Supporting Information

Cytomembrane-mimicking nanocarriers with a scaffold consisting of a CD44-targeted endogenous component for effective asparaginase supramolecule delivery

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Methods

1. Preparation of HA-g-PEG

General methods. NMR spectra were recorded with tetramethylsilane as the internal standard. ^{1}H NMR spectra were recorded at 600 MHz (Agilent, USA). Chemical shifts are reported in ppm downfield from $CDCl_3$ (δ = 7.26 ppm) for ^{1}H NMR spectroscopy. D_2O was used as solvent for HA and HA-g-PEG, and $CHCl_3$ for mPEG-glycine-Boc and mPEG-glycine. FT-IR was recorded at room temperature on a Thermo Scientific Nicolet iS50FT-IR spectrometer (USA) using KBr discs in the range of 400-4000 cm^{-1} region. Differential scanning calorimetry (DSC) and thermal gravimetric analysis (TGA) were performed on a simultaneous thermal analyzer STA 449C (NETZSCH, Germany) at the heating rate of 10 °C /min under N_2 atmosphere in the temperature range of 25-500 °C.

Synthesis of mPEG-glycine-Boc. Dry mPEG (5.0 g, 2.5 mmol) and *N*-Boc-glycine (0.47 g, 2.7 mmol) was dissolved in 30 mL dichloromethane (DCM), after which 4-(dimethylamino) pyridine (DMAP, 0.09 g, 0.75 mmol) and dicyclohexylcarbodiimide (DCC, 0.62 g, 3.0 mmol) were added in portions at 0 °C. The reaction mixture was then stirred at 0 °C for 24 h. After completion, the precipitated white solid was removed by filtration. The filtrate was then evaporated under vacuum. The obtained residue was purified by recrystallization using acetone. The crystallized whited solid was removed by filtration and the filtrate was evaporated and dried to provide mPEG-glycine-Boc as pale yellow solid, 2.23 g, 41.1% yield.

Synthesis of mPEG-glycine. mPEG-glycine-Boc (0.5 g, 0.25 mmol) was dissolved in DCM/trifluoroacetic acid (TFA, 1:1) and stirred for 2-3 h. After completion, most of the solvents were removed by evaporation under vacuum. The oily residue was then dissolved in 10 mL NaCl (15%). Adjust the pH of the solution to ~5.0 using 1.0 M NaOH. The insoluble white solid was filtrated and the filtrate was then extracted using $CHCl_3$ (10 mL X 3 times). The organic layer was combined, dried over Na_2SO_4, and evaporated under vacuum. The residue was dried over P_2O_5 to provide mPEG-glycine as pale yellow oil, 0.27 g, 60.3% yield.

Synthesis of HA-g-PEG. *N*-hydroxysuccinimide (NHS, 14.386 mg, 0.125 mmol) and 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide (EDC, 47.925 mg, 0.25 mmol) were dissolved in 5 mL hyaluronic acid (HA) aqueous solution (2%, w/v) and stirred for 1 h to form a homogeneous mixture. After that 0.859 mL mPEG-glycine aqueous solution (2%, w/v) was added, stirred under room temperature for 12 h. The reaction mixture was purified by dialysis in water for 72 h (dialysis bag: MWCO 8000-14000). The solution was then evaporated under vacuum and the obtained solid was dried, washed by acetone and CHCl3 (5 mL, 3 times each). Dry under vacuum to provide the product as pale yellow solid, 0.09 g, 75.9% yield.

2. Preparation of Asp-Cy5.5 and A-S-CmN-Cy5.5

Asp-Cy5.5 was prepared by stirring method, in short, 16 μL of dimethylformamide containing 0.670 μmol Cy5.5-NHS was mixed with 64 μL of Tris-HCL buffer (50 mmol/L, pH 7.3) consisting of 0.017 μmol Asp. The mixture was allowed to stir magnetically at room temperature in the dark for 24 h. The final mixture was dialyzed against deionized water for 72 h, and then freeze-dried to obtain Asp-Cy5.5.

A-S-CmN-Cy5.5 was prepared according to the preparation method of A-S-CmN (Asp was replaced by Asp-Cy5.5).

3. H & E and TUNEL staining

To dewax the paraffined sections to water, the sections were sequentially placed into dewaxing solution (Wuhan servicebio technology G1128, China) (10 min × 3) – anhydrous ethanol (5 min × 2) – water in turn. Then staining was performed using H & E staining kit (Wuhan servicebio technology G1076, China) and TUNEL staining kit (Wuhan servicebio technology G1507, China), respectively, following the manufacturer's protocols.

