Conference paper

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Chitosan nanoparticles based nanovaccines for cancer immunotherapy

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Abstract: Cancer immunotherapy based on tumor vaccine is very promising and intriguing for carcinoma treatment. Herein, antitumor nanovaccines consisting of self-assembled chitosan (CS) nanoparticles and two-component mucin1 (MUC1) glycopeptide antigens were reported. Two different kinds of polyanionic electrolyte [sodium tripolyphosphate (TPP) and γ-poly-L-glutamic acid (γ-PGA)] were combined with chitosan polymers to fabricate the diameter of nearly 400–500 nm CS nanoparticles by electrostatic interactions. The nanovaccines were constructed by physically mixing MUC1 glycopeptide antigens with CS nanoparticles, which reduced vaccine constructing complexity compared with traditional chemical total synthetic vaccines. Immunological studies revealed that the CS/γ-PGA nanoparticle could dramatically enhance the immunogenicity of peptide epitope and produce significantly high titers of IgG antibody which was even better than Freund's adjuvant-containing vaccines.

Keywords: cancer immunotherapy; chitosan nanoparticle; ICS-28; IgG antibody; immune response; MUC1 glycopeptide; nanovaccine; peptide antigen.

Introduction

Nanovaccines, which consist of nano-scale based particles, are now been widely studied because of the adjuvant effects, biodegradablity, enhanced antigen bioavailability, controlled release and reduced side effects [1, 2]. These nanoparticle vaccines could be classified according to chemical components: liposome, inorganic nanoparticles, bio-degradable or non-degradable polymers, and amphipathic peptides et al. [3–5]. Recently, nanovaccines are widely applied in the cancer immunotherapy [6–13].

Immunotherapies against tumorous mucin1 (MUC1) are widely focused due to their safety and efficiency for cancer therapy. MUC1 glycoproteins are overexpressed on many epithelial tumor cells and specifically glycosylated as tumor-associated antigens [14]. 20-mer repeats of extracellular domain of human MUC1 glycoprotein, HGVTSAPDTRPAPGSTAPPA, have five potential glycosylation sites of Thr or Ser [15]. There is a beta-turn conformation in PDTRP domain [16, 17], and early research demonstrated that glycosylation in PDTRP domain played a critical role in immune response against MUC1 glycopeptide [18–20]. In design of MUC1 glycopeptide vaccines, the main challenge is how to improve low immunogenicity of MUC1 glycopeptide.

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Multi-component is an important strategy to improve the immunogenicity of MUC1 glycopeptide by building immune-stimulatory components into vaccine structure [21–23]. Conjugation of MUC1 glycopeptide and carrier protein can elicit a significant level of immune response due to high immunogenicity of carrier itself [19, 24–26]. But undesired antibodies might be produced against carrier protein, which may elicit significantly side effect. To solve this problem, T-helper cell epitope was separated from carrier protein and conjugated to MUC1 glycopeptide, which may increase the immune response of vaccines. Kunz and co-workers synthesized two-component vaccines containing a MUC1 glycopeptide and a T-helper cell epitope derived from ovalbumin, which increased the immune response [27]. In our previous work, two-component vaccines were developed to increase the immunogenicity of MUC1 antigens by adding T-helper cell epitope from tetanus toxoid into structures of vaccines [28]. Covalent multi-components, such as Toll-like receptor agonists, could increase the level of immune response of vaccines by activating innate immune system [29–40]. However, covalent construction of multi-component vaccines may lead to more complexity in structure of vaccines, which is not beneficial for chemical synthesis and further clinical applications.

It was believed that nanoparticles could be effectively up-taken by immune cells and activate innate immune system [41–45]. To simplify the structure of MUC1 glycopeptide vaccine, we prefer nanotechnology to construct multi-component antitumor vaccine. Chitosan (CS) is a positive charged polymer, which is derived from deacetylation of chitin. CS can dissolve in dilute acid to form a polycationic polymer due to protonation of amino group at C2 positon of glucosamine blocks [46]. And CS polycationic polymer could self-assemble to form spherical aggregates by electrostatic interactions when anionic compounds were carefully dropped into solution [47, 48]. Besides, CS was found to activate both humoral and cellular immune system [49], and CS nanoparticles were proven to be a safe and effective drug delivery system [50, 51], but still have not been applied in cancer immunotherapy. In this study, two kinds of polyanionic compounds [sodium tripolyphosphate (TPP) and γ-poly-L-glutamic acid (γ-PGA)] were carefully combined to CS solution to form CS nanoparticles, respectively.

