Supporting information

Anti-Plasma Adhesion Peptides from One-Bead One-Compound Technique for Drug Delivery

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Materials and Methods

TentaGel Resin was purchased from Rapp Polymere (Germany, loading 0.31 mmol/g). Piperidine, 4-methylmorpholine (NMM), 8-Diazabicyclo[5.4.0]undec-7-ene(DBU), trifluoroacetic acid (TFA), Triisopropylsilane, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluoro phosphate (HBTU) were purchased from Macklin. N, N'-dimethylformamide (DMF), Dichloromethane (DCM) was from JING CHUN, Cyanogen bromide (CNBr) was from J&K Chemical (China). Fluorescein isothiocyanate (FITC) and triethylamine were purchased from Sigma-Aldrich Chemical Co. All Fmoc-amino acids and Wang resins were obtained from GL Biochem. NHS-Biotin, HRP, ELISA kit, Heparin sodium, and BCA Protein Assay Kit were purchased from Solarbio Science & Technology Co. CCK-8 was purchased from Beyotime Institute of Biotechnology, China. The HUVECs cells were from the cell culture center of the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing, China). Cell culture medium and fetal bovine serum were purchased from Wisent Inc. (Multicell, Wisent Inc., St. Bruno, Quebec, Canada). 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride(EDC), Goat anti-Mouse IgG Fc Secondary Antibody, HRP, Goat anti-Mouse IgM (heavy chain) Secondary Antibody, HRP were purchased from Thermo Fisher Scientific, Hemocyanin (KLH) was purchased from Shanghai yuan ye Bio-Technology Co., Ltd. Zeba spin desalting columns were from Pierce, mPEG-SH was from Shanghai Aladdin Biochemical Technology Co., Ltd. 2-Morpholinoethanesulphonic acid(MES), N-Hydroxy succinimide (NHS) were purchased from Shanghai Acmec Biochemical Co., Ltd. Maleimidoacetic acid N-*hydroxysuccinimide ester (AMAS), L-ASP were purchased from Med Chem Express, Mouse plasma was purchased from Guangzhou Hongquan Biological Technology Co., Ltd. BALB/c mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd.

The construction of OBOC library

In the research, the solid-phase peptide synthesis method was first used to construct a one-bead-one-compound AAPs library with a library capacity of 19^7. In the process of peptides library construction, each time the solid phase carrier TentaGelS resin was mixed and divided homogeneously in a cycle, and then different amino acids were added separately, and the process was repeated six times in total, and the peptide synthesis was carried out using anhydrous DMF and DCM as the main solutions. In the deprotection stage, the deprotection reagent was firstly prepared by mixing DBU and DMF in the volume of (DBU:DMF = 2:98); anhydrous piperazine with a mass fraction of 5.0% was weighed into the solution containing DBU and DMF and dissolved under ultrasonication. Subsequently, a deprotection reagent was added to remove the Fmoc protective groups from the TentaGel S resin, and the deprotection time was generally 15 min. In the coupling step, N-methylmorpholine and DMF were mixed according to the volume ratio of DMF: N-methylmorpholine (DMF: N-methylmorpholine =19:1); subsequently, according to the loading ratio of the resin and the weighed mass of the resin, a certain mass of amino acid and a 10-fold equivalent of HBTU were weighed and were mixed. After each deprotection and coupling step, a small amount of resin was dipped into a centrifuge tube using a capillary glass tube, and then ninhydrin, phenol, ascorbic acid, and other color-developing reagents were added sequentially, and the centrifuge tubes were placed in a water bath under boiling water for about one minute to determine the success of the deprotection and amino acid coupling process based on the color change. After completion, the tubes were rinsed three times alternately with DCM and DMF and then proceeded to the next cycle. After the last amino acid coupling was completed, the Fmoc protecting groups were removed by deprotection reagent, and the peptide beads were washed and drained under vacuum overnight. After the OBOC peptide library was prepared, the side-chain protective groups were removed by lysis reagent (95.0% TFA, 2.5.0% deionized water, 2.5.0% triisopropylsilane, v/v), and then used for the screening of anti-adhesion peptide beads.

Selection of anti-adhesion beads

A certain amount of fluorescein isothiocyanate (FITC) was weighed and dissolved in DMSO solution, followed by thorough mixing and storage in the dark. A certain volume of mouse plasma was taken up, diluted in a certain proportion with PBS solution, and stored in a serum bottle. Subsequently, a certain volume of FITC solution was aspirated into the serum glass bottle containing plasma, the pH was adjusted to about 9 by adding an appropriate amount of triethylamine, and the mixture was stirred and kept overnight at 4 °C in the dark. Subsequently, the plasma after the completion of fluorescence labeling was taken out for dialysis to remove the excess FITC, and the water needed to be changed per 1 h during the dialysis process for two days. A portion of peptide beads from the heptapeptide library established in the previous steps were randomly taken and placed into a petri dish, and incubated with the fluorescently labeled plasma at 4 °C overnight.

At the end of the co-incubation with plasma, the peptide beads in the Petri dish were washed 2-3 times with PBS solution to remove plasma that did not bind to the peptide beads with each other, and the PBS solution in the Petri dish was subsequently discarded. The petri dishes were placed under a fluorescent structured illumination microscope to select anti-adhesion polypeptide beads. Under the fluorescence structure illumination microscope, these peptide beads with lower fluorescence brightness were selected and aspirated out with a lance tip. These aspirated peptide beads were put into different centrifuge tubes one by one, and a certain volume of PBS solution was added, the peptide beads with higher brightness were selected as the control group for comparison.

