

## Supporting Information

### **$\beta$ -Glucan as Immune Activator and Carrier in the Construction of a Synthetic MUC1 Vaccine**

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## **I. General Information**

### **Materials and Methods**

All commercial materials (Aldrich, NovaBioChem, GL Biochem, TCI, Adamas, Acros, J&K, Aladdin) were used without further purification. All solvents were reagent grade or HPLC grade (Fisher, Sigma, Acros). Anhydrous THF, diethyl ether, DCM, toluene, and DMF were commercially available and were purified and dried by passing through a PURE SOLV® solvent purification system (Innovative Technology, Inc.). All reactions were performed under an atmosphere of dry argon (g). Ultra-pure argon ( $\geq 99.999\%$ ) was used in all ligation reactions unless otherwise stated.

All separations involved a mobile phase of 0.05% TFA (v/v) in water (solvent A) and 0.04% (v/v) TFA in acetonitrile (solvent B).

Analytical LC-MS chromatographic separations were performed using a Waters Alliance e2695 Separations Module, an SQ Detector, and a Waters 2489 UV/Visible (UV/Vis) Detector equipped with a Beim Brueckle C4 column ( $5.0\ \mu\text{m}$ ,  $4.6 \times 150\ \text{mm}$ ) at a flow rate of  $0.4\ \text{mL/min}$ . The wavelengths of UV-detector were set to 210 nm and 220 nm.

Preparative HPLC separations were performed using a Hanbon Sci. & Tech. NP7005C solvent delivery system equipped with a Hanbon Sci. & Tech. NU3010C UV detector. HPLC separations were performed using a Beim Brueckle C4 column ( $10.0\ \mu\text{m}$ ,  $20 \times 250\ \text{mm}$ ) at a flow rate of  $16\ \text{mL/min}$ . The wavelengths of UV-detector were set to 210 nm and 220 nm.

Solid-state  $^{13}\text{C}$  CP/MAS NMR experiments were carried out at 9.4 T on a Bruker-Avance III HD 400 spectrometer, using a 4 mm double-resonance probe with Larmor frequencies of 400.2 and 100.65 MHz for  $^1\text{H}$  and  $^{13}\text{C}$ , respectively.

## II. General Procedures for Peptide Synthesis

### 2.1 Preparation of amino acid pre-loaded resin and determination of resin loading

#### *Pre-load an amino acid to 2-chlorotritylchloride resin*

The first Fmoc-amino acid residue was loaded to 2-chlorotritylchloride resin before Fmoc-SPPS following the general procedure below.

To a mixture of Fmoc-amino acid (1.0 equiv) and 2-chlorotritylchloride resin was added dry DCM (approx. 10 mL per gram of resin) and DIEA (4.0 equiv). The reaction was agitated for 2 hours. The resin was collected and washed with 17/2/1 (v/v/v) of DCM/MeOH/DIEA (×3), DCM (×3), DMF (×2), DCM (×3), and dried in vacuo for 12 hours before the loading test.

#### *Determination of resin loading<sup>S1</sup>*

Dry Fmoc amino-acid resin (approx. 5 μmol with respect to Fmoc) was weighted into a clean test tube, followed by the addition of 2 mL of 2% DBU in DMF. The mixture was agitated gently for 30 min, and then diluted to 10 mL with CH<sub>3</sub>CN. 2 mL of the resulting solution was taken out and diluted to 25 mL in a 50 mL centrifuge tube as the test solution. A reference solution was prepared in the same manner without the addition of resin.

Two matched silica UV cells were filled with reference solution to blank the U.V. spectrophotometer. The solution in one of the silica UV cells was changed to the test solution after washing with the test solution for three times. The optical density at 304 nm was recorded for three times and the average value was calculated as Abs<sub>sample</sub>. The Fmoc loading of resin could be calculated using the equation below:

$$\text{Fmoc loading: mmole/g} = \text{Abs}_{\text{sample}} \times 16.4 / (\text{mg of resin}).$$

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<sup>S1</sup> Peptide Synthesis, 2010/2011 Catalog, Merck.

## 2.2 Automated Solid-Phase Peptide Synthesis

Automated peptide synthesis was performed on a CS Bio peptide synthesizer (CX136XT).

Peptide synthesis was performed following the general protocol using DMF as solvent, deblocking (5 min  $\times$  2) in piperidine/DMF (20:80, v/v) containing Oxyma (0.1 M), and coupling for 25 min using HATU. For amino acids after steric hindered residues, the coupling cycle was repeated as needed.

The following *N*-Fmoc-protected amino acids from NovaBiochem were employed in SPPS: Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Pro-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Val-OH. The employed 2-chlorotritylchloride resin (1.147 mmol/g) in SPPS was purchased from GL Biochem.

### **III. Preparation of Peptide- $\beta$ -Glucan Conjugate**

#### **3.1 Preparation of MUC1 PEG-Linked Peptide**

Upon completion of automated synthesis of MUC1 peptide on a 0.02 mmol scale, the resin was washed into a peptide synthesis vessel with DCM. After removing the solvent, a solution of 9-[(9H-Fluoren-9-ylmethoxy)carbonylamino]-4,7-dioxanonanoic acid (1.2 eq.), HATU (1.2 eq.) and DIEA (1.2 eq.) in 2 mL of DMF was added, and the resulting mixture was agitated for 24 h. The reaction solution was then removed, and the resin was washed with DCM and DMF. The coupling of PEG linker was repeated once and reacted for another 12 h. The resin was then separated, washed with DCM, and subjected to a cleavage cocktail TFA/TIS/H<sub>2</sub>O (95:2.5:2.5, v/v) for 2 h. The resin was then removed by filtration, and the filtrate was concentrated under an argon atmosphere.