In detail, for TUNEL, the dewaxed slice was added with protease K solution (Wuhan servicebio technology G1205, China) and incubated for 25 min at 37 °C to recover the tissue. After washing in PBS (pH = 7.4, 5 min × 3) and slightly dried, the slice was balanced in buffer, then stained in a mixed solution of terminal deoxynucleotidyl transferase (TdT, reagent 1) and deoxyuridine triphosphate (dUTP, reagent 2) (1: 2: buffer = 1: 5: 50) at 37 °C. After washing in PBS (pH = 7.4, 5 min × 3), the slice was placed in 3% hydrogen peroxide solution at room temperature away from light and incubate for 15 min and washed in PBS (pH = 7.4, 5 min × 3). Then the slice was placed into reagent 3 working liquid (3: TBST = 1: 200) at 37 °C for 30 min and washed in PBS (pH = 7.4, 5 min × 3) and slightly dried. Freshly prepared solution of diaminobenzidine (DAB, Wuhan servicebio technology G1212, China) was added for color development and the slice was observed under a microscope till the cell nucleus were stained brown when terminated with water. Hematoxylin solution (Wuhan servicebio technology G1004, China) was used to re-stain the nucleus for 3 min, and sequentially rinsed with hematoxylin differentiation solution (Wuhan servicebio technology G1039, China), hematoxylin return liquid (Wuhan servicebio technology G1040, China), then washed with water. The slice was dehydrated using 100% alcohol (5 min × 4), n-butanol (5 min) and soaked in xylene for 10 min for transparency, finally sealed with neutral gum and observed under a microscope.

For H & E, the dewaxed slice was first put into pretreatment solution for 1 min. Then the slice was stained in hematoxylin solution for 3-5 min and washed with water. The section was sequentially treated with differentiation solution and hematoxylin bluing solution, then washed with water and dehydrated in 95% ethanol for 1 min. After that the slice was stained with eosin dye for 15 s. The dehydration and transparency were applied sequentially using 100% alcohol (2 min × 3) - n-butanol (2 min × 2) - xylene (2 min × 2). Finally, the stained slice was sealed with neutral gum and observed under a microscope.

Table S1-S4

Table S1. Bioequivalence evaluation of A-S-CmN and free Asp.

Table S2. The fluorescence intensity at maximum wavelength when the mixture of Asp and BSA was set at different ratios.

Table S3. The fluorescence intensity at maximum wavelength when the mixture of B-A-S-CmN and BSA was set at different ratios.

Table S4. The fluorescence intensity at maximum wavelength when the mixture of A-S-CmN and BSA was set at different ratios.

Table S1. Bioequivalence evaluation of A-S-CmN and free Asp.

Parameter	90% confidential interval calculated	Bioequivalence standard	*P* value calculated	Bioequivalence
$AUC_{(0\sim48h)}$ (U/mL·h)	77.0%~78.5%	80~125%	-	No
$AUC_{(0\sim\infty)}$ (U/mL·h)	77.0%~78.5%	80~125%	-	No
C_{max} (U/mL·h)	94.4%~96.0%	70% ～143%	-	Yes
T_{max} (h)	-	> 0.05	0.001	No

In total: Free Asp was not equivalent to A-S-CmN, and A-S-CmN was better.

Table S2. The fluorescence intensity at maximum wavelength when the mixture of Asp and BSA was set at different ratios.

Asp			**Mixture of Asp and BSA**			**F_3[e]**
Concentration	**λ_{max}[a]**	**F_1[b]**	**Ratio (BSA:Asp)**	**λ_{max}[c]**	**F_2[d]**	
-	-	-	1:0 (BSA 0.5 μM)	343 nm	177.376	-
2 μM	328 nm	59.157	1:4	338 nm	243.221	236.533
3 μM	328 nm	87.720	1:6	335 nm	270.969	265.096
4 μM	319 nm	119.192	1:8	333 nm	289.929	296.568
5 μM	320 nm	147.455	1:10	333 nm	322.423	324.831
6 μM	319 nm	176.866	1:12	335 nm	352.706	354.242

[a]Fluorescence maximum wavelength of Asp

[b]Fluorescence intensity of Asp at maximum wavelength

[c]Fluorescence maximum wavelength of BSA or the mixture of Asp and BSA

[d]Fluorescence intensity of mixture of Asp and BSA at maximum wavelength

[e]Fluorescence intensity of Asp plus fluorescence intensity of BSA at maximum wavelength

Table S3. The fluorescence intensity at maximum wavelength when the mixture of B-A-S-CmN and BSA was set at different ratios.