Synthesis of AAPs

Peptides were synthesized through standard Fmoc-based solid-phase peptide synthesis using Wang resin. After completing the deprotection process for the Fmoc group of the last amino acid using a deprotection reagent after six cycles, the resin was washed three times with DCM and DMF, and then washed three times with methanol to shrink the resin. Subsequently, after evacuating the water from the resin using vacuum, the Wang resin containing the target peptide was transferred to a glass vial to which the lysis solution was subsequently added. The lysate and resin were stirred in an ice water bath for 2.5 h. The lysis solution and resin in the glass bottle were transferred to a syringe and separated through degreasing cotton. The lysis solution was then blown dry with nitrogen gas. After drying, ice-cold ether was used to precipitate the peptide. The ether solution containing peptides was transferred to a centrifuge tube and centrifuged at 8000 rpm for 3 min, and the supernatant was discarded and dried to obtain the peptide powder. The peptides were purified by reversed-phase high-performance liquid chromatography (HPLC). Peptides were determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF-MS, Brook Dalton, USA) and liquid chromatography-mass spectrometry (LC-MS 8050, Shimadzu, Japan).

Secondary screening of AAPs by ELISA

Although OBOC as a simple, fast, and efficient technical method to screen the target peptides, its screening results still have some false positive appearances. So ELISA, a classical method, was used to test the performance of the pre-screened AAPs. Firstly, the plasma and albumin were diluted to a specific concentration using coating buffer. 100 μL of the diluted solution was added to each well of the ELISA plate. The plate was then incubated overnight at 4 °C for coating. After that, the coating solution was removed, and the plate was washed three times with washing buffer. Then blocking buffer was added to each well (100 μL/well), and the plate was incubated at room temperature for two hours to block any unoccupied binding sites. For the treatment of peptides, ten peptides were weighed to a certain mass, and the peptides were labeled with biotin. Adding these labeled peptides into the wells of the enzyme plate prepared earlier, 100 μL/well, and incubating at 37 °C for 2 h. A certain mass of peptide was weighed and labeled with biotin. The labeled peptide was added in the wells of the prepared ELISA plate with 100 μL of peptide for each well. The plate was incubated at 37 °C for 2 h. After incubation, the plate was washed three times with buffer solution. After each wash, the plate was patted dry to remove any residual buffer from the wells. Then streptavidin-horseradish peroxidase (HRP) working solution, diluted to a ratio of 1:1000, was added to each well, with 100 μL per well. The plate was incubated at 37 °C for 30 min to allow the streptavidin-HRP to bind the biotin-labeled peptide and washed with buffer solution 3 times, after adding 100 μL/well of TMB, and incubating at 37 °C for 15 min, then adding 50 μL/well of H_2SO_4 to terminate the reaction, and the ELISA plate was read within 5 min at wavelength of 450 nm to measure the absorbance. The ten peptides obtained so far were subjected to secondary screening to verify the reliability of the OBOC screening results and to judge the anti-adhesion effects of the ten peptides.

Stability of AAPs in plasma

The test was carried out by HPLC, a certain mass of peptide samples were weighed and prepared into a master batch of 1 mg/mL. Afterwards, the samples were sequentially diluted to the concentrations of 800, 600, 500, 400, 200, 100, 50, 20, and 10 μg/mL, and these samples at different concentrations were measured and standard curves were made. Subsequently, AAP1, AAP2, AAP3, AAP4, and AAP8 were dissolved into 1 mL of mouse plasma at a concentration of 1 mg/mL at 37 °C. On the time points of 0, 0.08, 0.16, 0.25, 0.33, 0.5, 1, 2, 4, 6, 8, 24, 48, and 72 h, 100 μL of plasma was taken out and then 40 μL of termination solution was added (50.0% TFA, 50.0% deionized water, v/v). The supernatant was collected after centrifugation and subsequently analyzed using high-performance liquid chromatography (HPLC), and the amount of anti-adhesion peptide in solution was calculated based on the standard curve produced.

Protein adsorption evaluation of AAPs by SPR

The modified High-affinity streptavidin (SA) sensor chip with biotinylated AAP4, AAP8, PEG was for SPR interaction analysis on Biacore systems. SPR analysis was performed on a Biacore8K spectrometer using PBST (pH 7.4, with 0.05.0% Tween 20) as the running buffer. All solutions for SPR measurement were freshly prepared and filtered through 0.22 μm filters prior to use. The flow rate for the whole experiment was kept at 30 μL/min. The baseline was first stabilized by running a PBST buffer. Then a solution of 10.0% blood plasma flowed over the modified chip for 6 min, after that by flushing the surface with PBST to remove the unabsorbed plasma. Protein adsorption on the surface flushed with PBST was used as control. The amount of adsorbed protein was calculated by the change in response unit (RU) before and after adsorption, 1 RU corresponds to a protein surface coverage of 1 pg/mm^2.

