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TITLE:

Targeted Antibody Blocking by a Dual-Functional Conjugate of Antigenic Peptide and Fc-III Mimetics (DCAF)

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KEYWORDS:

DCAF, targeted therapy, antibody, native chemical ligation, solid phase peptide synthesis, protein expression and purification, ELISA

SUMMARY:

Development of a dual-functional conjugate of antigenic peptide and Fc-III mimetics (DCAF) is novel for the elimination of harmful antibodies. Here, we describe a detailed protocol for the synthesis of DCAF1 molecule, which can selectively block 4G2 antibody to eliminate antibody dependent enhancement effect during Dengue virus infection.

ABSTRACT:

Elimination of harmful antibodies from organisms is a valuable approach for the intervention of antibody-associated diseases, such as Dengue hemorrhagic fever and autoimmune diseases. Since thousands of antibodies with different epitopes are circulating in blood, no universal method, except for the dual-functional conjugate of antigenic peptide and Fc-III mimetics (DCAF), was reported to target specific harmful antibodies. The development of DCAF molecules makes significant contribution to the progress of targeted therapy, which were demonstrated to eliminate the antibody dependent enhancement (ADE) effect in a Dengue virus (DENV) infection model and to boost the acetylcholine receptor activity in a myasthenia gravis model. Here, we describe a protocol for the synthesis of a DCAF molecule (DCAF1), which can selectively block 4G2 antibody to attenuate ADE effect during Dengue virus infection, and illustrate the binding of DCAF1 to 4G2 antibody by an ELISA assay. In our method, DCAF1 is synthesized by the conjugation of a hydrazine derivative of a Fc-III peptide and a recombinant expressed long α-helix with antigenic sequence through native chemical ligation (NCL). This protocol has been successfully applied to DCAF1 as well as other DCAF molecules for targeting their cognate antibodies.

INTRODUCTION:

Antibodies play important roles in humoral immune response for the neutralization of pathogenic bacteria and viruses[1]. However, some antibodies exhibit harmful impacts to the organisms, such as cross-reactive antibodies in the ADE effect during DENV infection and

over-reactive antibodies in myasthenia gravis, which is an autoimmune diseases[2,3]. ADE effect is mediated by the cross-reactive antibodies that make the bridge to connect DENV and Fc receptor presenting cells[4,5], while myasthenia gravis is caused by the excessive antibodies that attack acetylcholine receptors between the cell-cell junctions in muscle tissue[6,7]. Although partially effective approaches have been developed to treat these diseases[8,9], undoubtedly direct elimination of these harmful antibodies would make progress for the interventions.

Recently, DCAF molecules, which have dual-functional groups, have been developed for targeted antibody blocking[10]. DCAF is a long peptide that is composed of 3 parts: 1) an antigen part that can specific recognize the cognate antibody, 2) an Fc-III or Fc-III-4C tag for strongly binding to the Fc region of the antibody to inhibit either Fc receptor or complement component proteins, 3) a long α-helical linker that conjugates these two functional groups[10]. The linker part, designed from Moesin FERM domain, was optimized by Rosseta software to ensure the antigen part and Fc-III part in a DCAF molecule can bind to the Fab and Fc regions of IgG simultaneously. Four DCAF molecules have been synthesized to target 4 different antibodies, among them DCAF1 was used to eliminate 4G2 antibody, which is a cross-reactive antibody during DENV infection to contribute to ADE effect; and DACF4 was designed for the rescue of acetylcholine receptors by blocking mab35 antibody in *myasthenia gravis*[10].

In the present study, taken DCAF1 as the example, we showed the protocols for the synthesis of DCAF molecule and the detection of the interaction between a DCAF and its cognate antibody. The DCAF1 is semi-synthesized by NCL approach[11-14], which conjugates the hydrazine derivative of a Fc-III peptide and the expressed linker-antigen parts together. The NCL approach has significant advantages over fully chemical synthesis and fully recombinant expression for DCAF1 synthesis, because both these methods lead to low yield and high cost. The current approach is not only the most cost-effective way to get the full-length DCAF, but also can maintain the conformation of the linker part similar as its native form. Since different DCAF molecules have similar sequences except for the antigen parts, our methods for DCAF1 synthesis and the interaction assay between DCAF1 and 4G2 antibody can be applied to other DCAF molecules to targeted block their cognate antibodies as well.

PROTOCOL:

1. Chemical synthesis of the hydrazine derivative of a Fc-III peptide

1.1. Converting 2-Cl-(Trt)-Cl resin to 2-Cl-(Trt)-$NHNH_2$ resin

1.1.1. Weigh 625 mg of 2-Cl-(Trt)-Cl resin (0.25 mmol) into a 25 mL peptide synthesis vessel.

1.1.2. Add 5 mL of N,N-dimethylformamide (DMF) to the resin from the top of the vessel, put the cap back, gently shake the vessel for 15 s and then drain it. Repeat DMF washing for two more times.

1.1.3. Add 5 mL of dichloromethane (DCM) to the vessel, put the cap back, gently shake the vessel for 15 s and then drain it. Repeat DCM washing for two more times.

1.1.4. Repeat step 1.1.2 three times.

1.1.5. Use 6 mL of 50% (vol/vol) DMF/DCM to swell the resin for 30 min, and then drain it.

1.1.6. Transfer 6 mL 5% (vol/vol) NH_2NH_2 in DMF to the reaction vessel, shake the mixture at 120 rpm, 30 °C for 30 min, and then drain the solution by vacuum pump.

NOTE: Add 0.3 mL of hydrazine hydrate to 5.7 mL of DMF to get 5% (vol/vol) NH_2NH_2. Freshly prepare NH_2NH_2 before use.

CAUTION: Hydrazine hydrate is a dangerous substance and may cause cancer. Wear a lab coat, gloves and a mask. Perform the experiments in a fume hood.

1.1.7. Add 5 mL of DMF to the vessel, gently shake it for 15 s and then drain it.

1.1.8. Repeat step 1.1.6 and then wash the resin by repeating steps 1.1.2-1.1.4 for two times.

1.1.9. Add 6 mL of 5% (vol/vol) MeOH/DMF to the vessel, gently shake it for 10 min and then drain it.

NOTE: This step is very important to ensure the unreacted sites on the resin are capped.

1.1.10. Wash the resin thoroughly by repeating steps 1.1.2-1.1.4.

1.1.11. Add 5 mL of DCM to the resin, shake it gently for 15 s and then drain it to obtain 2-Cl-(Trt)-$NHNH_2$ resin.

NOTE: The resin becomes yellow or light green when the substitution is successful.

1.2. Peptide elongation

1.2.1. Add 1 mmol of the Fmoc-Ala-OH (934 mg) and 0.95 mmol of 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo-[4,5-b] pyridinium hexafluorophosphate 3-oxide (HATU) (360 mg) into a 5 mL tube containing 3 mL of DMF.

CAUTION: Exposure to HATU can cause an allergic reaction. Wear a lab coat, gloves and a mask.

1.2.2. Add 2 mmol of N,N-diisopropylethylamine (DIEA) (0.36 mL) to the dissolved solution and vortex for 0.5–1 min.

NOTE: Critical Step: The activation should take no more than 5 min, as the activated amino acids isomerize from the more active O-forms to the less active N-forms over time.

1.2.3. Add the activated Fmoc-Ala-OH to the reaction vessel containing the 2-Cl-(Trt)-$NHNH_2$ resin, prepared from step 1.1. Gently shake at 120 rpm, 30 °C for 20 min in a constant-temperature shaker, and then drain the solution by vacuum pump.

1.2.4. Add 5 mL of DMF to wash the resin, gently shake it for 15 s and then drain it.

1.2.5. Add 1 mmol of the Fmoc-Ala-OH (934 mg) and 0.95 mmol of 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU) (390 mg) into a 5 mL tube containing 3 mL of DMF.