Herein, we developed a series of glycopeptide epitope combined with CS nanoparticles to form antitumor nanovaccines. The MUC1 peptide was glycosylated by Tn antigen at the PDTRP domain (M3, Fig. 1a), which is the most immunogenic motif in MUC1 glycopeptide. And Tn modification of Thr in PDTRP increases the immunogenicity by stabilizing beta-turn comformation of PDTRP [52]. The T-helper cell epitope from tetanus toxoid ($TT_{947–967}$: FNNFTVSFWLRVPKVSASHLE) (T21, Fig. 1b) [28], was proven to improve the immune response of MUC1 glycopeptide vaccines in previous work of our group. The two-component peptide epitope

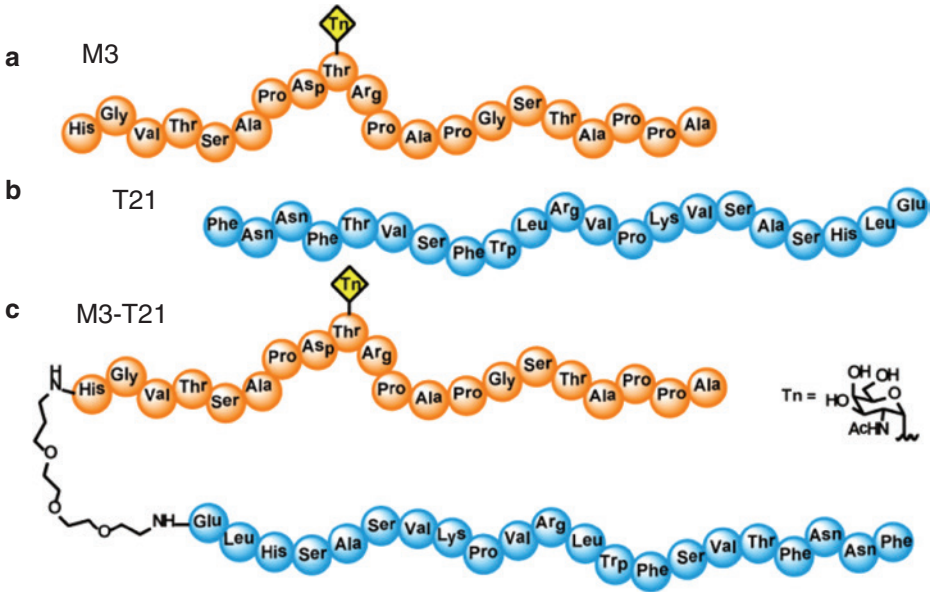

Fig. 1: Structures of antigens of M3 (a), T21 (b) and M3 – T21 (c).

(M3-T21) was the covalent link of M3 and T21 by a triethylene glycol spacer (Fig. 1c). The nanovaccines were constructed by physically mixing peptide antigens with CS nanoparticles, and then immunized with mice for immunological studies.

Experimental

Synthesis and characterization of glycopeptides

All the chemicals in this experiment were obtained from Sigma, Aldrich or Acros without further purification. Amino acids, coupling reagents and resins were purchased from GL Biochem. The synthesis of glycosylated amino acid building block Fmoc-Thr(αAc$_3$GalNAc)-OH was described previously [19, 53]. The 1H-NMR spectra was recorded by an Jeol-ECA-300 spectrometer using $CDCl_3$ as solvent with frequency of 300 MHz and the analytic data was reported in the Supplementary Material.

Peptides synthesis was performed by standard protocol on a Liberty CEM microwave peptide synthesizer. The natural amino acid building blocks were coupled to resin with 2-(1H-benzotriazole-1-yl)-1,1,3,3- tetramethylhexafluorophosphate (HBTU, 6.0 equiv)/N-hydroxybenzotriazole (HOBt, 6.0 equiv) as activator, N,N-Diisopropylethylamine (DIEA, 12.0 equiv) as activator base, DMF as solvent. For coupling glycoamino acid building block, 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU, 2.0 equiv)/1-Hydroxy-7-azabenzotriazole (HOAt, 2.0 equiv) were used as activator and N-methylmorpholine(NMM, 5.0 equiv) as activator base and N-Methylpyrrolidone(NMP) as solvent. The peptide was detached from resin by TFA/TIS/H_2O (20/1.2/1.2, v/v/v) for 2 h. After removing TFA, peptide was precipitated in diethyl ester and centrifuged with 6000 r/min for 10 min. The crude peptide was purified and analyzed by RP-HPLC on a Waters-600-2487 with solution A (80 % acetonitrile/water with 0.06 % trifluoroacetic acid) and solution B (100 % water with 0.06 % trifluoroacetic acid). Absorption signal of 215 nm were detected by UV detector. The MALDI-TOF MS spectra of peptides were recorded by an Applied Bio-systems 4700 Proteomics Analyzer 283 with the matrix of 2, 5-dihydroxybenzoic acid (DHB) or α-cyano-4-hydroxycinnamic acid (CHCA). The details of analytic data were shown in Supplementary Material.