Modification of KLH by AAPs

KLH is a large protein molecule with high immunogenicity commonly used in the laboratory. The immunogenicity of the peptide itself was tested by ELISA after coupling KLH with a small molecule anti-adhesion peptide. Hemocyanin (KLH) was weighed and dissolved in PBS buffer. The peptide was dissolved in MES buffer. A quantity of EDC and NHS was added to the peptide solution and reacted for 15 min at room temperature, followed by the addition of 2-mercaptoethanol to quench the EDC, and the prepared KLH solution was added to the peptide solution and reacted at room temperature for 2 h. Finally, hydroxylamine hydrochloride was added to make the final concentration of KLH. Finally, a certain amount of hydroxylamine hydrochloride was added to a final concentration of 10 mM, and the excess hydroxylamine hydrochloride was removed by using a Zeba spin desalting chromatography column to obtain the peptide coupled with KLH. Thin-layer chromatography experiments were used for separating and characterizing samples. AMAS was used for coupling between KLH and PEG (molecular weight 2000). A certain mass of peptide was weighed, KLH and BSA were dissolved separately in 0.1 mM conjugate buffer (PBS buffer pH 6.0-7.5). AMAS was first dissolved in 1 mL of 0.1.0% DMSO (10 mM excess), and then a volume of cross-linker was added to dissolved KLH and BSA protein solutions, respectively, to reach a final concentration of 1 mM (10-fold molar excess). The reaction mixtures were incubated at room temperature for 30 min. The excess cross-linker was removed using a desalting chromatography column equilibrated with conjugate buffer. The mPEG-SH and KLH/BSA were conjugated and mixed according to the desired molar ratio of the final conjugate and according to the relative amounts of sulfhydryl groups and activated amines present on the two proteins. The reaction mixture was incubated for 30 min at room temperature and used for subsequent experiments.

Immunogenicity testing of AAP-modified KLH

BALB/c mice were used in this experiment. After the sample solution was drawn through a syringe, it was injected into the mice using intraperitoneal injection. Subsequently, the injection was given once on the 1st day of every week (Day 1, 8 and 15). On the day 28, Blood was collected and centrifuged to obtain the supernatant as serum, and then frozen at -20 °C for subsequent determination. Each well of a 96-well plate was coated with 100 μL of antigen solution (protein concentration of 10 μg/mL) prepared with 0.1M sodium carbonate buffer (pH 10.5). Plates were incubated overnight at 4 °C to achieve complete antigen encapsulation. After discarding the antigen solution, all wells were washed five times with PBS solution (pH 7.4) and injected with the blocking solution (1.0% skimmed milk solution, 0.1 M Tris buffer, pH 8.0). After incubation for 1 hour at room temperature, the blocking solution was removed, and all wells were washed five times with PBS. Serum samples were serially diluted in PBS containing 1.0% skimmed milk. After incubation at 37 °C for 1 h, all solutions were removed and the plate was washed five times with PBS. The HRP-conjugated secondary antibody (100 μL/well) was then added. The 96-well plate was left at 37 °C for 1 h to complete antibody binding. The plate was washed five times with PBS before the addition of TMB, 100 μL/well. The plate was incubated at 37 °C for 15 min and 50 μL of termination solution (2 M H2SO4) was added to each well. The absorbance at 450 nm and 570 nm was read by the microplate reader. To test anti-AAPs and anti-PEG antibodies, BSA-AAPs and BSA-PEG conjugates were used as antigens in the procedure. BSA-AAPs were prepared in the same way as KLH-AAPs as described above.

Modification of ASP by AAPs

L-Asparaginase (ASP) is a clinical intravenous drug that is usually used in the treatment of acute granulocytic leukemia. The solution was prepared by dissolving ASP in PBS buffer. A certain mass of EDC and NHS were weighed and added to 1 mL of peptide solution, and reacted at room temperature for 15 min. Subsequently, a certain molar concentration of 2-mercaptoethanol was added to quench EDC. ASP solution was added to the peptide solution and the two reacted at room temperature for 2 h. Finally, hydroxylamine hydrochloride was added, excess hydroxylamine hydrochloride was removed using zeba spin desalting chromatography column to obtain the peptide coupled with ASP. Repeating the above steps for ASP coupled with 1-3 layers of anti-adhesion peptide. A sample solution of the peptide coupling ASP-AAPs was pipetted into a cuvette and placed into the dynamic light scattering apparatus to test for changes in the particle size of the sample.

***In vitro* enzymatic activity testing of AAP-modified ASP**

The BCA and Cu reagents were dispensed at a 50:1 volume ratio and mixed thoroughly. Samples of one layer to five layers of AAP4 and one layer to five layers of AAP8 were added to a 96-well plate. Each well was added with 200 μL of BCA working solution and placed at 37 °C for 15-30 min. The 96-well plate was read at the wavelength of 562 nm, and the protein concentration was calculated from the standard curve. The ASP proportion was calculated with concentration of ASP and AAPs utilized for the preparation of the ASP-AAPs-n. The enzyme activity of ASP-AAPs was also tested with ASP activity assay kit, and *in vitro* enzyme activity of ASP and the percentage of ASP in the coupling were calculated in summary.

Cytotoxicity testing of AAP-modified ASP

Peptide-coupled drugs enter the organism through intravenous injection and first contact with endothelial cells in the blood vessels, so HUVECs was chosen as the experimental subjects to test the cytotoxicity of anti-adhesive peptides in this study. HUVECs was inoculated in Petri dishes and incubated with prepared DMEM medium. These cells were observed through the microscope and after the incubation was completed, they were washed twice with PBS solution and subsequently discarded.