CAUTION: Exposure to HCTU can cause an allergic reaction. Wear a lab coat, gloves and a mask.

1.2.6. Add 2 mmol of DIEA (0.36 mL) to the tube and vortex for 0.5–1 min to dissolve it.

1.2.7. Add the activated Fmoc-Ala-OH to the reaction vessel. Gently shake at 120 rpm, 30 °C for 40-60 min in a constant-temperature shaker, and then drain the solution by vacuum pump.

1.2.8. Wash the resin by repeating the steps 1.1.2-1.1.4.

1.2.9. Add 4 mL of 20% (vol/vol) piperidine in DMF to the resin, gently shake it for 5 min at room temperature and then drain the solution.

CAUTION: Pyridine is highly flammable and toxic. Perform the experiments in a fume hood.

1.2.10. Add another 4 mL of 20% (vol/vol) piperidine in DMF to the resin, gently shake it for 15 min at room temperature and then drain the solution.

1.2.11. Follow the steps 1.2.1-1.2.10 to couple and deprotect the Fmoc-Ala-OH, Fmoc-Thr-OH, Fmoc-Cys-OH, Fmoc-His-OH and Fmoc-Ala-OH. Repeat steps 1.2.5-1.2.10 to couple and deprotect the Fmoc-Trp-OH, Fmoc-Val-OH, Fmoc-Leu-OH, Fmoc-Glu-OH, Fmoc-Gly-OH and Fmoc-Asp-OH.

1.2.12. Repeat steps 1.1.2-1.1.4 to wash the resin thoroughly.

1.2.13. Repeat step 1.1.3 to wash the resin and then vacuum-dry the resin for 5 min.

1.3. Cleavage of the hydrazine derived Fc-III crude peptide

1.3.1. Add 12 mL of cocktails trifluoroacetic acid (TFA)/2,2′-(ethylenedioxy)diethanethiol (DODT)/triisopropylsilane (TIPS)/H_2O (92.5/2.5/2.5/2.5) to the resin, and then use a constant-temperature shaker to cleave the peptide from the resin at 200 rpm, 37 °C about 2 h. Filter the mixture into a 50 mL centrifuge tube.

1.3.2. Add 1 mL of cocktails into the resin to wash it and collect the filtrate into the 50 mL centrifuge tube. Repeat two times.

1.3.3. Concentrate the mixture to 2 mL by a vaper rotor in a fume hood, and then add 20 mL of precooled diethyl ether into the tube to precipitate crude peptides.

NOTE: The anhydrous ether should be precooled to 0 °C to avoid violent heat release, which

may cause side reactions of the crude peptide.

1.3.4. Incubate the peptide precipitation at -20 °C for 1-2 h.

1.3.5. Centrifuge at 5,000 x *g*, 4 °C for 10 min and remove the solvent from the centrifuge tube carefully.

1.3.6. Add 20 mL of precooled diethyl ether into the tube two times according to the steps 1.3.3-1.3.5 to wash the precipitation. Air-dry the peptide product in a watch-glass for about 15 min. Store the dried crude peptides at 4 °C for further analysis.

1.4. Purification of the hydrazine derivative of a Fc-III peptide

1.4.1. Use the high-performance liquid chromatography (HPLC) system to purify the peptide by a 30 min gradient elution (0-1 min, 5% B; 1-20 min, 50% B; 20-25 min, 85% B; 25-30 min, 85% B) at a flow rate of 1 mL/min.

NOTE: The column is in 4.6 mm ID, 250 mm length, packed with C-18 resin. Mobile phase A consisted of 0.1% TFA in water, and mobile phase B consisted of 100% acetonitrile and 0.1% TFA.

2. Protein expression and purification of linker and antigen parts

2.1. Plasmid construction

2.1.1. Synthesize the whole gene sequence that can express SUMO-linker-antigen (see **Table of Materials**).

2.1.2. Cut 10 ng of PET-28a empty vector and 50 ng of the synthesized SUMO-linker-antigen DNA fragment by using NcoI and XhoI in a 37 °C water bath for 3 h.

2.1.3. Purify the double-enzyme digested vector and DNA fragment by DNA Gel Extraction Kit.

2.1.4. Add 5 µL of DNA digested DNA fragment, 5 µL of digested vector, 1 µL of T4 DNA ligase, 2 µL of 10x ligase buffer and 7 µL of ddH_2O to a reaction tube, and then incubate it overnight at 16 °C.

2.1.5. Mix 10 µL of the ligation product from step 2.1.4 into 100 µL of competent cells (DH5α) and put it on an ice bath for 30 min. Put the competent cells to a 42°C water bath for 90 s and fast transfer it to an ice bath for 2 min. Add 800 mL of LB liquid medium (without antibiotics) to the tube and shake it at 37 °C, 180 rpm for 1 h. Coat the competent cells onto a Kanamycin LB plate to make them equally distributed, and put the plate into a 37 °C incubator overnight.

2.1.6. Pick 5 single colonies from the plate and culture the bacteria in 5 mL of LB medium in a test tube using a 37 °C shaker.

2.1.7. Extract plasmids from the bacteria harvested from step 2.1.6 by using Plasmid

Extraction Kit.

2.1.8. Send the plasmids for gene sequencing and choose the positive colony that can express SUMO-linker-antigen fusion protein. Transform the positive plasmid to BL21(DE3)plysS competent cells following step 2.1.5.

2.2. Protein purification

2.2.1. Pick a single colony of transformants from step 2.1.7 into 5 mL of LB liquid medium containing kanamycin (50 μg/mL). Shake the cultures overnight at 200 rpm, 37 °C.

2.2.2. Inoculate 1 L of prewarmed LB medium containing kanamycin (50 μg/mL) in a flask with 5 mL of the overnight cultures, and grow at 37 °C with vigorous shaking, until the OD_{600} is 0.6.

2.2.3. Add isopropyl β-D-thiogalactoside (IPTG) into the medium to the final concentration of 0.4 mM, and shake the flask at 200 rpm overnight at 20 °C.

NOTE: Low temperature for protein induction is important to avoid inclusion body formation, so make sure the temperature is no more than 22 °C.

2.2.4. After harvesting the cells, add 50 mL of lysis buffer to the cell pellets to resuspend the cells. Sonicate the cell lysates twice for 5 min at 200 W, ultrasound 3 s, interval 3 s on ice. Centrifuge it at 15, 000 x *g* for 30 min at 4 °C to collect the supernatants.

NOTE: The lysis buffer is composed of 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole. Adjust the pH value to 8.0 by using NaOH.

2.2.5. Add 2 mL of the 50% Ni-NTA Sephorase, equilibrated by lysis buffer, to the cleared supernatants and mix gently by shaking (200 rpm on a rotary shaker) at 4 °C for 1 h.

2.2.6. Load the lysate and Ni-NTA mixture into a column with the bottom outlet capped, and remove bottom cap and discard the column flow-through.

2.2.7. Wash three times with 6 mL of wash buffer, and then elute the targeted protein 3 times with 1.5 mL of elution buffer. Collect the eluate in one tube.

NOTE: The wash buffer is composed of 50 mM NaH_2PO_4, 300 mM NaCl, 30 mM imidazole. Adjust the pH value to 8.0 by using NaOH. The elution buffer is composed of 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole. Adjust the pH value to 8.0 by using NaOH.

2.2.8. Put the eluate into a dialysis bag in 1 L of 50 mM PBS at 4 °C to desalt the protein. Replace new phosphate buffered saline (PBS) for 3 times.

2.3. Cleavage of the SUMO tag

2.3.1. Add 2 mL of Small Ubiquitin-Like Modifier (SUMO) tagged protein at the concentration of 1 mg/mL, 1 mL of SUMO Protease (see **Table of Materials**), 1 mL of 10x SUMO Protease

Buffer and 6 mL of ddH_2O to prepare the reaction solution.