The residue was triturated with cold diethyl ether to give a white solid, which was then dissolved in a mixture of acetonitrile and water containing 5% acetic acid. The resulting solution was ready for HPLC purification after filtration. After purification, the peptide was lyophilized to give PEG-linked peptide **3** as a white powder (6.0 mg).

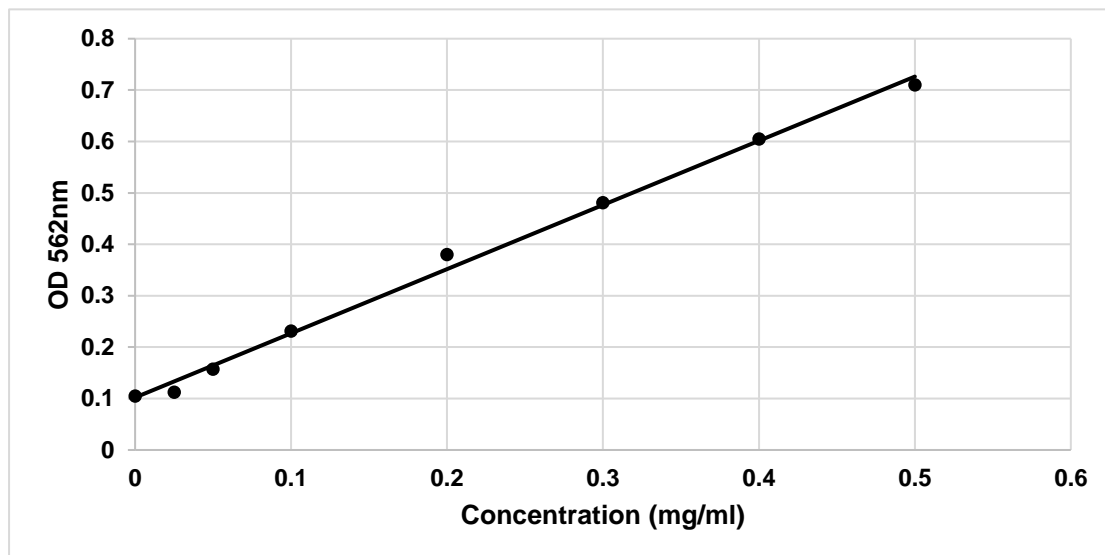
#### **3.2 Synthesis of Peptide- $\beta$ -Glucan Conjugate**

8 mg of commercially available linear  $\beta$ -1,3-glucan from yeast with MW ~20 kDa, was dispersed in 1 mL of DMSO, and activated with 1 mL of 1,1'-carbonyl-dimidazole solution (0.5 M in DMSO), followed by the addition of 2.0 mg of MUC1-PEG linked peptide. After stirring at 25 °C for 24 h, the reaction was diluted with 4 mL of water, and dialyzed for eight rounds within 48 h using a dialysis bag (1.5 kDa cut off) against water. The obtained solution was lyophilized to afford 1.8 mg of the  $\beta$ -glucan MUC1 conjugate as a white powder.

### 3.3 BCA method to Measured the Loading

The BCA Kit was employed to measure the loading of peptide antigen on the glucan.

The standard curve was established.

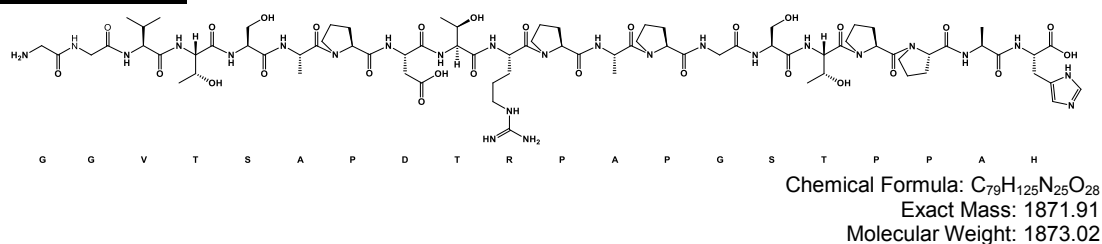


**Figure S1.** The standard curve of BCA method

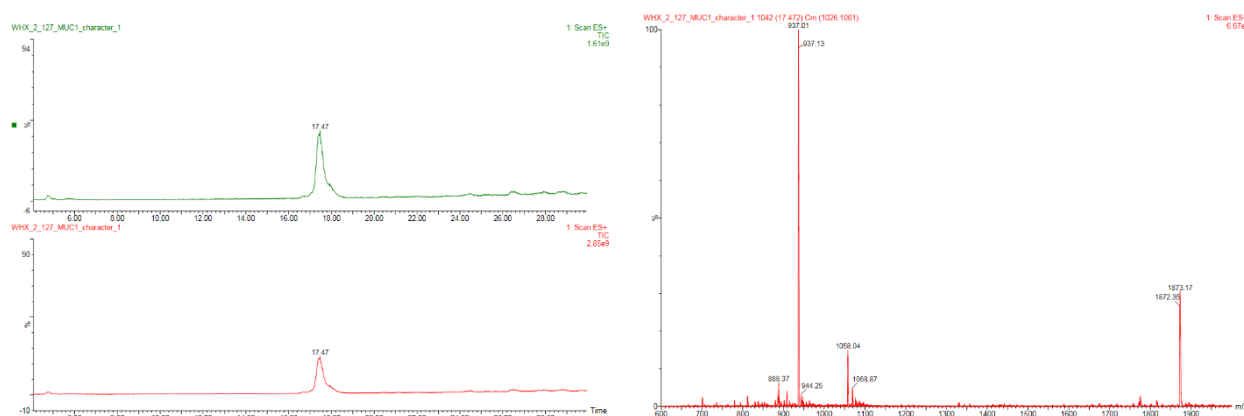
The calculated loading is 7.7  $\mu$ g/100  $\mu$ g