Trypsin solution was added and the cells were incubated at 37 °C for 2 min with 5.0% CO_2 to dislodge the adherent cells. After removing the petri dish, the medium solution was added and the solution was blown and transferred to a centrifuge tube, and centrifuged at 1000 rpm for 2 min. After counting these cells, they were diluted to 10,000 cells/well with DMEM medium inoculated into 96-well plates and incubated for 24 h. Different concentrations of peptide solutions were added to the 96-well plates and incubated for 24 h. After incubation, the plates were washed twice with PBS solution, then CCK-8 (10.0%) solution was added to the 96-well plates and incubated for 4 h. The cytotoxicity of the cells was measured and calculated by using enzyme marker.

In vivo enzyme activity testing of AAP-modified ASP

To evaluate the enzyme activity of the AAP-modified ASP *in vivo* by using healthy mice, mice were injected with ASP and ASP-AAPs 200 μL via the tail vein on the first day of each week (days 1, 8, and 15). The concentration of ASP was consistent with ASP-AAPs and blood samples were collected after each injection at 5 min, 1, 4, 8, 24, 48, and 72 h into the anticoagulation tubes. The blood samples collected in the previous step were centrifuged at 1200×g for 10 min, and the supernatant was taken in a new centrifuge tube, followed by centrifugation again at 1300×g for 2 min, then the plasma was taken and then frozen at -20 °C for subsequent determination. The enzyme activity in the plasma of the samples was subsequently detected using an ASP activity assay kit.

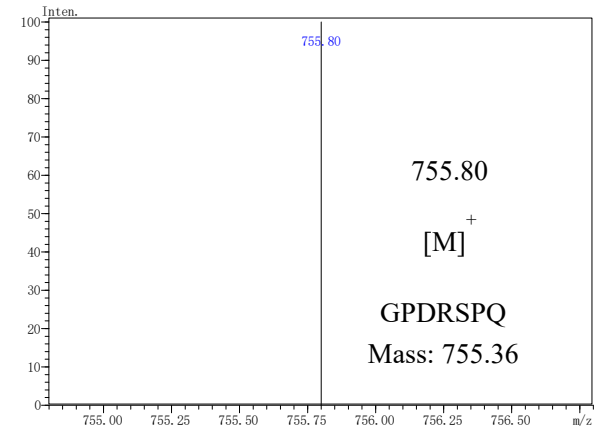
In vivo immunogenicity testing of AAP-modified ASP

On the 28th day after the first injection of the above experiment, blood samples were collected from above mice. The collected blood samples were placed at room temperature for 30 min, then centrifuged with 1800×g for 10 min, and the supernatant was taken into a new centrifuge tube (Non-anticoagulated centrifuge tube), then centrifuged again with 1300×g for 2 min, serum was taken and then frozen at -20 °C for the subsequent determination. The immunogenicity of ASP and ASP-AAPs was determined by indirect ELISA with the detection of IgG and IgM antibody titers. Absorbance was determined by using the enzyme marker spectraMAX M2.

Biosafety evaluation of ASP-AAPs

The safety of ASP-AAP4-1, ASP-AAP4-3 and ASP-AAP8-1, ASP-AAP8-3 on tissues and organs was detected by hematoxylin-eosin (H&E) staining. On the 28th day after the first injection of ASP-AAP4-1, ASP-AAP4-3, and ASP-AAP8-1, ASP-AAP8-3 solution (200 μL, 200 U/kg), mice were dissected and livers, spleens, and pancreas were collected. These tissues were fixed using 4.0% paraformaldehyde PBS buffer, and subsequently embedded in paraffin for H&E staining.

