8** Cell viability assay of RGD4C-AaLS itself toward KB cells (A) and SP94-AaLS itself toward HepG2 cells (B) after 48 hour of incubation time. These two protein cages did not show significant effect on the cell viability. All results are expressed as means +SE (Standard Error) of five individual experiments.