B-A-S-CmN			Mixture of B-A-S-CmN and BSA			F_3[e]
Concentration	λ_{max}[a]	F_1[b]	**Ratio (BSA:B-A-S-CmN)**	λ_{max}[c]	F_2[d]	
-	-	-	1:0 (BSA 0.5 μM)	343 nm	177.376	-
2 μM	330 nm	12.089	1:4	343 nm	666.690	189.465
3 μM	330 nm	15.775	1:6	343 nm	626.148	193.151
4 μM	330 nm	13.244	1:8	343 nm	500.572	190.62
5 μM	330 nm	17.378	1:10	343 nm	592.572	194.754
6 μM	331 nm	15.102	1:12	343 nm	508.136	192.478

[a]Fluorescence maximum wavelength of B-A-S-CmN

[b]Fluorescence intensity of B-A-S-CmN at maximum wavelength

[c]Fluorescence maximum wavelength of BSA or the mixture of B-A-S-CmN and BSA

[d]Fluorescence intensity of mixture of B-A-S-CmN and BSA at maximum wavelength

[e]Fluorescence intensity of B-A-S-CmN plus fluorescence intensity of BSA at maximum wavelength

Table S4. The fluorescence intensity at maximum wavelength when the mixture of A-S-CmN and BSA was set at different ratios.

A-S-CmN			Mixture of A-S-CmN and BSA			F_3[e]
Concentration	λ_{max}[a]	F_1[b]	**Ratio (BSA: A-S-CmN)**	λ_{max}[c]	F_2[d]	
-	-	-	1:0 (BSA 0.5 μM)	343 nm	177.376	-
2 μM	329 nm	94.109	1:4	340 nm	771.152	271.485
3 μM	330 nm	105.828	1:6	338 nm	668.905	283.204
4 μM	329 nm	172.411	1:8	338 nm	831.375	349.787
5 μM	330 nm	167.34	1:10	335 nm	733.619	344.716
6 μM	328 nm	250.999	1:12	334 nm	960.857	428.375

[a]Fluorescence maximum wavelength of A-S-CmN

[b]Fluorescence intensity of A-S-CmN at maximum wavelength

[c]Fluorescence maximum wavelength of BSA or the mixture of A-S-CmN and BSA

[d]Fluorescence intensity of mixture of A-S-CmN and BSA at maximum wavelength

[e]Fluorescence intensity of A-S-CmN plus fluorescence intensity of BSA at maximum wavelength

Figure S1-S4

Figure S1. MALDI-TOF spectra of HA-g-PEG.
Figure S2. Size and Zeta potential of A-S-CmN.
Figure S3. Protein-protein docking analysis of interaction between Asp and BSA.
Figure S4. H&E and TUNEL staining of tumor tissues treated by Asp and A-S-CmN.