a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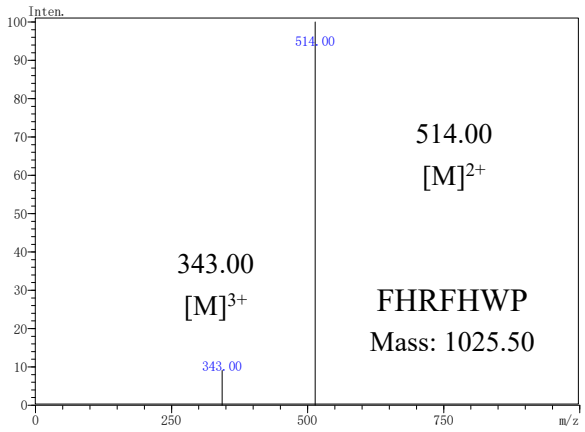
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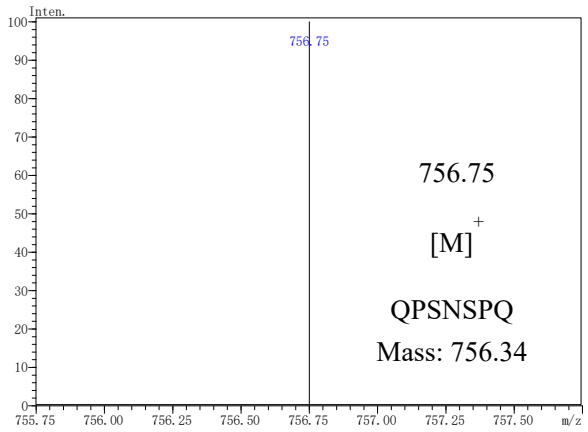
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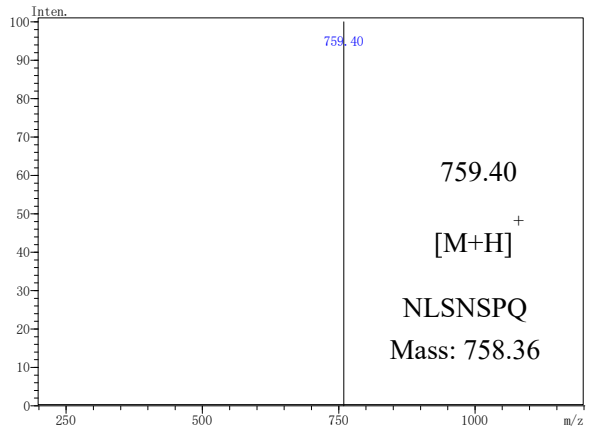
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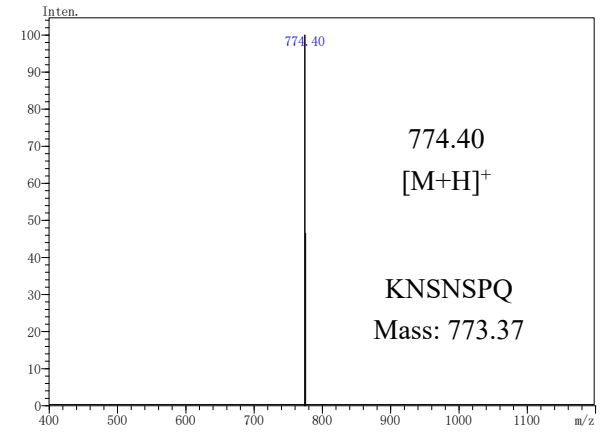
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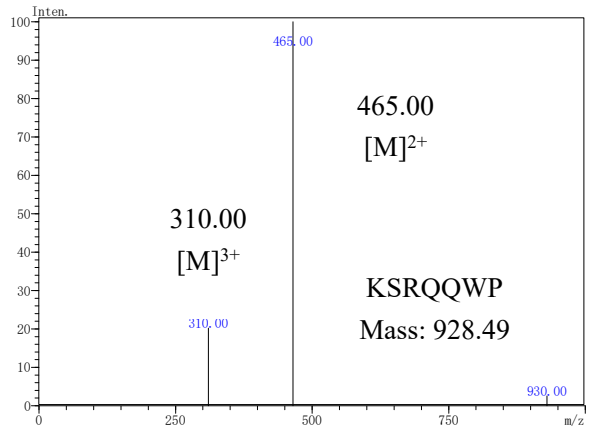
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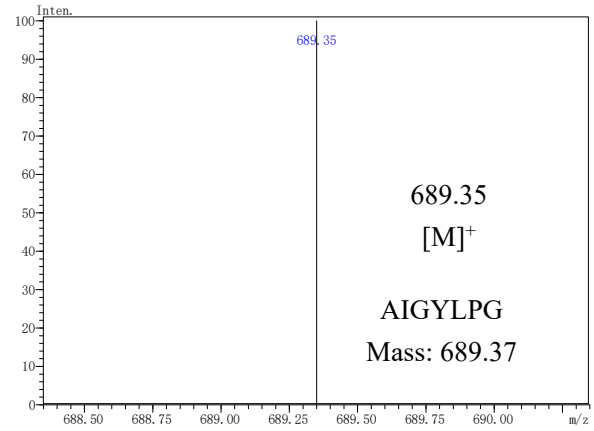
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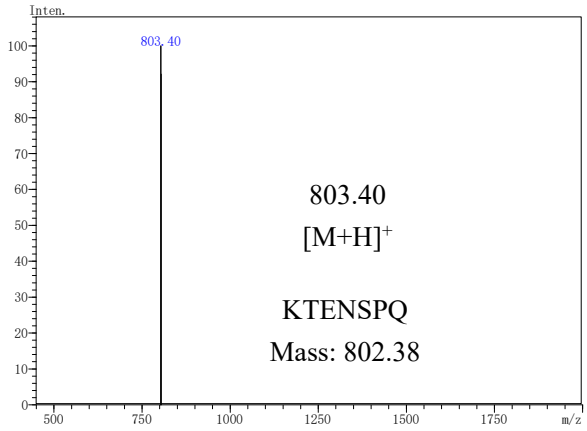
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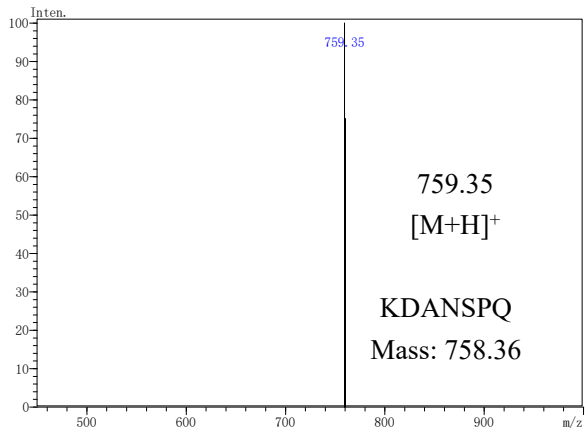

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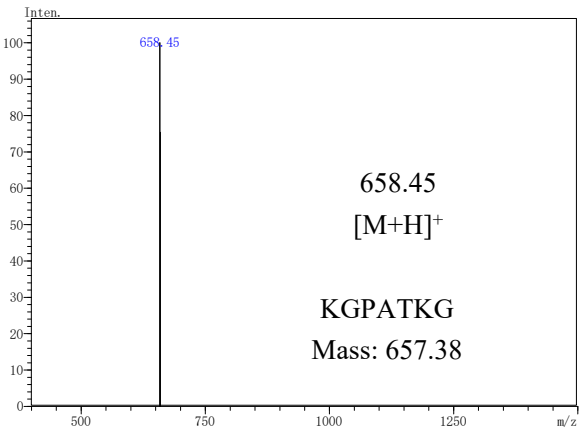

Fig. S1. Mass spectra via MALDI-TOF or LC-MS of anti-adhesion peptides.