NOTE: SUMO tag protein concentration is about 1 mg/mL. Excessive concentrations after enzyme digestion may cause the coagulation of the protein.

2.3.2. Incubate the mixture for 12-16 h at 4 °C.

2.3.3. Centrifuge the mixture at 15,000 x *g* for 10 min at 4 °C and discard the aggregates. Add 0.5 mL of the 50% Ni-NTA slurry, equilibrated by 50 mM PBS, to the 10 mL cleared supernatants and mix gently by shaking (200 rpm on a rotary shaker) at 4 °C for 60 min.

2.3.4. Load the lysate and Ni-NTA mixture into a column with the bottom outlet capped. Remove the bottom cap and then save the flow through into a 15 mL centrifuge tube.

NOTE: Histagged SUMO protein is binding on the column. The linker-antigen part, which starts with Cys for NCL reaction, is in the flow through.

2.3.5. Centrifuge the flow through at 15,000 x *g* for 10 min at 4 °C and discard the aggregates. Purify the linker-antigen part using HPLC by a 25 min gradient elution (0-1 min, 5% B; 1-3 min, 15% B; 3-20 min, 45% B; 20-21 min, 85% B; 21-25 min, 85% B) at a flow rate of 1 mL/min.

NOTE: The column is in 4.6 mm ID, 250 mm length, packed with C-18 resin. Mobile phase A consisted of 0.1% TFA in water, and mobile phase B consisted of 100% acetonitrile and 0.1% TFA.

2.3.6. Collect the purified product from the HPLC, and then freeze-dry overnight to get the linker-antigen product.

3. Assembling of DCAF1 by native chemical ligation

3.1. Conjugating Fc-III peptide and linker-antigen part together

3.1.1. Weigh 1.8 mg (1 µmol) of hydrazine derivative of a Fc-III peptide into a 2 mL EP tube and add 0.8 mL of 6 M GnHCl in 0.2 M NaH_2PO_4 (pH 3.0) solution to dissolve it by vortexing.

NOTE: 6 M GnHCl in 0.2 M NaH_2PO_4 (pH 3.0) solution is prepared by adjusting the pH value with HCl or NaOH.

CAUTION: NaOH and HCl are highly corrosive. Wear a lab coat, gloves and a mask.

3.1.2. After centrifuging the tube at 7,200 x *g* for 1 min at room temperature, put the tube in an ice-salt bath and use the magnetic stirring to agitate the solution for 15 min gently.

3.1.3. Add 40 µL of 0.5 M $NaNO_2$ to the solution to oxidize the hydrazine group. Gently agitate the solution for another 15 min in the ice-salt bath.

3.1.4. Weigh and add 11 mg of purified linker-antigen part prepared from step 2.3 and 13.6

mg of 4-mercaptophenylacetic acid (MPAA) into the reaction tube in the ice-salt bath, stir for 5 min, and then adjust the pH value to 6.8-7.0 at room temperature with 6 M NaOH.

NOTE: Adjusting the pH value to neutral is the critical step to initiate the ligation reaction.

3.1.5. After 12 h, add 0.4 mL of 0.1 M tris(2-carboxyethyl)phosphine hydrochloride (TCEP) neutral solution (pH 7.0) to the reaction system and stir for 20 min to terminate the reaction.

NOTE: 0.1 M TCEP neutral solution (pH 7.0) is prepared by dissolving TCEP in 6 M GnHCl in 0.2 M NaH_2PO_4 (pH 3.0) solution, and using NaOH to adjust the pH value to 7.0.

3.1.6. Centrifuge the tube at 15,000 x *g* for 10 min, then analyze and purify the ligation product with HPLC by a 26 min gradient elution (0-1 min, 5% B; 1-21 min, 45% B; 21-22 min, 85% B; 22-26 min, 85% B) at a flow rate of 1 mL/min. The expected yield for the ligation reaction is over 50%.

NOTE: The column is in 4.6 mm ID, 250 mm length, packed with C-18 resin. Mobile phase A consisted of 0.1% TFA in water, and mobile phase B consisted of 100% acetonitrile and 0.1% TFA.

3.2. Desulfurization of the conjugate

3.2.1. Dissolve the conjugate (3.4 mg, 0.3 µmol) prepared from step 3.1.6 in 50 µL of 6 M GnHCl in 0.2 M NaH_2PO_4 (pH 7.0) solution.

3.2.2. Add 50 µL of 1 M TCEP, 10 µL of tBuSH and 5 µL of 0.1 M VA-044 solution to the above mixture.

NOTE: 0.1 M VA-044 solution is prepared by dissolving 9.7 mg of VA-044 powder into 0.3 mL of 6 M GnHCl in 0.2 M NaH_2PO_4 (pH 7.0) solution. The VA-044 solution should be prepared freshly before use, as VA-044 is easily oxidized by exposure to air.

3.2.3. Adjust the final pH of the solution to 6.9 and keep the solutiomat 37 °C with stirring for about 5 h.

3.2.4. Centrifuge the tube at 15,000 x *g* for 10 min and remove the insoluble substance. Analyze and purify the ligation product with HPLC by a 26 min gradient elution (0-1 min, 20% B; 1-21 min, 45% B; 21-22 min, 85% B; 22-26 min, 85% B) at a flow rate of 1 mL/min. The expected yield for the desulfurization reaction is about 40%.

NOTE: The column is in 4.6 mm ID, 250 mm length, packed with C-18 resin. Mobile phase A consisted of 0.1% TFA in water, and mobile phase B consisted of 100% acetonitrile and 0.1% TFA.

3.3. Removal of Acm to get the final product DCAF1

3.3.1. Dissolve the peptide (1.35 mg, 0.11 µmol) prepared from step 3.2.4 in 200 µL of 32 mM

AgOAc, and then stir the reaction mixture at room temperature for 4 h.

3.3.2. Add 3 µL of 1M Dithiothreitol (DTT) in 6 M GnHCl in 0.2 M NaH_2PO_4 (pH 7.0) solution to convert the silver thiolates on peptide to free thiols.

3.3.3. After violently stirring for 1 min, centrifuge the tube at 15,000 x *g* for 10 min to discard the insoluble substance. Analyze and purify the final product with HPLC by a 26 min gradient elution (0-1 min, 20% B; 1-21 min, 45% B; 21-22 min, 85% B; 22-26 min, 85% B) at a flow rate of 1 mL/min. The expected yield for the Acm deprotection reaction is about 30%.

NOTE: The column is in 4.6 mm ID, 250 mm length, packed with C-18 resin. Mobile phase A consisted of 0.1% TFA in water, and mobile phase B consisted of 100% acetonitrile and 0.1% TFA.

3.4. After HPLC purification, freeze-dry the final product overnight, and dissolve the powder in PBS (50 mM NaH_2PO_4, 150 mM NaCl, pH 8.0). Centrifuge the tube at 15,000 x *g* for 10 min at 4 °C and discard the pellet.

3.5. Adjust the concentrations of final product (from step 3.4) and linker-antigen part (from step 2.3.6) to 10 µM, record the circular dichroism (CD) spectra from 260 to 195 nm of these two products using a CD spectrometer at 25 °C with 1 mm path length.

NOTE: CD spectra are used to demonstrate the amount of α helical structure in the final product is as similar as that in the recombinant expressed one.

4. Detection of the products by mass spectrometry

4.1. Separate the product from each chemical reaction by a 60 min gradient elution (0-8 min, 2% B; 8-10 min, 5% B; 10-35 min, 30% B; 35-50 min, 50% B; 50-52 min, 80% B; 52-58 min, 80% B; 58-59 min, 2% B; 59-60 min, 2% B) at a flow rate of 0.300 µL/min with a nano-HPLC system which is directly interfaced with the high resolution mass spectrometer.