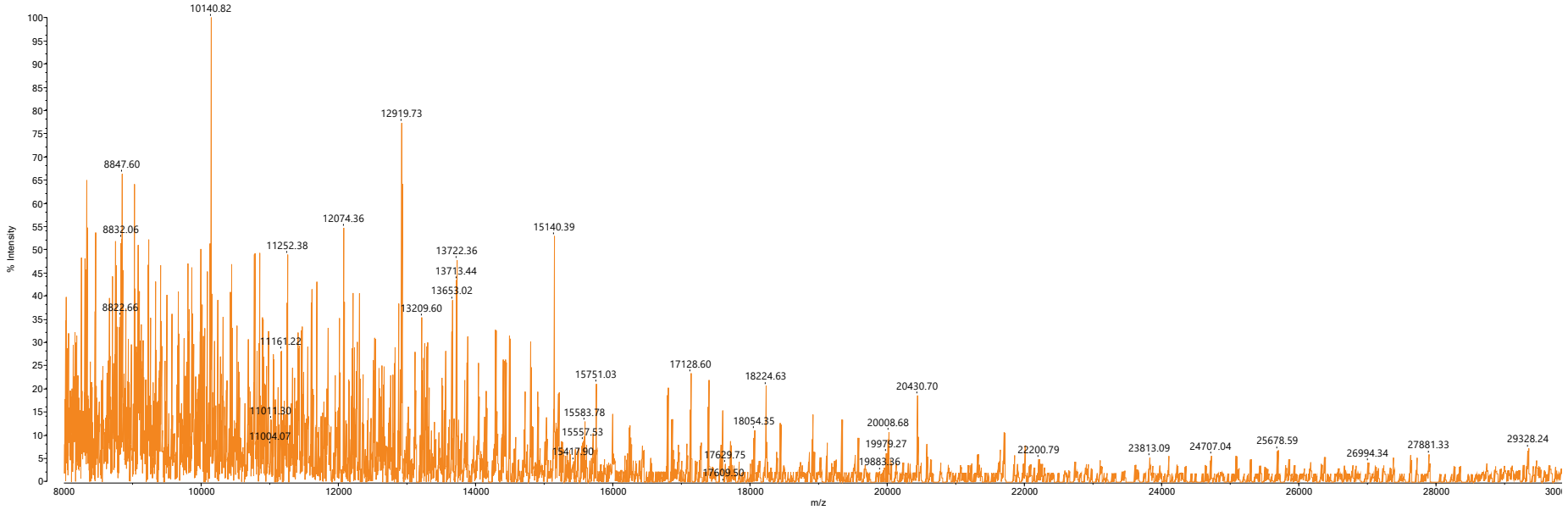

Figure S1. MALDI-TOF spectra of HA-g-PEG.

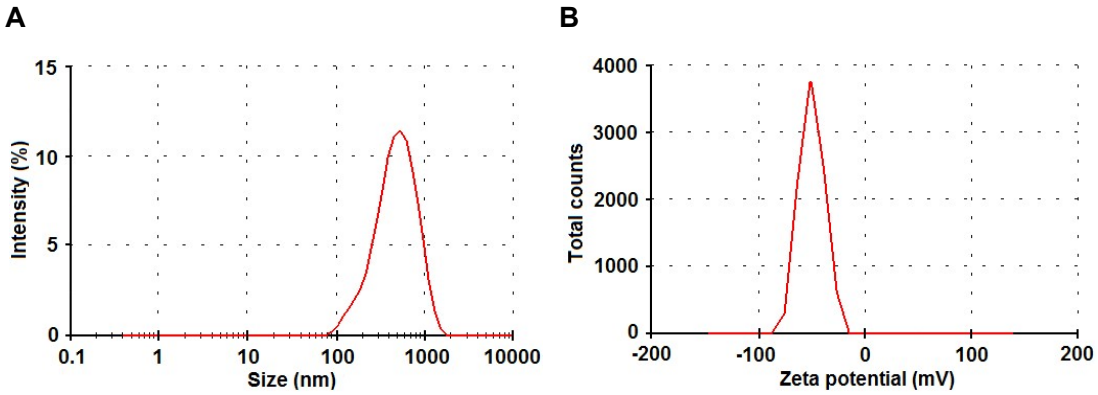

Figure S2. Size and Zeta potential of A-S-CmN. (A) Size distribution of A-S-CmN. (B) Zeta potential of A-S-CmN.

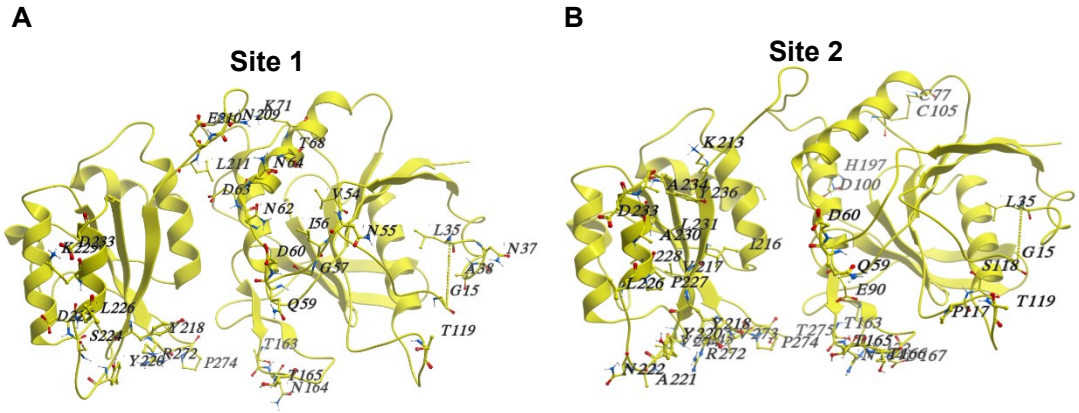

Figure S3. Protein-protein docking analysis of interaction between Asp and BSA. (A,B) Binging interface of Asp to BSA binding site (A) 1and (B) 2, residues at the interface are shown as the “CPK” illustration.