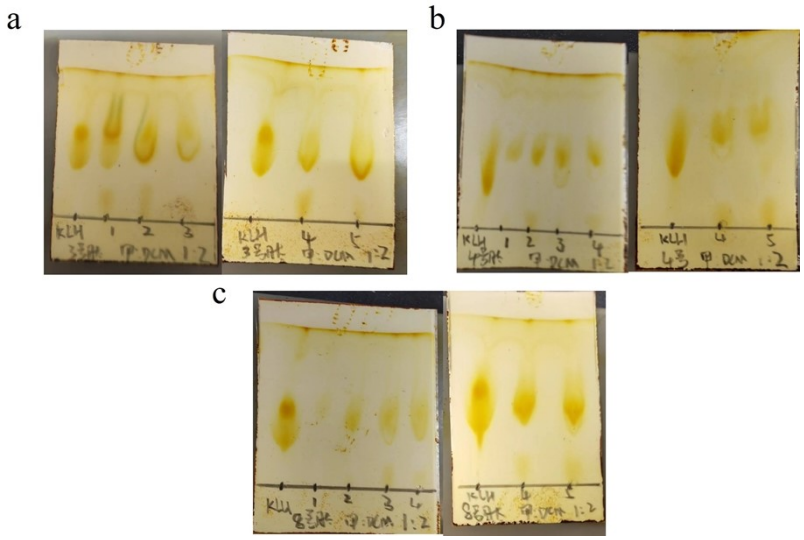

Fig. S2. Results of thin-layer chromatographic. (a) AAP3 modification of 1-5 layers on the KLH surface; (b) AAP4 modification of 1-5 layers on the KLH surface; (c) AAP8 modification of 1-5 layers on the KLH surface.

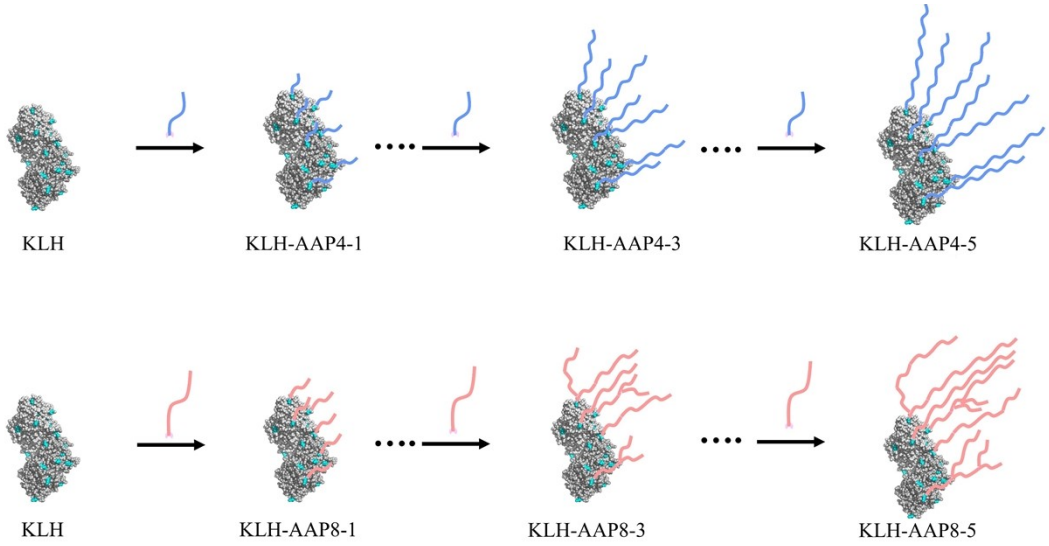

Fig. S3. Schematic illustration of one, three, and five layers of AAP4, and AAP8 modifications on KLH.

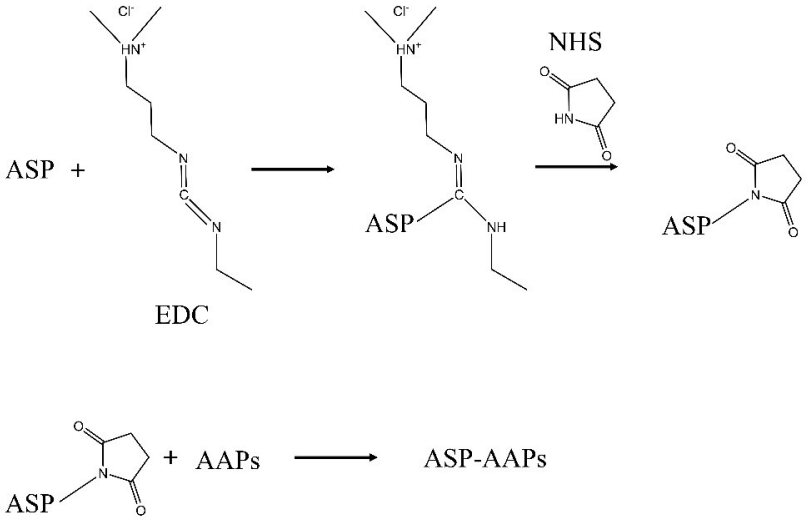

Fig. S4. The coupling reaction process of anti-adhesion peptides with ASP.