Preparation and characterization of nanoparticle

Ten microgram of the purchased CS (sigma, medium molecular weight, 190~310 kDa, 75–85 % deacetylation degree) was dissolved in 9.7 mL 1 % acetic acid solution. Then 10 M NaOH solution was added slowly until the pH was 5.5 and the solution was filtrated by 0.2 μm filter membrane. Twenty seven microliter 1.5 % (w/v) sodium tripolyphosphate (TPP) solution or 24 μL 1.5 % (w/v) γ-poly-L-glutamic acid (γ-PGA) was dropwise mingled with a stirred 970 μL CS solution to form nanoparticles suspension. And under these amounts, the charge ratio of positive and negative charge was 2:1 in both nanoparticles. Keep stirring 2 h without separation.

After the nanoparticle suspensions were stirred for 15 min, 100 μL antigen solution contains 100 μg M3 or 100 μg M3 with 120 μg T21 or 228 μg M3 – T21 was dropwise added into the two nanoparticle suspensions, respectively. Keep stirring for another 2 h to form the nanovaccines. Otherwise, peptide antigens in 100 μL solution were also added into 900 μL CS solution as negative control experiments. Moreover, 480 μL CS solution and 600 μL Freund's adjuvant (FA) were combined with 100 μL peptide antigens solution to form water-in-oil emulsion as positive control (Table 1).

M3, M3/T21, M3-T21 were three kinds of peptide antigens, of which M3 was the MUC1 glycopeptide, M3/T21 was mixtures of M3 and a T-helper cell epitope T21, M3-T21 was covalent peptide antigen of M3 and T21. The CS/TPP NPs and CS/γ-PGA NPs were used as adjuvants to form nanovaccines. FA was Freund's adjuvant that used as positive control. CS were used as negative control.

Table 1: Immune groups were designed for immunological evaluation.

Vaccine	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12
Antigen	M3	M3	M3	M3	M3/T21	M3/T21	M3/T21	M3/T21	M3-T21	M3-T21	M3-T21	M3-T21
Adjuvant	CS	CS/TPPNPs	CS/γ-PGA NPs	CS/FA	CS	CS/TPP NPs	CS/γ-PGA NPs	CS/FA	CS	CS/TPP NPs	CS/γ-PGA NPs	CS/FA

Transmission electron microscopy analysis

Eight microliter CS/TPP or 8 μL CS/γ-PGA suspension solution was applied to copper grids of carbon support films for 1 min. Eight microliter phosphotungstic acid (15 mg/mL, pH 6–7) was added for 1 min for negative-stain. After drying overnight, the copper grids were imaged by Hitachi H-7650B transmission electron microscopy.

Dynamic light scattering and zeta potential analysis

Dynamic light scattering and zeta potential of CS/TPP and CS/γ-PGA nanoparticles suspensions were measured by the Malvern ZEN3690 Zetasizer apparatus.

Immunization

Balb/c mice of 4–6 weeks were purchased and bred in Animal Facility of Center of Biomedical Analysis, Tsinghua University. M3, M3/T21, M3-T21 antigens were dissolved in pure water and then added into CS solution, CS/TPP, CS/γ-PGA, and FA respectively (Table 1). From V1 to V12 group, each group was immunized with 200 μL solution of 10 nmol peptide antigens with or without FA by intraperitoneal injection. The first immunization was combined with Complete Freund's adjuvant, while boosted with Incomplete Freund's adjuvant. Each group consists of four Balb/c female mice. Mice were injected at intervals of 2 weeks for five times and the serum was collected 1 week after last immunization. Serum without immunization was negative control.

All animal use protocols performed in this study are according to the Institutional Animal Care and Use Committee(IACUC), and the Laboratory Animal Facility at the Tsinghua University is accredited by AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International).