NOTE: The analytical column is in 75 µm ID, 150 mm length, packed with C-18 resin. Mobile phase A consisted of 0.1% formic acid in water, and mobile phase B consisted of 100% acetonitrile and 0.1% formic acid.

4.2. Operate the mass spectrometer in the full-scan mode and set the m/z range at 300-2,000 and resolution at 60,000.

4.3. Open the mass spectra data and find the peaks of the product in each step to confirm the chemical reaction is successfully preformed.

5. ELISA assay of the interaction between DCAF1 and 4G2 antibody

5.1. Coat a 96-well microtiter plate with 1 pmol anti-GST antibody (see **Table of Materials**) in 100 µL of coating buffer/well, seal the plate and incubate it at 4 °C overnight on a shaker.

5.2. Block each well with 200 µL of 1% BSA in PBS, seal the plate and incubate it at room temperature for 1 h on a shaker.

5.3. Wash each well 4 times by using PBS with 0.05% Tween 20.

5.4. Add the GST-fused antigen protein (0.05 pmol in 100 µL of blocking solution) to the wells, seal the plate and incubate for 2 h at room temperature.

NOTE: GST-fused antigen protein is expressed in bacteria and purified by GSH Sepharose.

5.5. Repeat step 5.3 to wash the plate.

5.6. Add 1 pmol 4G2 antibody or 1 pmol 4G2 antibody plus the other ligands: antigen, Fc-III or DCAF1 at series amounts (0.1 pmol, 1 pmol, 10 pmol, 100 pmol and 1000 pmol) in 100 µL of blocking solution/well, seal the plate and incubate 2 h at room temperature on a shaker.

5.7. Repeat step 5.3 to wash the plate.

5.8. Add Anti-mouse IgG, HRP-linked Antibody (1:20,000 dilution) in 100 µL of blocking solution/well, seal the plate and incubate 30 min at room temperature on a shaker.

5.9. Wash each well 6 times by using PBS with 0.05% Tween 20.

5.9. Mix TMB reagent A and B 1:1 in volume immediately prior to use, add 100 µL/well, and then incubate 30 min at room temperature in the dark.

5.10. Add 100 µL/well of stop solution, and use a plate reader to measure the OD_{450} values for each well within 30 min.

6. ELISA assay of the interaction between Fc-III and IgG molecule

6.1. Coat a 96-well microtiter plate with 1 pmol anti-CA antibody (see **Table of Materials**) in 100 µL of coating buffer/well, seal the plate and incubate it at 4 °C overnight on a shaker.

6.2. Repeat steps from 5.2 to 5.3.

6.3. Add the Fc-III or Fc-III-4C fused CA protein (0.01, 0.05, 0.1, 0.5 and 1 pmol in 100 µL of blocking solution) to the wells, seal the plate and incubate for 2 h at room temperature.

NOTE: Fc-III or Fc-III-4C fused CA protein is expressed in bacteria and purified by Ni-NTA.

6.4. Repeat the steps from 5.7 to 5.11 and check the results.

REPRESENTATIVE RESULTS:

The flowchart for the synthesis route by native chemical ligation in this article is shown in **Figure 1**. **Figures 2-6** show the chromatograms (**A**) and mass spectra (**B**) of chemical

synthesized hydrazine derivative of a Fc-III peptide, recombinant expressed linker and antigen part, the purified product from NCL reaction, the purified product from desulfurization reaction and the purified final product DCAF1, respectively. The chromatograms show the purifies of all the products are over 90%, while the mass spectra indicate the molecular weight of the product after each reaction. The deconvolutional molecular weights are also shown in **Figures 3-6**, which reflect the accurate molecular weight of each product. **Figure 7** shows the principle of ELISA assay (A) and the results of antigen peptide, Fc-III and DCAF1 competitively inhibiting 4G2 antibody binding. Both antigen peptide and DCAF1 significantly block 4G2 binding, whereas the Fc-III peptide does not affect the antigen-antibody interaction.

FIGURE AND TABLE LEGENDS:

Figure 1. The workflow of the native chemical ligation method for the semi-synthesis of DCAF1 molecule. First, the hydrazine derivative of a Fc-III peptide is obtained by solid phase peptide synthesis. Then, the SUMO tag fused linker and antigen part is expressed and purified from bacteria, followed by the SUMO tag cleavage. After NCL reaction of the two fragments, the product undergoes desulfurization and removing Acm groups to become DCAF1 molecule.

Figure 2. The chromatogram and mass spectrum of the hydrazine derivative of a Fc-III peptide. This figure shows that the LC profile of the purified peptide (**A**) and the mono-isotope peak at m/z 915.92 matched the duple charged peptide (**B**). This figure has been modified from reference 10, Figure 2.

Figure 3. The chromatogram and mass spectrum of the linker and antigen part. This figure shows the LC profile of the purified fragment (**A**) and the deconvolutional molecular weight of this fragment is calculated as 9429.0 from the mass spectrum (**B**). This figure has been modified from reference 10, Figure S2.

Figure 4. The chromatogram and mass spectrum of the conjugation product by NCL reaction. This figure shows the LC profile monitoring the ligation product at 0 h (top), 12 h (middle) and the purified peptide (bottom) (**A**) and the deconvolutional molecular weight of this product is calculated as 11227.0 from the mass spectrum (**B**). This figure has been modified from reference 10, Figure S2.

Figure 5. The chromatogram and mass spectrum of the product after desulfurization reaction. This figure shows the LC profile of the purified product (**A**) and the deconvolutional molecular weight of this product is calculated as 11195.0 from the mass spectrum (**B**). This figure has been modified from reference 10, Figure S2.

Figure 6. The chromatogram, mass spectrum and CD spectra of the DCAF1 molecule. This figure shows the LC profile of the purified DCAF1 (**A**), the deconvolutional molecular weight of this molecule calculated as 11053.0 from the mass spectrum (**B**) and the CD spectra of the final product (solid line) and the linker-antigen part (dash line) in FCAF1. This figure has been modified from reference 10, Figure 2.

Figure 7. ELISA assay of DCAF1 molecule. (**A**) The workflow of the sandwich ELISA assay. First anti-GST antibody is coated on a well plate. Then GST-fused antigen peptide is incubated. Then 4G2 antibody with different concentrations of DCAF1, antigen or Fc-III is added for

colorimetric analysis. (**B**) The ELSIA results of antigen, Fc-III and DCAF1 demonstrating inhibition effects of 4G2 binding using different ligand concentrations.

DISCUSSION:

The protocol here describes the semi-synthesis and detection of DCAF1 by using NCL approach, which is shown in **Figure 1**. Briefly, the two fragments of DCAF1 are chemical synthesized and recombinantly expressed, respectively; then, the full length DCAF1 molecule is assembled, modified and purified. For the hydrazine derived Fc-III fragment synthesis, using low-capacity 2-Cl resin is quite important, because high-capacity has a negative effect for hydrazine generation and leads to low yield of the product. The linker and antigen part are expressed with SUMO tag for two reasons: first, SUMO tag can enhance the solubility of the fusion proteins; second, SUMO protease is a conformation-recognized protease, which allows the released product to start with Cys residue for the further ligation reaction. One of the most critical steps in protein expression and purification is the SUMO protease cleavage. The concentration of SUMO tag-fused protein should be adjusted to the proper range. A too high or too low concentration would lead to protein aggregation or difficulty for the further purification after digestion. Another critical step of this protocol is adjusting the pH value during NCL reaction, which is based on thiol-ester exchange that should happen in neutral buffer. Fine control of the pH value in the reaction buffer is helpful to enhance the ligation efficiency.