## IV. Preparation and Characterization of Peptide Segments

### MUC1 Peptide



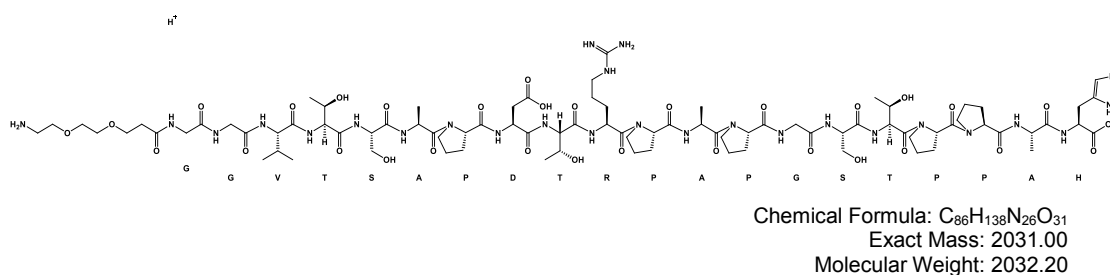
The MUC1 peptide was prepared following the General Procedure described above. The crude material was purified using preparative RP-HPLC (linear gradient 5-30% solvent B over 30 min, Beim Brueckle C4 column),  $t_R = 17.47$  min. The fractions were collected, and concentrated via lyophilization to provide product (24.0mg, 26%) as a white solid.



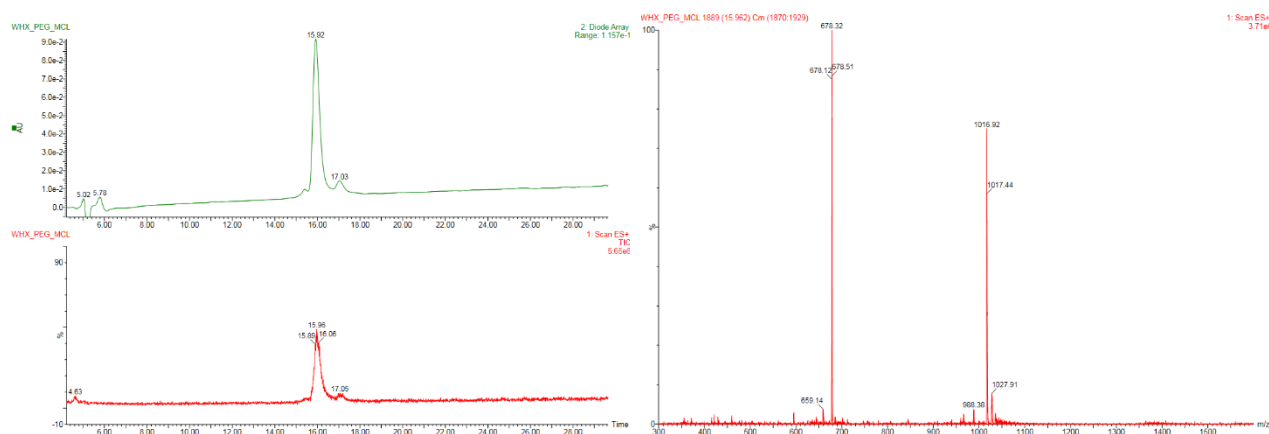
**Figure S2.** Left: UV and MS traces from LC-MS analysis of MUC1; Right: ESI-MS data of MUC1. Calcd for  $C_{79}H_{125}N_{25}O_{28}$ : 1871.91,  $(m/z)$   $[M+H]^+ = 1872.92$ ,  $[M+2H]^{2+} = 937.46$ ; found  $[M+H]^+ = 1873.17$ ,  $[M+2H]^{2+} = 937.13$ .



### MUC1 PEG-linked Peptide 3



The MUC1 PEG-linked peptide was prepared following the General Procedure described above. The crude material was purified using preparative RP-HPLC (linear gradient 5-30% solvent B over 30 min, Beim Brueckle C4 column),  $t_R = 15.92$  min. The fractions were collected, and concentrated via lyophilization to provide product **3** (6.0 mg, 15%) as a white solid.