Antibody titer measurement

High-binding 96-Well ELISA plates (Costar 3590) were coated with 20 μg/mL glycopeptide M3 (100 μL per well) in 0.1 M $NaHCO_3$ solution (pH = 9.6) overnight at 4 °C. Then each well was washed by PBS with 0.05 % (v/v) Tween-20 for 3 times, and then blocked by incubating 400 μL PBS with 0.25 % (w/v) gelation for 3 h at room temperature [52]. After another 3-times washing, the antiserum was diluted and added to each well for 100 μL and incubated for 1.5 h under 37°C. Washed 3 times, the 1:2000 diluted rabbit anti-mouse IgG-Peroxidase antibodies (Secondary antibody, sigma) was added and incubated for 1 h at 37 °C. After washed for 4 times, 100 μL OPD substrate (1 mg/mL o-Phenylenediamine in critic acid buffer (pH = 5), 1.5 μL/mL 30 % H_2O_2) was added to each well and incubated for 30 min at room temperature. Optical absorption was measured at 450 nm. Titers are defined as the highest dilution with 0.1 optical absorption or greater than the absorbance of negative control sera. Each sample was repeatedly measured for 3 times.

Isotype determination

High-binding 96-Well ELISA plates were coated with 20 μg/mL glycopeptide M3 (100 μL per well) in 0.1 M $NaHCO_3$ solution (pH = 9.6) overnight at 4 °C. Then each well was washed by the PBS with 0.05 % (v/v) Tween-20 for 3 times, and then blocked by incubating 400 μL PBS with 0.25 % (w/v) gelation for 3 h at room temperature [50]. After another 3-times washing, the antiserum was diluted to 1/50 and added to each well for 100 μL, then incubated for 1.5 h under 37°C. After washing, 100 μL of the 1:1000 diluted goat anti-mouse isotype antibodies (Secondary antibody, sigma), including goat antimouse IgG1, IgG2a, IgG2b, IgG3, IgM, was added and incubated for 1 h at 37 °C. After washed for 3 times, 100 μL of the 1:1000 diluted rabbit anti-goat IgG-Peroxidase antibodies (Third antibody, sigma) was added and incubated for 1 h at 37 °C. After washing for 4 times, 100 μL OPD substrate was added to each well and incubated for 30 min at room temperature. Optical absorption was measured at 450 nm. Each sample was repeatedly measured for 3 times.

Cell culture and flow cytometry analysis

Human breast cancer cell line MCF-7 purchased from Biodee Inc, Beijing were cultured in dulbecco's modified eagle medium(DMEM) culture medium supplemented with 10 % FBS at 37 °C. 1/25 diluted antisera of V10, V11, V12 and control groups were incubated with 2.0×10^5 cells per sample for 1 h on ice. After washing for 3 times, 1/50 diluted FITC-conjugated rabbit anti-mouse IgG antibody (DAKO) were added and incubated for 1 h on ice. After another 3-times washing, FACS analysis was measured on BD Calibur Flow cytometry in Center of Biomedical Analysis, Tsinghua University.

Statistical analysis

Data were presented as mean ± standard deviation (SD). A one-way ANOVA with Bonferroni's multiple comparison test was used to perform the statistical analysis between data of antibody isotypes. Significant differences were considered as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ and n.s. indicates no significant difference.

Results and discussion

As aforementioned, two kinds of polyanionic compounds were carefully combined to CS solution, respectively, to construct CS nanoparticles. The first was the solution of sodium tripolyphosphate (TPP), which represent the inorganic polyelectrolyte. The TPP solution was added to CS solution to form nano-aggregates by ionotropic gelation (CS/TPP) [54]. The other compound was γ-poly-L-glutamic acid (γ-PGA), which is a peptide-based polymeric polyelectrolyte. The CS nanoparticles were prepared by mixing solutions of CS with γ-PGA through complex coacervation (CS/γ-PGA) [55]. And make sure that the ratio of positive charge to negative charge was 2 to 1 in both kinds of nanoparticles [56]. Suspensions were formed in both methods when polyanionic solution was added into CS polycationic solution.