This protocol is suitable for the synthesis of other DCAF molecules with different antigen sequences, because the sequences of different DCAF molecules are quite similar and the antigen part usually contributes little to the whole structure and nature of DCAF. For example, we used this protocol to synthesize another three DCAF molecules to target their cognate antibodies. Among them, DCAF4 was designed to block mAb35 antibody, which can neutralize acetylcholine receptor and cause *myasthenia gravis* in a rat model. We used DCAF4 to reduce the clinical symptoms in rat with mAb35-induced *myasthenia gravis*, and rescue the acetylcholine receptor by inhibiting the complement component proteins.

The application of our technique is limited by several factors: first, the yield of DCAF molecule synthesized by the current approach is low (usually less than 10%) due to the multiple purification steps; second, antibodies with conformational determinants are difficult to target by DCAF; third, DCAF may induce extra immune response in organisms compared to traditional small compound drug. We envision that the application of this protocol to other antibody-induced pathological conditions will help synthesize more DCAF molecules to targeted eliminate harmful antibodies.

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DISCLOSURES:

The authors have nothing to disclose.

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Fig 1

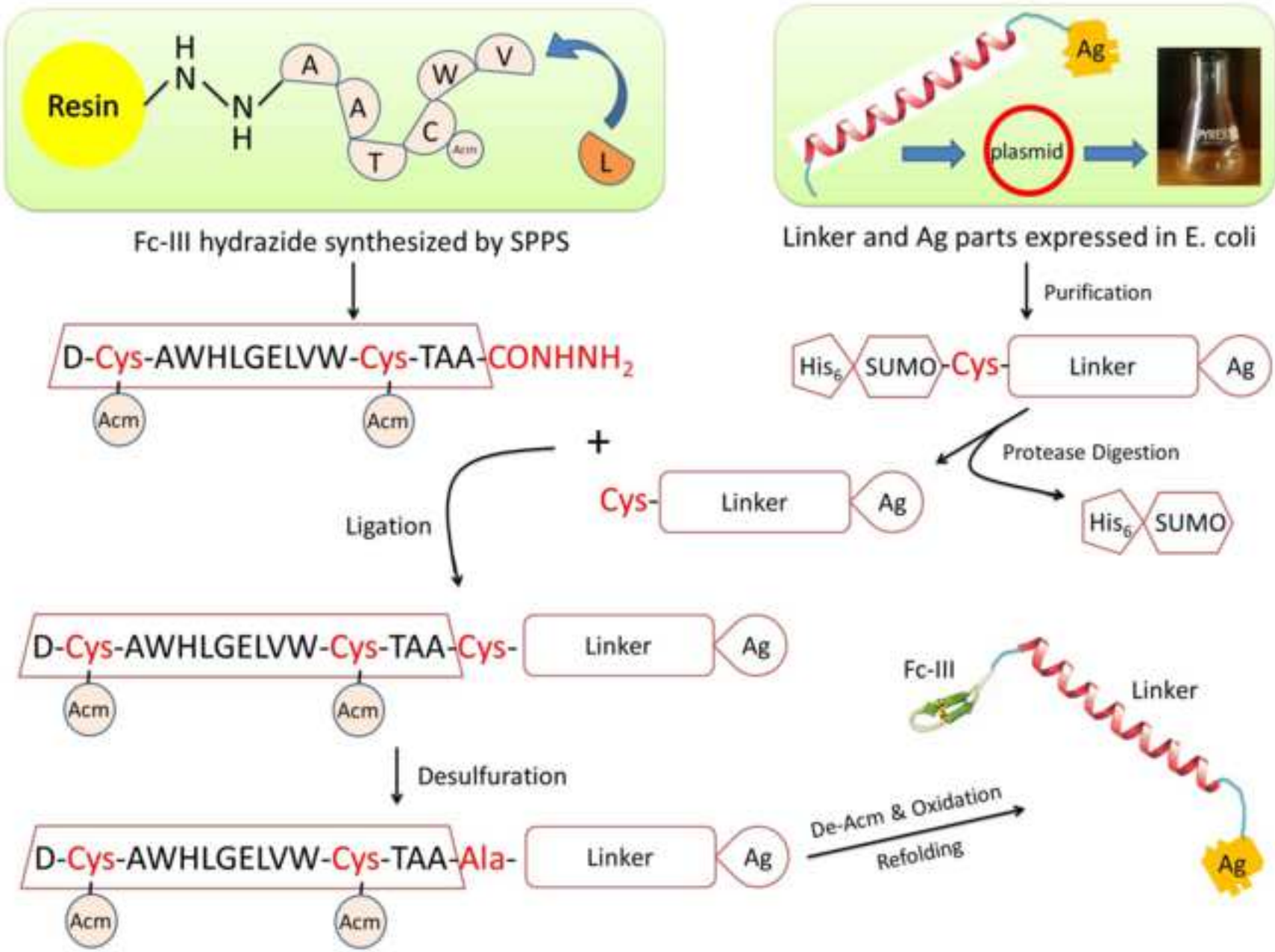


Fig 2

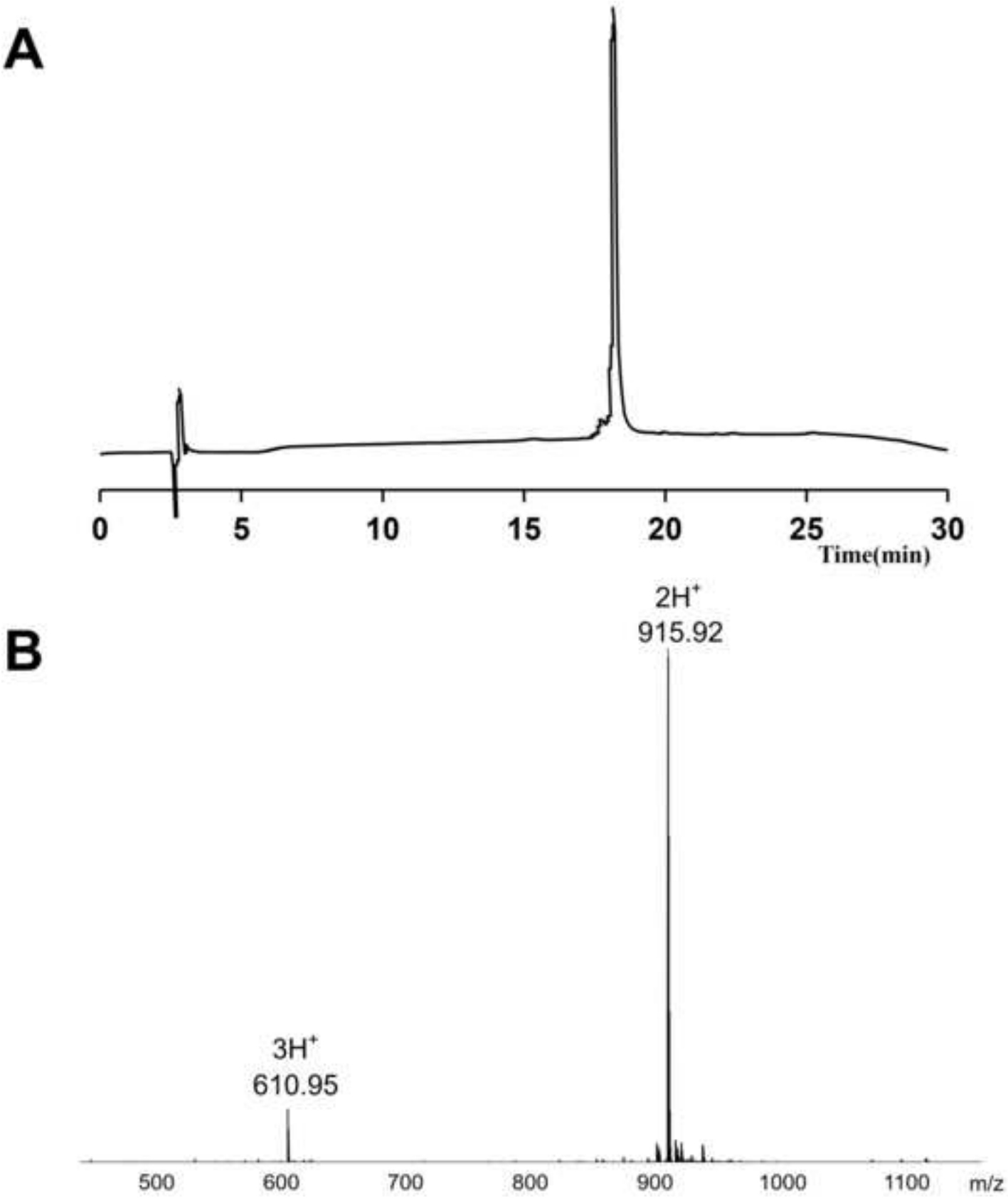


Fig 3

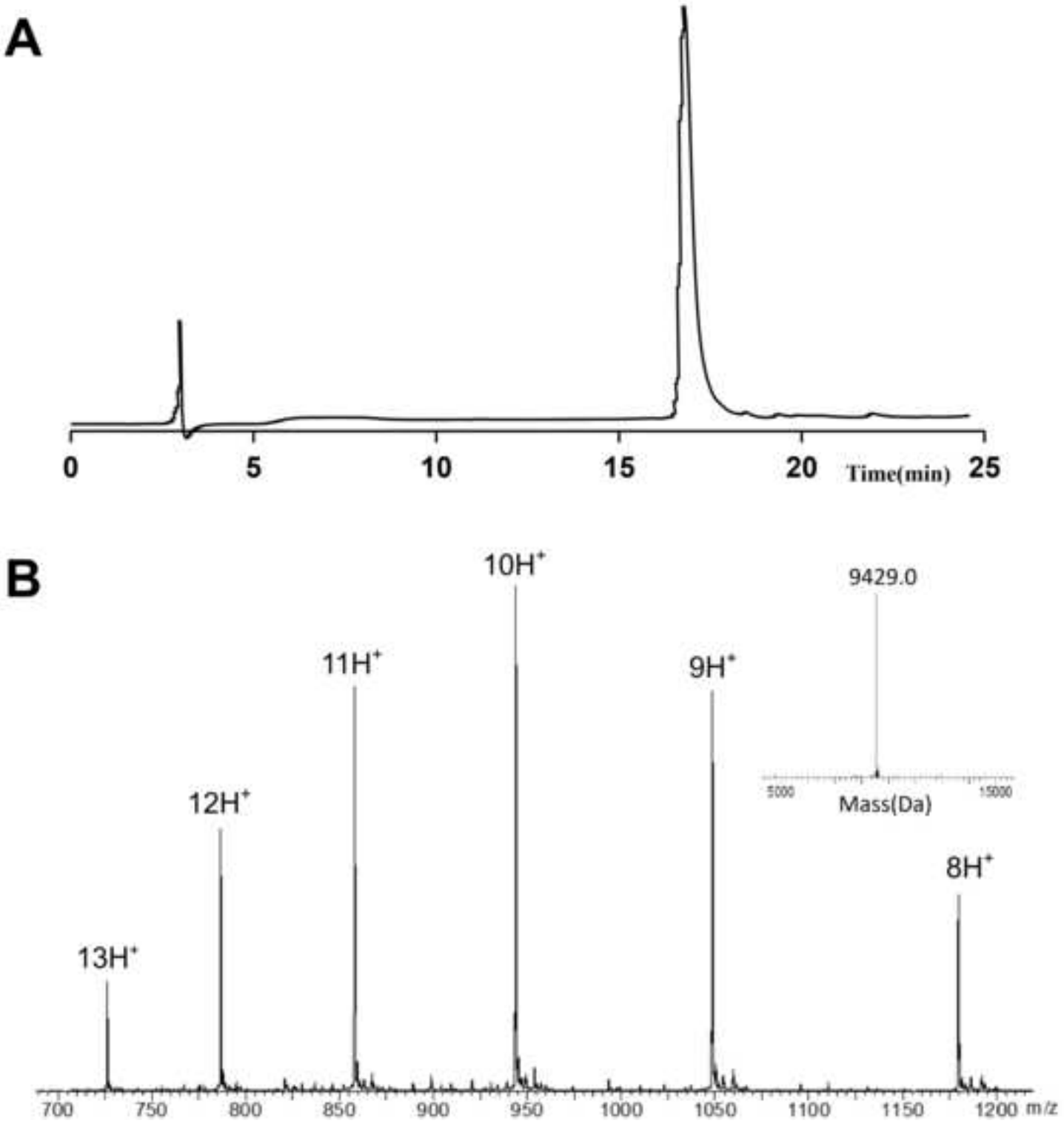


Fig 4

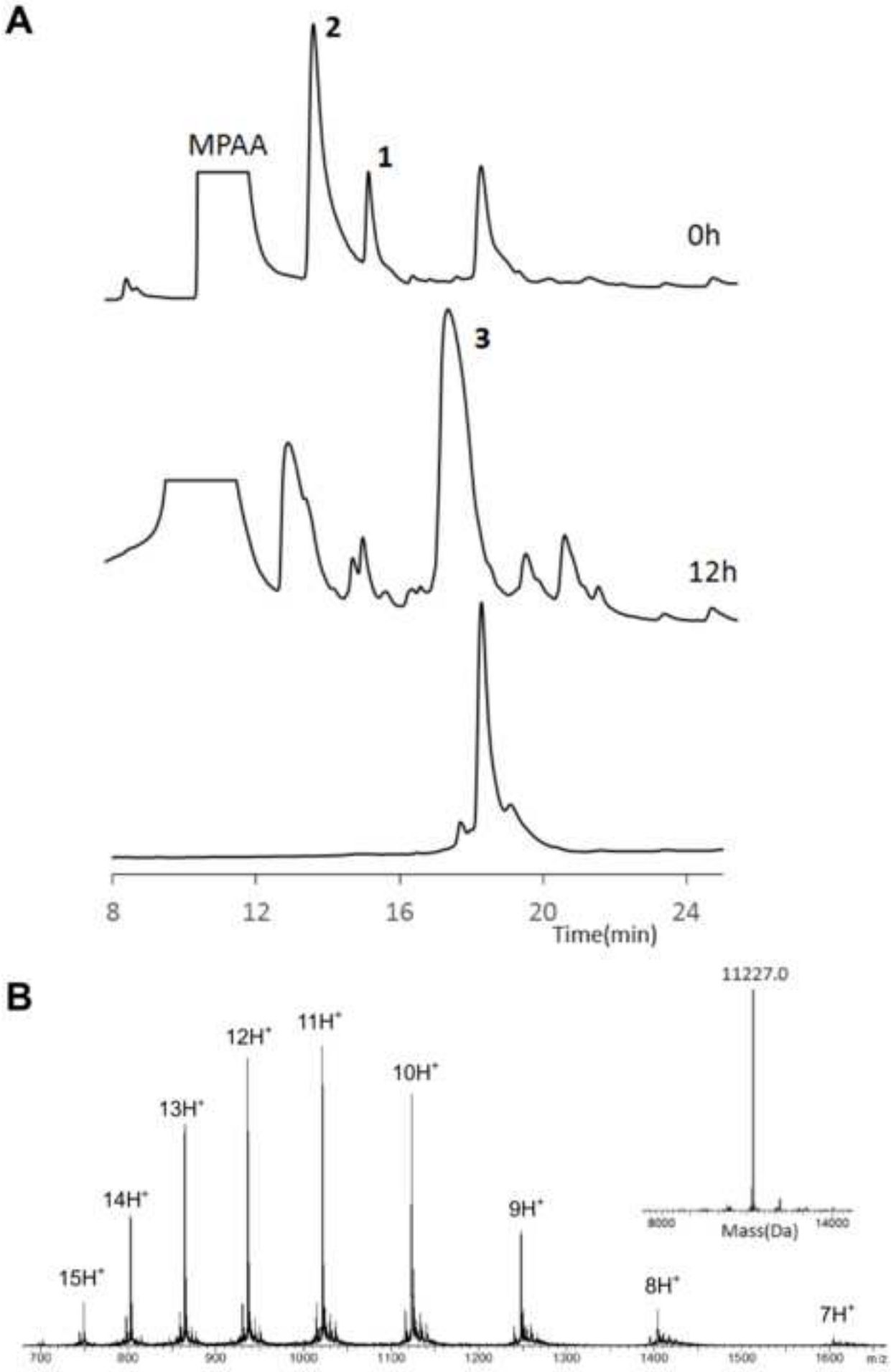
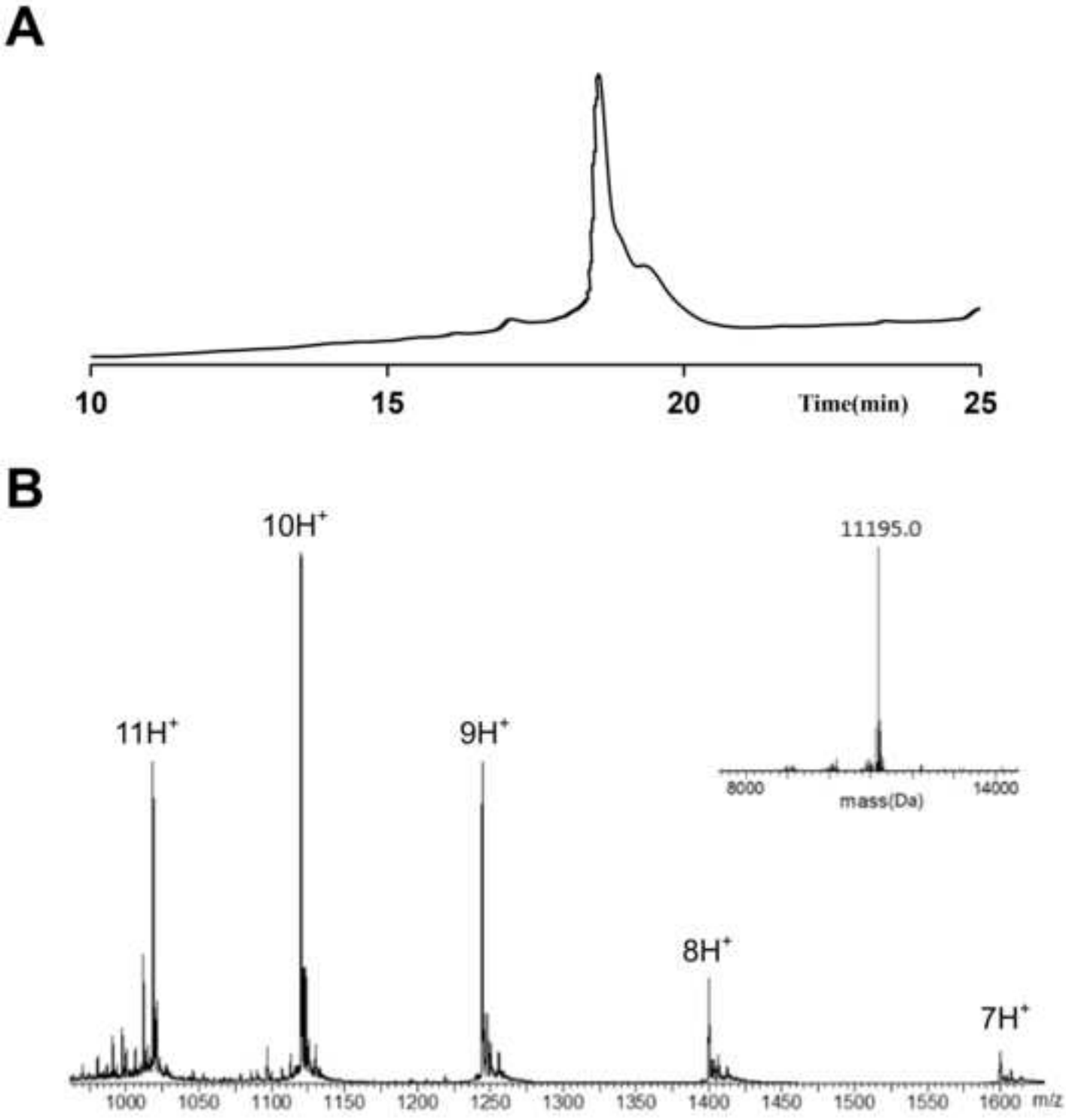