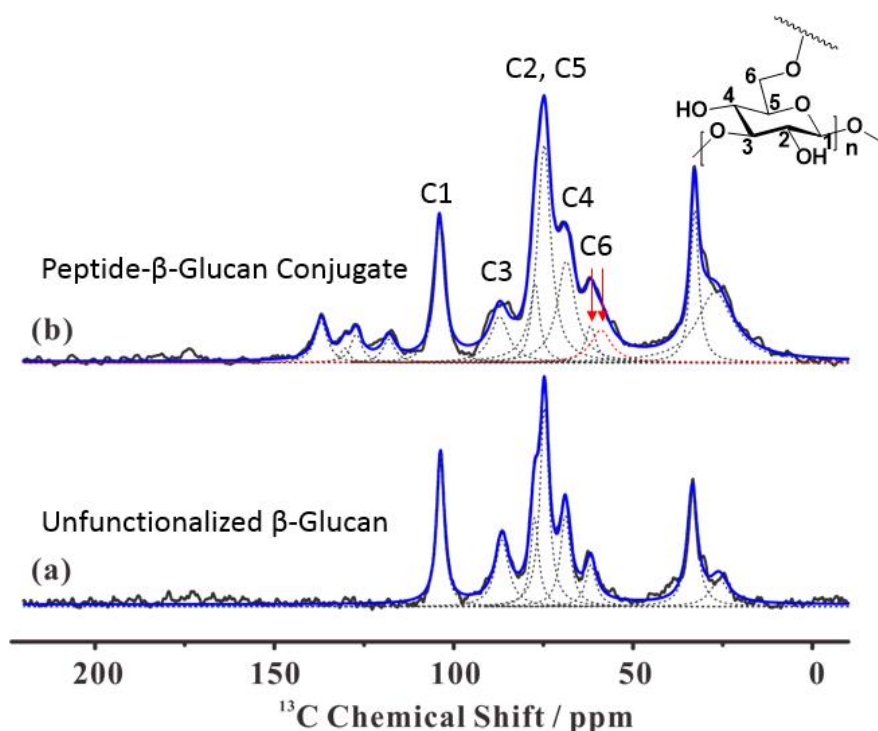


**Figure S3.** Left: UV and MS traces from LC-MS analysis of MUC1 PEG-linked peptide; Right: ESI-MS data of MUC1 PEG-linked peptide. Calcd for  $C_{86}H_{138}N_{26}O_{31}$ : 2031.00, ( $m/z$ )  $[M+H]^+ = 2032.20$ ,  $[M+2H]^{2+} = 1016.51$ ; found  $[M+2H]^{2+} = 1016.92$

## V. Characterization of Conjugate 1

### 5.1 Solid State Nuclear Magnetic Resonance (SSNMR) Experiment

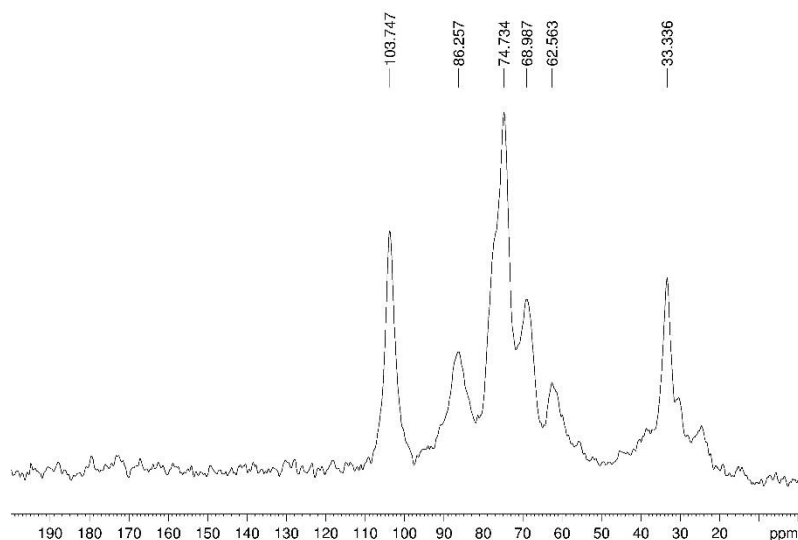
To confirm the covalent linkage between peptide and  $\beta$ -glucan,  $^{13}\text{C}$  solid-state Cross Polarization Magic-Angle Spinning (CP/MAS) NMR experiments were performed (Figures S5-7). By careful spectral deconvolution, the signals at ca. 104, 87, 77, 75, 69 and 62 ppm can be assigned to C1, C3, C2, C5, C4 and C6 of the glucose units in  $\beta$ -glucan or the conjugate, respectively (Figure S4),<sup>S1</sup> and obvious signals at ca. 137, 130, 127 and 118 ppm that correspond to the peptides could be observed (Figure S4b). Moreover, a small change of signals from C6 could be found after the introduction of peptide, where the peak at ca. 59 ppm becomes broader, and was assigned as the combined signals resulted from the C6 of glucose units with or without peptide derivatization.



**Figure S4.**  $^{13}\text{C}$  NMR spectra of (a)  $\beta$ -glucan, (b) Peptide- $\beta$ -glucan conjugate

<sup>S1</sup> E. R. Carbonero, A. H. P. Gracher, F. R. Smiderle, F. R. Rosado, G. L. Sasaki, P. A. J. Gorin and M. Iacomini, *Carbohydr. Polym.* **2006**, 66, 252-257.