Both nanoparticles were analyzed by transmission electronic microscope (TEM), dynamic light scattering (DLS) and Zeta potential. We found that both CS nano-aggregates were at nearly 400–500 nm in transmission electronic microscope (TEM) and the CS/γ-PGA nanoparticle seemed to be little larger than CS/TPP (Fig. 2a and b). Meanwhile, dynamic light scattering experiments showed that the hydrodynamic diameter of both nanoparticles were about 500 nm (Fig. 2c and d), which were consistent with the TEM results. The polydispersity indexes (PDI) were 0.271 and 0.488, respectively. The zeta potential revealed that the positive charge was partially neutralized after CS polymer was self-assembled into nanoparticle and the surface charge of CS/γ-PGA nanoparticles was much smaller than CS/TPP nanoparticles (Figure S1). Meanwhile, CS/γ-PGA nanoparticles were observed to be more stable than CS/TPP nano-aggregates as the precipitation

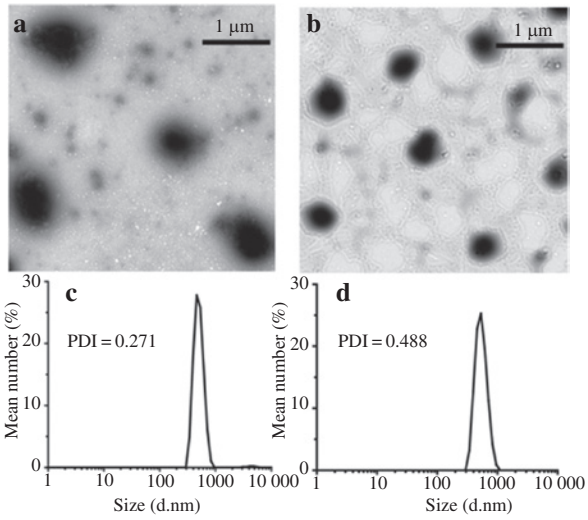

Fig. 2: Chitosan self-assembled into nanoparticles by adding TPP (a, c) and γ-PGA (b, d). TEM (a, b) and DLS (c, d) analysis of nanoparticles demonstrated that the size of both CS nanoparticles was nearly about 400–500 nm.

would be megascopic in CS/TPP suspension after 4 h, which may due to the less surface positive charges than CS/γ-PGA nanoparticles [57].

To explore the adjuvant property of the CS nanoparticles on glycopeptide based antitumor vaccine, we combined the CS/TPP and CS/γ-PGA nanoparticles suspensions with M3, M3/T21 and M3-T21 antigens to form antitumor vaccines, respectively. CS solution combine with antigens were used as negative controls. Freund's adjuvant combine with antigens were positive controls (Table 1). Firstly, we measured the DLS and zeta-potential of V2, V3, V6, V7, V10 and V11 groups, respectively (Table S1). The results indicated that there was not any influence on size and charge after combining different glycopeptide antigens into both CS nanoparticles. Meanwhile, none aggregates of M3 or M3/T21 or M3-T21 antigen epitopes were observed by TEM (Figure S2).

To address the immunological property of these nanovaccines, Balb/c mice were immunized by intraperitoneal injection on days 0, 14, 28, 42 and 56. Sera were collected on day 63. The antibody titer assay was administrated by enzyme-linked immunosorbent assay (ELISA). The results showed that all groups containing M3-T21 (V9–V12) elicited much stronger immune response than M3-containing groups (V1–V4) (Fig. 3),

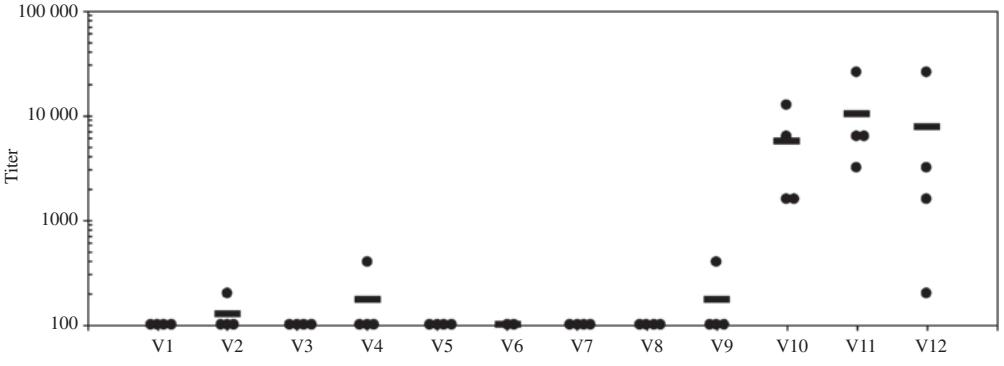

Fig. 3: Antibody titer assay of anti-MUC1 antibody elicited by vaccines from V1 to V12 by ELISA method. Each spot represents the serum of one mouse after fifth immunization. Black line represents the average value in each group. Vaccines containing M3 (V1 – V4) or M3 + T21 (V5–V8) elicited almost no immune responses. Vaccines containing M3-T21 elicited different level of immune responses. Titers are defined as the greatest dilution that yielded an optical absorption of at least 0.1 above that of negative control sera.