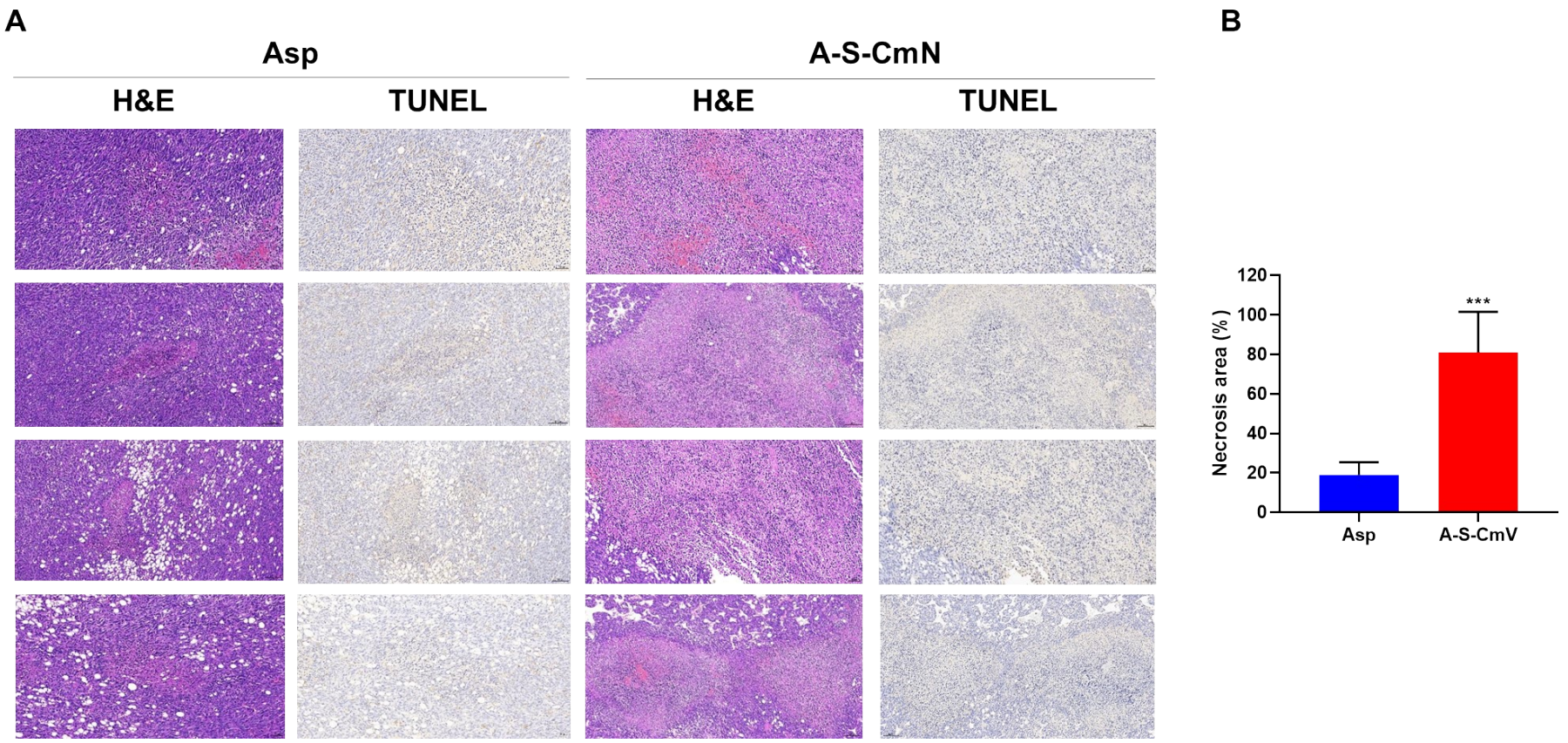

Figure S4. H&E and TUNEL staining of tumor tissues treated by Asp and A-S-CmN. (A) Representative figures of staining. (B) Quantitative analysis of the necrosis area (%) in the two groups. The area fraction was analyzed using ImageJ software. The analysis was performed in n = 4 scopes from 2 independent tumor samples. Statistical analysis: two-tailed t-test, ***p < 0.001.