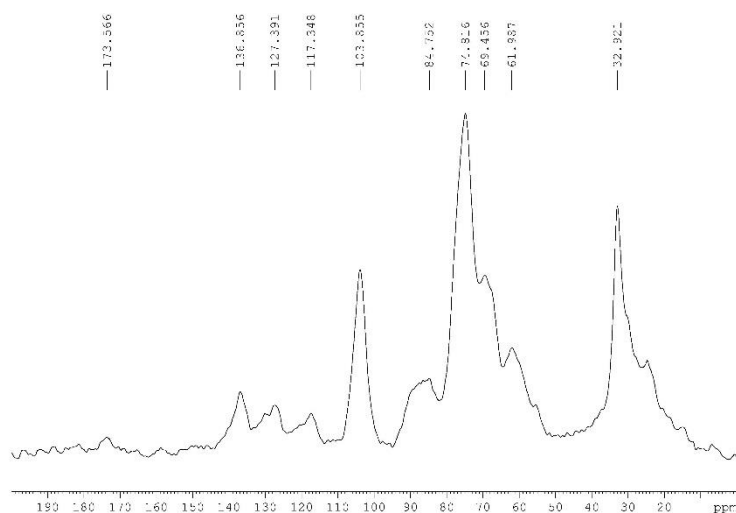
### Unfunctionalized $\beta$ -Glucan



**Figure S5.**  $^{13}\text{C}$  NMR spectra of  $\beta$ -glucan.

$^{13}\text{C}$  NMR (100 MHz):  $\delta$  103.747, 86.257, 74.734 (two carbons overlap), 68.987, 62.563, 33.336 (grease)

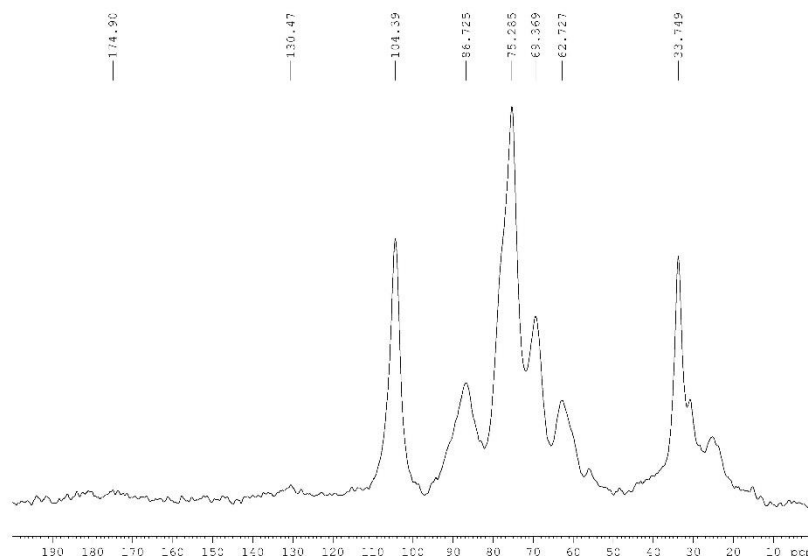
### Peptide- $\beta$ -Glucan Conjugate



**Figure S6.**  $^{13}\text{C}$  NMR spectra of peptide- $\beta$ -glucan conjugate

$^{13}\text{C}$  NMR (100 MHz):  $\delta$  173.566, 136.856, 127.391, 117.348, 103.855, 84.752, 74.816 (two carbons overlap), 69.456, 61.987 (two carbons overlap), 32.921 (grease)

### Peptide/ $\beta$ -Glucan Mixture



**Figure S7.**  $^{13}\text{C}$  NMR spectra of peptide/ $\beta$ -glucan mixture.

$^{13}\text{C}$  NMR (100 MHz):  $\delta$  174.90, 130.47, 104.39, 86.725, 75.285 (two carbons overlap), 69.369, 62.727, 33.749 (grease)

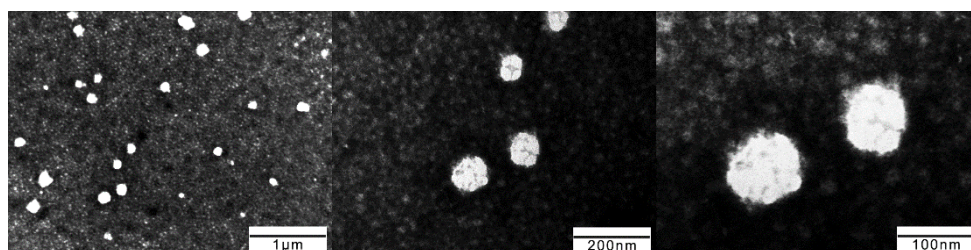
#### Experimental Section:

Solid-state  $^{13}\text{C}$  CP/MAS NMR experiments were carried out at 9.4 T on a Bruker-Avance III HD 400 spectrometer, using a 4 mm double-resonance probe with Larmor frequencies of 400.2 and 100.65 MHz for  $^1\text{H}$  and  $^{13}\text{C}$ , respectively. For the  $^1\text{H} \rightarrow ^{13}\text{C}$  CP/MAS NMR experiments, the Hartmann - Hahn condition was achieved by using hexamethylbenzene (HMB), and a total of 21500 free-induction-decay signals were accumulated with a contact time of 3.5 ms and a repetition time of 2.0 s. The chemical shifts for the  $^{13}\text{C}$  resonances were referred to HMB and the magic-angle spinning rate was set to 10 KHz.