which revealed the importance of T helper cell epitope in effectively stimulating immune system. The M3/T21 groups (V5–V8) neither elicited low antibody titer, demonstrating that the T helper cell epitope should be covalently linked with B cell epitope (Fig. 3). Among M3-T21 containing groups (V9–V12), V9 group did not activate immune system effectively while the V10 and V11 group elicited high level immune responses (Fig. 3). This indicated that the CS polymer could not stimulate innate immune system only if CS nanoparticles were formed. The antibody titer of CS/γ-PGA group was higher than CS/TPP group. This may because that the stability of CS/γ-PGA was better than CS/TPP and the γ-PGA was believed to have some adjuvant effect [58, 59]. Furthermore, CS/γ-PGA group almost did not lead ascites after several intraperitoneal injections compared to FA group, which indicated that CS/γ-PGA nanoparticle might be an effective and safe adjuvant for MUC1 glycopeptide vaccines and better than Freund's adjuvant. Antibody titer assays of each group were shown in the Supplementary Material Figure S3–S14.

To evaluate the characters of immune response elicited by M3-T21 combined with CS nanoparticles, we administrated antibody isotype assay for mice antisera by ELISA method. The results demonstrated that M3-T21 combined with CS/γ-PGA nanoparticles (V11) elicited the highest level of antibodies of all isotypes among CS/γ-PGA, CS/TPP and FA groups (Fig. 4a–e). CS/γ-PGA group elicited significant IgG1 antibody compared to IgG2a, which means CS/γ-PGA might mainly activate Th2 mediated immune pathway [60], and consequently lead to humoral immune response. IgG2a and IgG2b were associated with activation of Th1 cells and cellular immune response. IgM may be produced by fast response of immune system in the early stage. CS/γ-PGA might be used to modulate immune response, such as balance between cellular and humoral immune response, which plays an important role in immunotherapy for different diseases.

To evaluate the binding character of antisera to tumorous natural MUC1 protein, MUC1-expressing tumor cell line of MCF-7 cells were incubated with antisera and consequently FITC-labeled anti-mouse IgG. We analyzed fluorescence intensity of MCF-7 by flow cytometry analysis (FACS) and the results showed that mouse antisera of CS/γ-PGA, CS/TPP and FA groups could not only bind synthetic MUC1 glycopeptide but also bind with natural MUC1 protein (Fig. 5), which proved the possibility to target MUC1 glycoprotein in vivo using synthetic MUC1 glycopeptide.