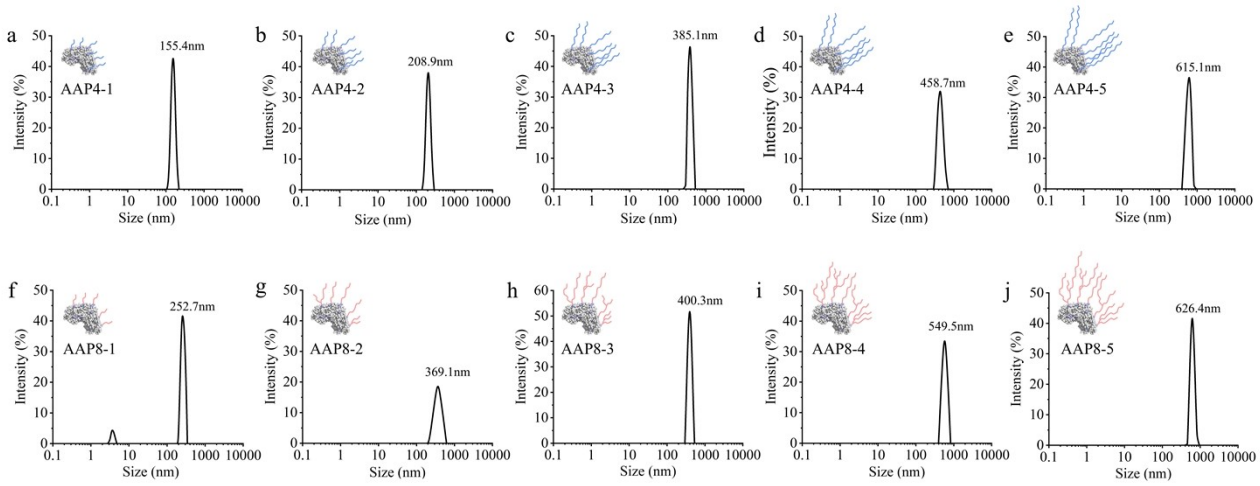

Fig. S5. The particle size of AAP4 (a-e) and AAP8 (f-j) modified of the one to five layers on ASP.

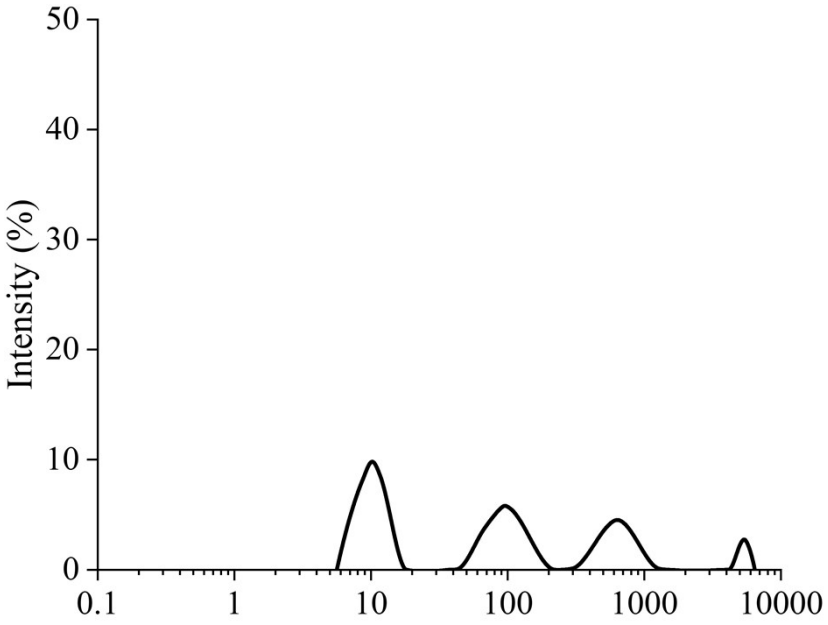

Fig. S6. Particle size of ASP.