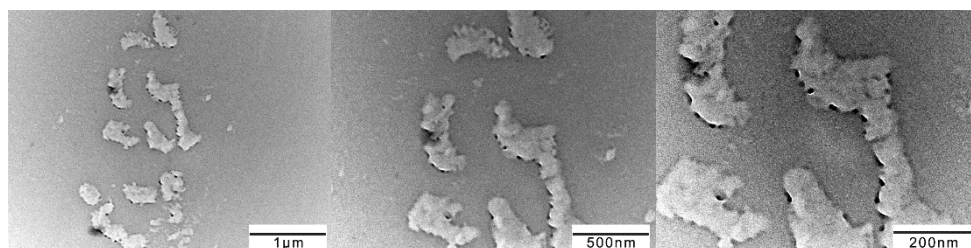
The deconvolution of  $^{13}\text{C}$  CP/MAS NMR spectra was performed using PeakFit (v4.12).

## 5.2 Electron Microscopy Analysis

20 $\mu$ L solution of conjugate and  $\beta$ -glucan on the copper grids of carbon support films for 1min 10second respectively. Then 20  $\mu$  L phosphotungstic acid was added for 1min. Awas added for 1min. After dried, copper grids were imaged on transmission electron microscopy.



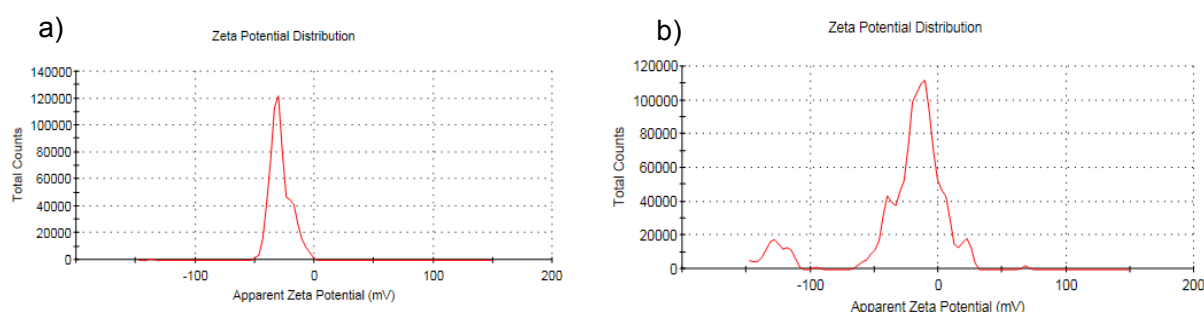
**Figure S8.** The TEM images of conjugate with different image scale. The uniform nano particles could be observed.



**Figure S9.** The TEM images of  $\beta$ -glucan with different image scale. The nano structure is irregular.

## 5.3 Dynamic Light Scattering and Zeta Potential Analysis

ZETASIZER NANO ZSP was employed to measure the dynamic light scattering of conjugate and  $\beta$ -Glucan. The result showed that the conjugate have better distribution than the  $\beta$ -glucan.

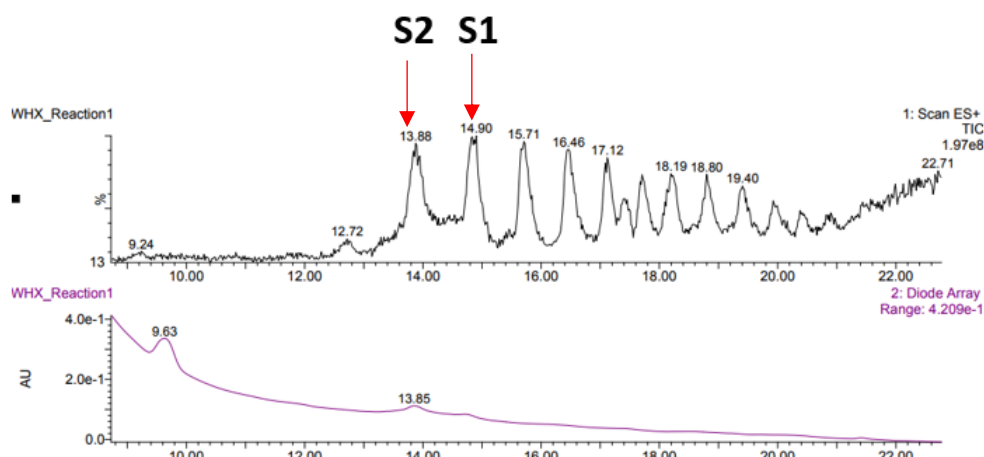


**Figure S10.** a) The Zeta potential of conjugate. b) The Zeta Potential of  $\beta$ -glucan. The absolute value and distribution showed that the conjugate is more stable and uniform than the  $\beta$ -glucan.

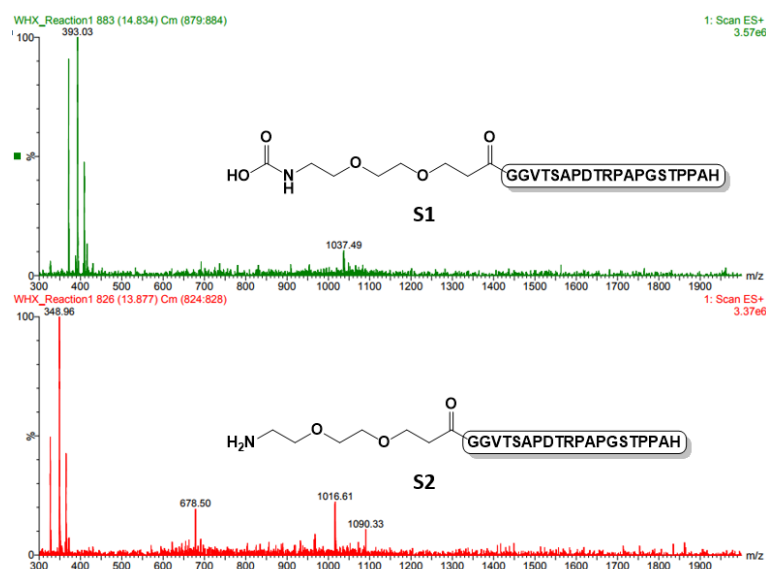
## VI. Hydrolysis Experiment of Peptide- $\beta$ -Glucan Conjugate