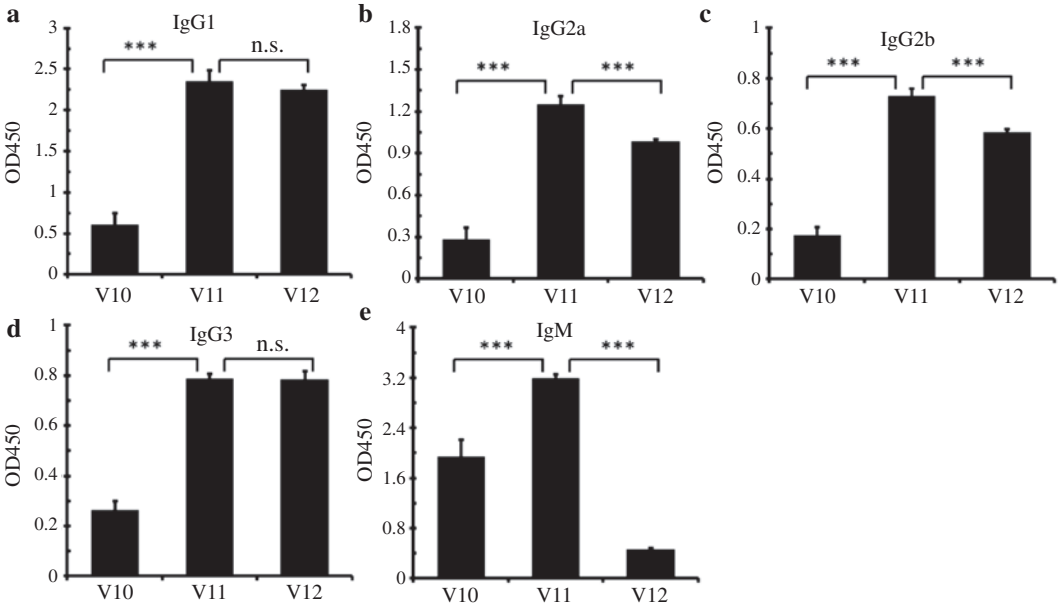

Fig. 4: Isotype of antibodies, IgG1 (a), IgG2a (b), IgG2b (c) IgG3 (d) and IgM (e), elicited by vaccines were analyzed by ELISA. The negative control of pre-immunization sera was cut off in the final results. All results represent the mean ± SD in each group (n = 3). Significant differences were considered as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ and n.s. indicates no significant difference.

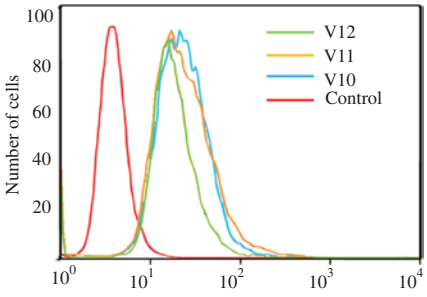

Fig. 5: The binding ability of antisera elicited by vaccines to MCF-7 cell line was analyzed by FACS.

Conclusions

In summary, we have developed a nanovaccine consisting of two-component MUC1 glycopeptide and self-assembled CS nanoparticles. We prepared CS nanoparticles by adding γ-PGA or TPP into CS solution and dissolved glycopeptide antigens into these suspensions to construct nanovaccines. Immunological studies revealed that simple combination CS nanoparticle and MUC1 glycopeptide antigen can dramatically improve the immunogenicity of peptide epitope and elicited high-level immune response and produced significant IgG1 antibodies. Moreover, CS/γ-PGA nanoparticle was more potent in stimulating immune system than CS/TPP and even better than Freund's adjuvant. Otherwise, covalently link T helper cell epitope with glycopeptide antigen was very important in eliciting strong specific immune response. Our results indicate that CS/γ-PGA nanoparticle could be a useful and safe adjuvant system for MUC1 glycopeptide based cancer vaccines.

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References

[1] T. D. Nandedkar. *J. Biosciences*. **34**, 995 (2009).
[2] T. M. Fahmy, S. L. Demento, M. J. Caplan, I. Mellman, W. M. Saltzman. *Nanomedicine-Uk*. **3**, 343 (2008).
[3] M. Skwarczynski, I. Toth. *Curr. Drug. Deliv.* **8**, 282 (2011).
[4] M. Skwarczynski, I. Toth. *Nanomedicine-Uk*. **9**, 2657 (2014).
[5] L. Yang, W. Li, M. Kirberger, W. Z. Liao, J.Y. Ren. *Biomater. Sci.* **4**, 785 (2016).
[6] J. M. Silva, M. Videira, R. Gaspar, V. Preat, H. F. Florindo. *J. Control. Release*. **168**, 179 (2013).
[7] Y. Krishnamachari, S. M. Geary, C. D. Lemke, A. K. Salem. *Pharmaceut. Res.* **28**, 215 (2011).
[8] A. L. Parry, N. A. Clemson, J. Ellis, S. S. Bernhard, B. G. Davis, N. R. Cameron. *J. Am. Chem. Soc.* **135**, 9362 (2013).
[9] K. K. L. Phua, S. K. Nair, K. W. Leong. *Nanoscale*. **6**, 7715 (2014).
[10] Y. Gao, Z. Y. Sun, Z. H. Huang, P. G. Chen, Y. X. Chen, Y. F. Zhao, Y. M. Li. *Chem-Eur. J.* **20**, 13541 (2014).
[11] Z. H. Huang, L. Shi, J. W. Ma, Z. Y. Sun, H. Cai, Y. X. Chen, Y. F. Zhao, Y. M. Li. *J. Am. Chem. Soc.* **134**, 8730 (2012).
[12] Z. Y. Sun, P. G. Chen, Y. F. Liu, B. D. Zhang, J. J. Wu, Y. X. Chen, Y. F. Zhao, Y. M. Li. *Chem. Commun.* **52**, 7572 (2016).
[13] S. Sungsuwan, Z. J. Yin, X. F. Huang. *ACS Appl. Mater. Interfaces*. **7**, 17535 (2015).
[14] R. E. Beatson, J. Taylor-Papadimitriou, J. M. Burchell. *Immunotherapy*. **2**, 305 (2010).
[15] R. Singh, D. Bandyopadhyay. *Cancer. Biol. Ther.* **6**, 481 (2007).
[16] K. Engelmann, S. E. Baldus, F. G. Hanisch. *J. Biol. Chem.* **276**, 27764 (2001).
[17] N. Ohyabu, H. Hinou, T. Matsushita, R. Izumi, H. Shimizu, K. Kawamoto, Y. Numata, H. Togame, H. Takemoto, H. Kondo, S. Nishimura. *J. Am. Chem. Soc.* **131**, 17102 (2009).
[18] J. A. Mollick, F. S. Hodi, R. J. Soiffer, L. M. Nadler, G. Dranoff. Cancer. *Immunity.* **3**, 3 (2003).
[19] H. Cai, Z. H. Huang, L. Shi, Z. Y. Sun, Y. F. Zhao, H. Kunz, Y. M. Li. *Angew. Chem. Int. Ed.* **51**, 1719 (2012).