Fig 5



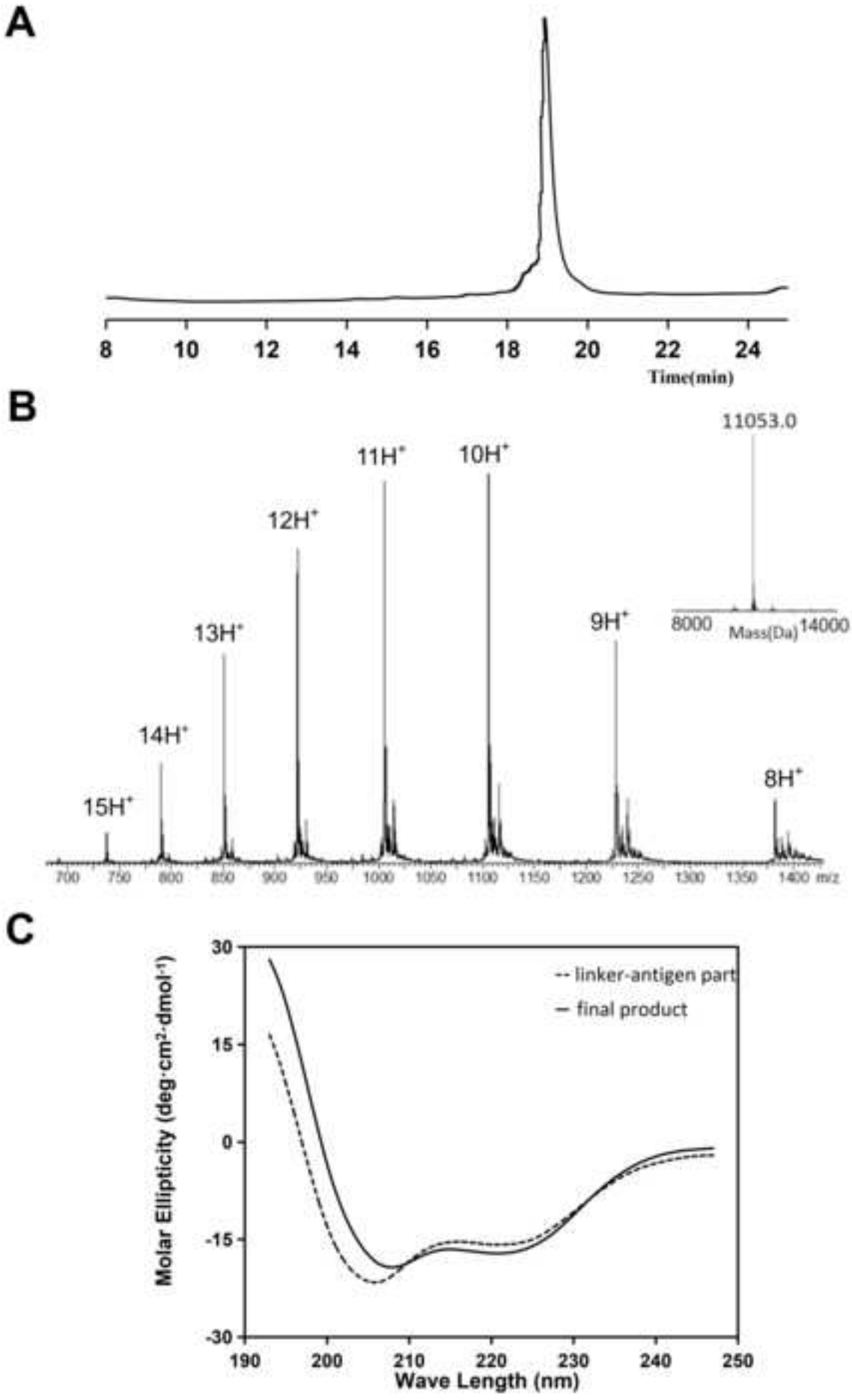
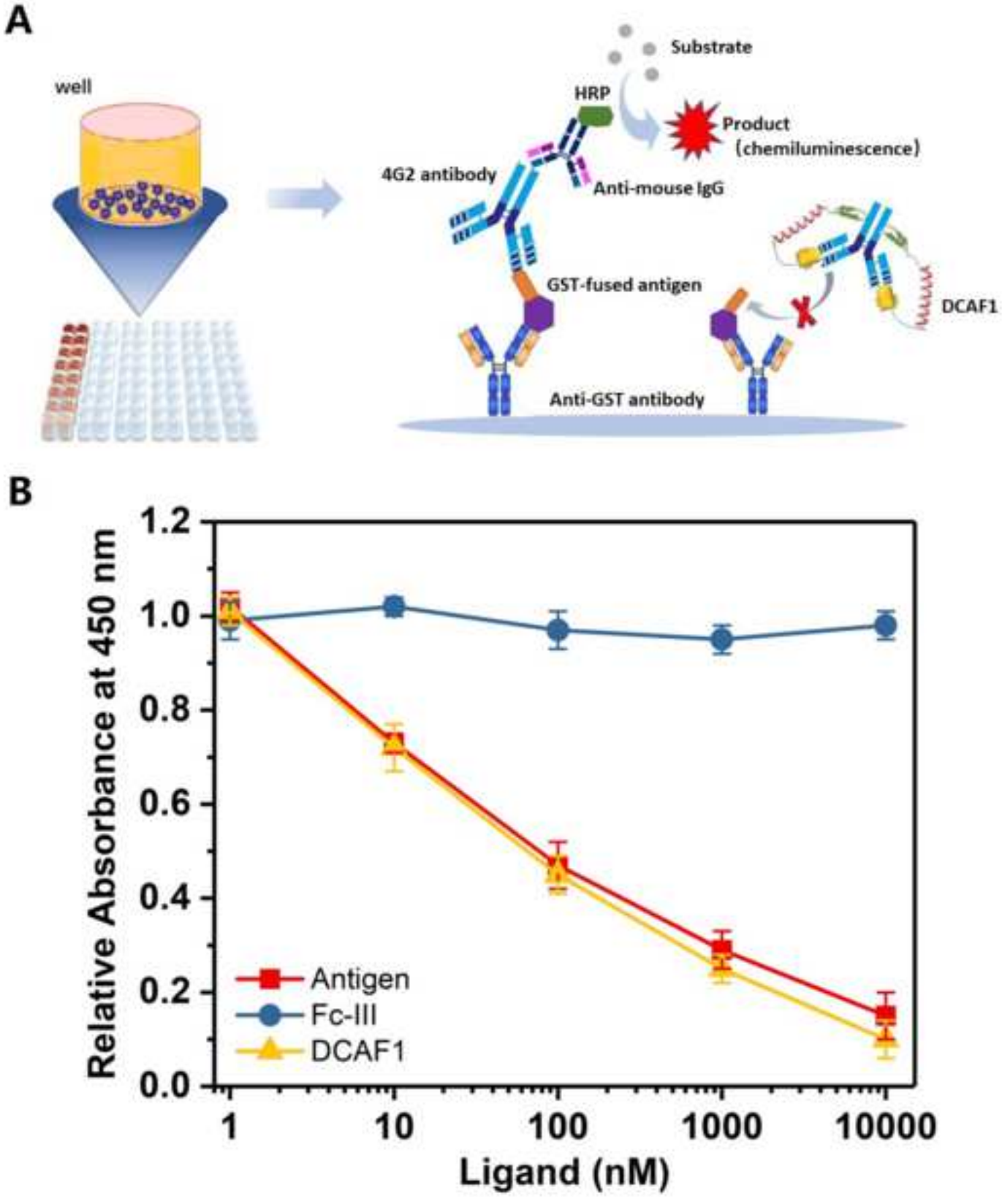


Fig 7



Name of Material/ Equipment	Company	Catalog Number
2-Chlorotrityl resin	Tianjin Nankai HECHENG S&T	
1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo-[4,5-b]pyridinium hexafluorophosphate 3-oxide	GL Biochem	00703
2-(6-Chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminiumhexafluorophosphate	GL Biochem	00706
2,2′ -Azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride	J&K Scientific	503236
4G2 antibody	Thermo	MA5-24387
4-mercaptophenylacetic acid	Alfa Aesar	H27658
96-well microtiter plates	NEST	701001
Acetonitrile	Thermo-Fisher	A955
AgOAc	Sinopharm Chemical Reagent	30164324
anti-GST antibody	Abclonal	AE001
Anti-mouse IgG, HRP-linked Antibody	Cell Signaling Technology	7076P2
BSA	Beijing DINGGUO CHANGSHENG BOITECHNOL	
CD spectrometer	Applied Photophysics Ltd	
dialysis bag	Sbjbio	SBJ132636
Dichloromethane	Sinopharm Chemical Reagent	80047360
diethyl ether	Sinopharm Chemical Reagent	10009318
DNA Gel Extraction Kit	Beyotime	D0056
Fusion Lumos mass spectrometer	Thermo	
GSH Sepharose	GE Lifesciences	
Guanidine hydrochloride	Sinopharm Chemical Reagent	30095516
Hydrazine hydrate	Sinopharm Chemical Reagent	80070418
Hydrochloric acid	Sinopharm Chemical Reagent	10011018
imidazole	SIGMA	12399-100G
Isopropyl β-D-Thiogalactoside	SIGMA	5502-5G
kanamycin	Beyotime	ST101
Methanol	Thermo-Fisher	A456
N, N-Diisopropylethylamine	GL Biochem	90600

N, N-Dimethylformamide	Sinopharm Chemical Reagent	8100771933
NcoI	Thermo	ER0571
PBS buffer	Solarbio	P1022
Peptide BEH C18 Column	Waters	186003625
piperidine	Sinopharm Chemical Reagent	80104216
Plasmid Extraction Kit	Sangon Biotech	B611253-0002
QIAexpress Kit	QIAGEN	32149
Rapid DNA Ligation Kit	Beyotime	D7002
Sodium dihydrogen phosphate dihydrate	Sinopharm Chemical Reagent	20040718
Sodium hydroxide	Sinopharm Chemical Reagent	10019762
Sodium nitrite	Sinopharm Chemical Reagent	10020018
sodium chloride	Sinopharm Chemical Reagent	10019318
Standard Fmoc-protected amino acids	GL Biochem	
sterilizing pot	Tomy	SX-700
SUMO Protease	Thermo Fisher	12588018
stop solution	Biolegend	423001
the whole gene sequence that can express SUMO-linker-antigen	Taihe Biotechnology Compay	
TMB reagent	Biolegend	421101
Trifluoroacetic acid	SIGMA	T6508
Triisopropylsilane	GL Biochem	91100
Tris(2-carboxyethyl)phosphine hydrochloride	Aladdin	T107252-5g
tryptone	OXOID	LP0042
Tween 20	Solarbio	T8220
Ultimate 3000 HPLC	Thermo	
vacuum pump	YUHUA	SHZ-95B
XhoI	Thermo	IVGN0086
yeast extract	OXOID	LP0021

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