The conjugate was dispersed in HOAc/NaOAc buffer (pH=5.0) at a concentration of 1.25mg/ml, and the control group of MUC1 peptide was dissolved in HOAc/NaOAc buffer (pH=5.0) at a concentration of 25  $\mu$ g/ml. 1.0 mg of dextranase was added to each group, and the reaction was stirred at 50°C for 24 h, and analyzed using LC-MS.

### Peptide- $\beta$ -Glucan Conjugate Hydrolysis

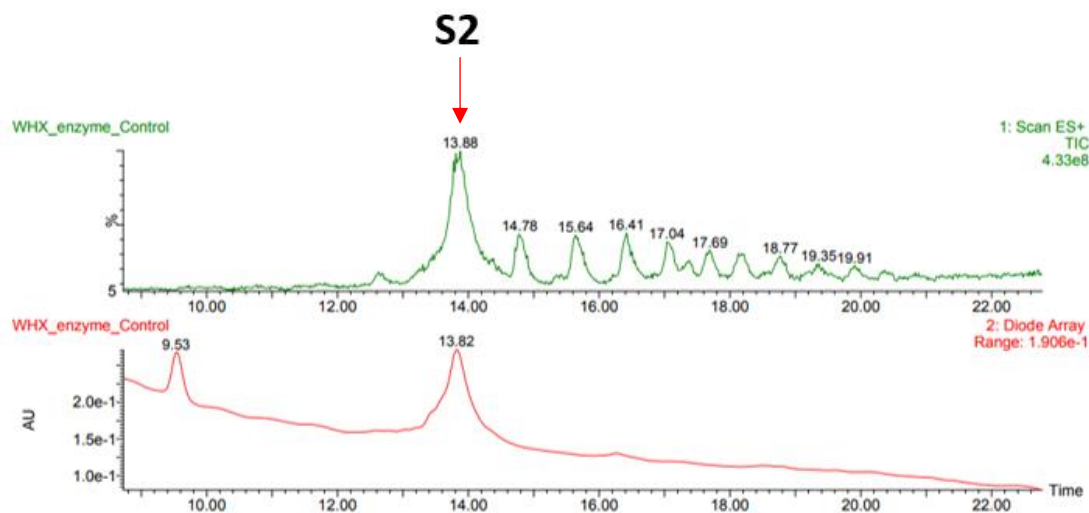


**Figure S11.** UV and MS traces from LC-MS analysis of hydrolysis experiment.

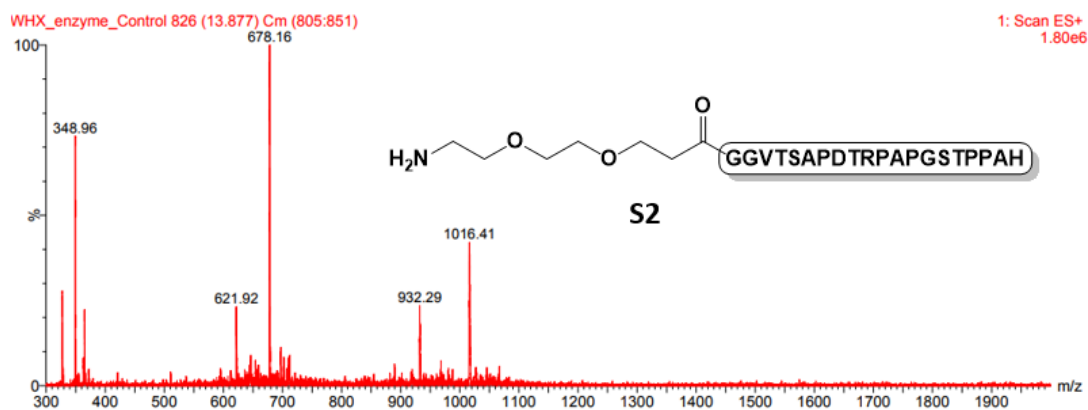


**Figure S12.** (a) ESI-MS data of peptide. Calcd for  $C_{86}H_{138}N_{26}O_{31}$ : 2031.00, ( $m/z$ )  $[M+H]^+ = 2032.20$ ,  $[M+2H]^{2+} = 1016.51$ ; found  $[M+2H]^{2+} = 1016.61$ , (b) ESI-MS data of peptidyl carbamic acid. Calcd for  $C_{87}H_{138}N_{26}O_{33}$ : 2074.99, ( $m/z$ )  $[M+H]^+ = 2076.00$ ,  $[M+2H]^{2+} = 1038.50$ ; found  $[M+2H]^{2+} = 1037.49$