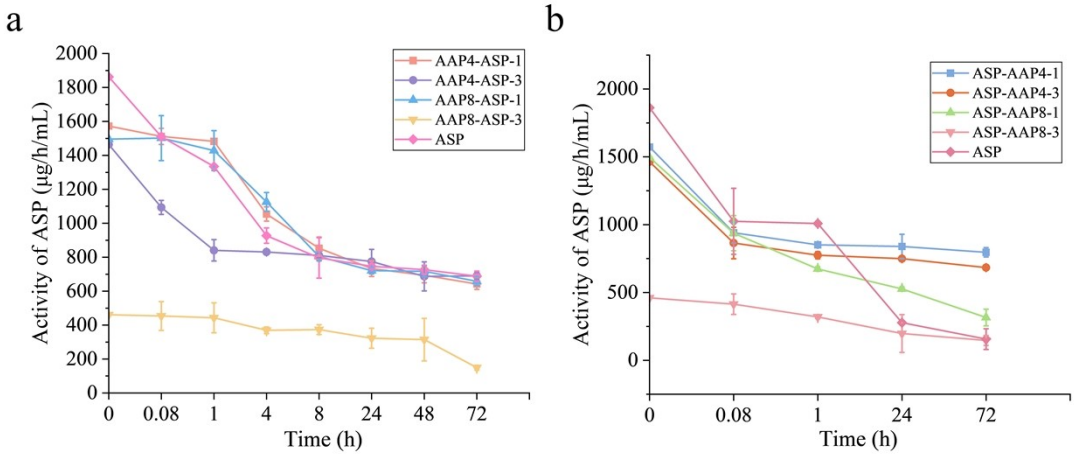

Fig. S7. (a) ASP enzymatic activity over 72 hours following the first injection (Day 1). (b) ASP enzymatic activity over 72 hours following the third injection (Day 15). Data are presented as mean ± SD

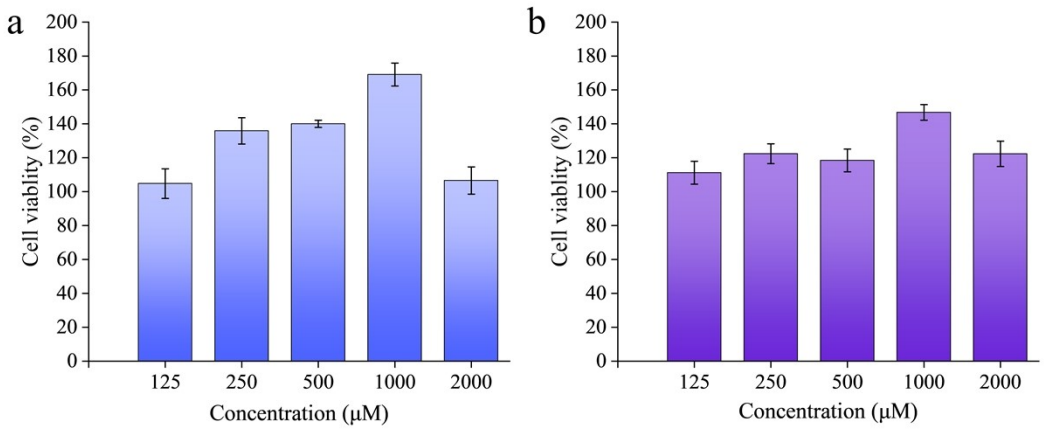

Fig. S8. Evaluation of the cytotoxicity of AAPs. (a) Co-incubation of AAP4 with HUVECs. (b) Co-incubation of AAP8 with HUVECs.

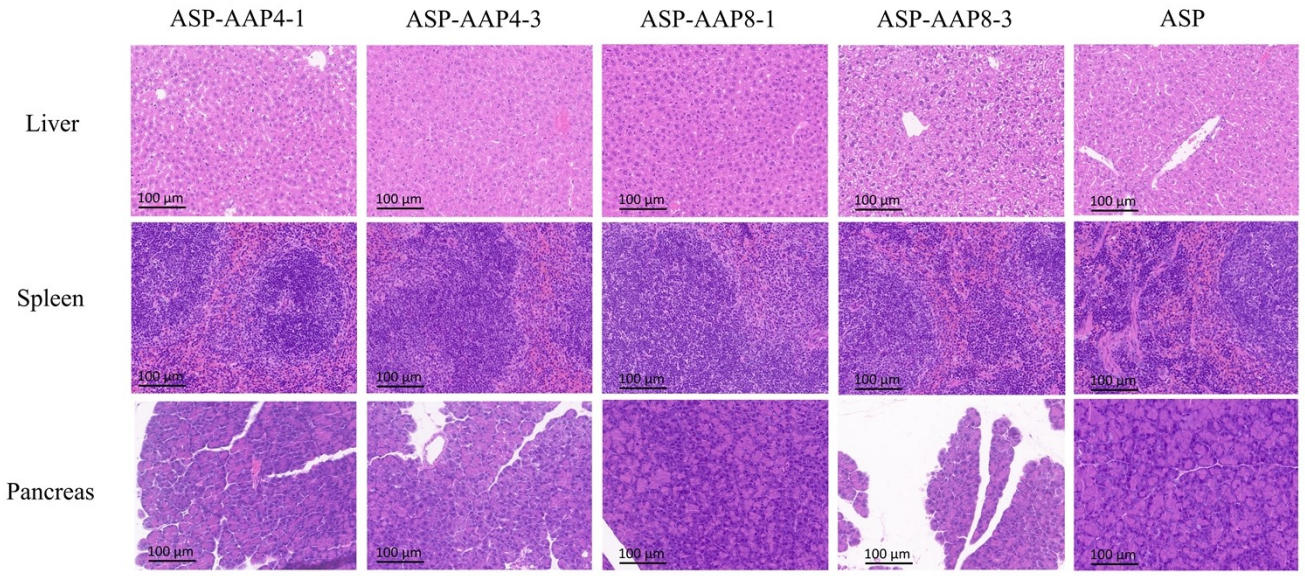

Fig. S9. H&E-stained sections of liver, spleen, and pancreas from mice after tail vein injection of ASP, ASP-AAPs-1, and ASP-AAPs-3.