## Peptide-Control Experiment



**Figure S13.** UV and MS traces from LC-MS analysis of control group.



**Figure S14.** (a) ESI-MS data of peptide. Calcd for  $\text{C}_{86}\text{H}_{138}\text{N}_{26}\text{O}_{31}$ : 2031.00, ( $m/z$ )  $[\text{M}+\text{H}]^+ = 2032.20$ ,  $[\text{M}+2\text{H}]^{2+} = 1016.51$ ; found  $[\text{M}+2\text{H}]^{2+} = 1016.41$

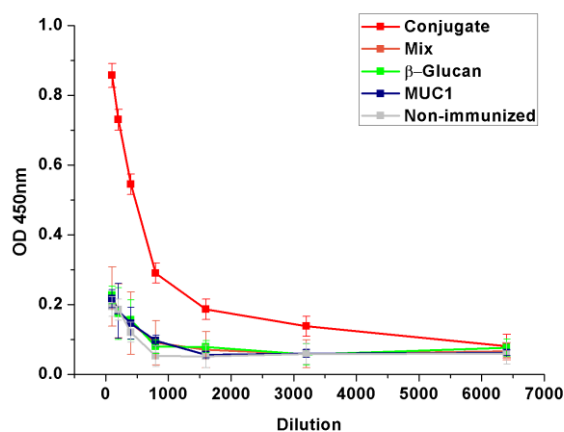
## VII. Evaluation of the Immune Response

### 6.1 Vaccination

6 weeks C57BL/6 mice were purchased from Peking University Health Science Center. 25 mice were divided into 5 groups, each group give intravenous injection of conjugate, mixture of MUC1 and  $\beta$ -glucan, MUC1,  $\beta$ -glucan and PBS buffer, 20  $\mu$ g of antigens per mice. Vaccination for four times at days 0, 7, 14, 21, the mice serum was collected to measure the anti MUC1 IgG and cytokines. Animals were well cared for and approved by Peking University Health Science Center.

### 6.2 Antibody (IgG) Titers

High-binding 96-Well ELISA plates were coated with MUC1 peptide (20 $\mu$ g/ml, 150 $\mu$ l/well) in Na<sub>2</sub>CO<sub>3</sub>, NaHCO<sub>3</sub> buffer (PH=9.6) overnight at 4°C. After washed three times by 0.05% Tween-PBS solution, the plates were blocked with 3% BSA solution. Then, the antiserum was diluted into different concentration and added to the plates (100 $\mu$ l/well). Incubated for 2h at 37°C. After washed three times, the plates were incubated with rabbit anti-mouse IgG-Peroxidase antibodies (SBA, 1:2500 dilution) for 1h at 37°C. Then, the plates were washed for three times and TMB substrate (Solarbio) was added and reacted at room temperature for 20min in dark. The absorbance at 450 nm was measured (Infinite M200 Pro). The antibody titers was calculated, and the groups immunized with synthetic conjugate showed a significant higher IgG titer.



**Figure S15.** Antibody titers of each groups



### 6.3 IL-6 and IFN- $\gamma$ ELISA Test

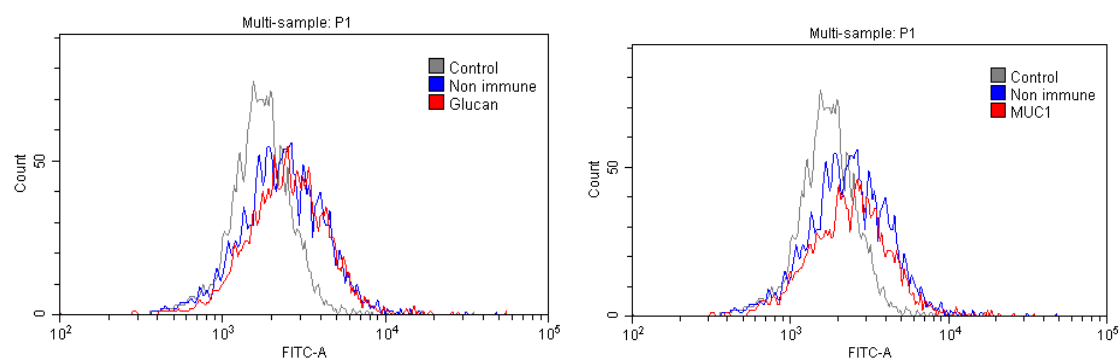
The ELISA kit was provided by JSKTSC and JIMEI. Prepared the serum into five fold dilution. Added the serum. Follow the procedure, the OD was measured (Infinite M200 Pro) the standard curve was established and the amount of IL-6 in the serum was measured.

### 6.4 Antibody isotypes analysis

The ELISA kit was provided by Proteintech. Prepared the serum into 50 fold dilution and added the serum to the ELISA kit. Follow the procedure, the OD was measured (Infinite M200 Pro).

### 6.5 FACS analysis

Human breast-tumor cells (MCF-7) were cultured in DMEM supplemented with 10%FBS.  $2.0 \times 10^5$  cells each sample were incubated with 1/20 antisera from each groups for 1.5h at 0°C. After washed three times with PBS, Fluorescein (FITC)-conjugated Affinipure Goat Anti-Mouse IgG(H+L) (Proteintech) was added for 1h. After washed three times with PBS, the fluorescence was measured by flow cytometry (cytoflex).



**Figure S16.** FACS analysis of the binding of antisera to MCF-7 cells. Grey line means the PBS solution, blue line means the non-immunized sera, and red line means antisera induced by the MUC1 only and  $\beta$ -glucan only respectively.