[20] T. Matsushita, N. Ohyabu, N. Fujitani, K. Naruchi, H. Shimizu, H. Hinou, S. Nishimura. *Biochemistry*. **52**, 402 (2013).
[21] T. Buskas, P. Thompson, G. J. Boons. *Chem. Commun*. 5335 (2009).
[22] N. Gaidzik, U. Westerlind, H. Kunz. *Chem. Soc. Rev.* **42**, 4421 (2013).
[23] D. M. McDonald, S. N. Byrne, R. J. Payne. *Front. Chem.* **3**, 60 (2015).
[24] A. Hoffmann-Roder, A. Kaiser, S. Wagner, N. Gaidzik, D. Kowalczyk, U. Westerlind, B. Gerlitzki, E. Schmitt, H. Kunz. *Angew. Chem. Int. Ed.* **49**, 8498 (2010).
[25] B. Palitzsch, N. Gaidzik, N. Stergiou, S. Stahn, S. Hartmann, B. Gerlitzki, N. Teusch, P. Flemming, E. Schmitt, H. Kunz. *Angew. Chem. Int. Ed.* **55**, 2894 (2016).
[26] A. Xiao, X. J. Zheng, C. C. Song, Y. Gui, C. X. Huo, X. S. Ye. Org. Biomol. Chem. **14**, 7226 (2016).
[27] S. Dziadek, A. Hobel, E. Schmitt, H. Kunz. *Angew. Chem. Int. Ed.* **44**, 7630 (2005).
[28] H. Cai, M. S. Chen, Z. Y. Sun, Y. F. Zhao, H. Kunz, Y. M. Li. *Angew. Chem. Int. Ed.* **52**, 6106 (2013).
[29] S. Ingale, M. A. Wolfert, J. Gaekwad, T. Buskas, G. J. Boons. *Nat. Chem. Biol.* **3**, 663 (2007).
[30] H. Cai, Z. Y. Sun, Z. H. Huang, L. Shi, Y. F. Zhao, H. Kunz, Y. M. Li. *Chem-Eur. J.* **19**, 1962 (2013).
[31] H. Cai, Z. Y. Sun, M. S. Chen, Y. F. Zhao, H. Kunz, Y. M. Li. *Angew. Chem. Int. Ed.* **53**, 1699 (2014).
[32] B. L. Wilkinson, S. Day, L. R. Malins, V. Apostolopoulos, R. J. Payne. *Angew. Chem. Int. Ed.* **50**, 1635 (2011).
[33] A. Kaiser, N. Gaidzik, T. Becker, C. Menge, K. Groh, H. Cai, Y. M. Li, B. Gerlitzki, E. Schmitt, H. Kunz. *Angew. Chem. Int. Ed.* **49**, 3688 (2010).
[34] D. M. McDonald, B. L. Wilkinson, L. Corcilius, M. Thaysen-Andersen, S. N. Byren, R. J. Payne. *Chem. Commun.* **50**, 10273 (2014).
[35] V. Lakshminarayanan, P. Thompson, M. A. Wolfert, T. Buskas, J. M. Bradley, L. B. Pathangey, C. S. Madsen, P. A. Cohen, S. J. Gendler, G. J. Boons. *Proc. Natl. Acad. Sci. USA*. **109**, 261 (2012).
[36] T. H. Wright, A. E. S. Brooks, A. J. Didsbury, J. D. MacIntosh, G. M. Williams, P. W. R. Harris, P. R. Dunbar, M. A. Brimble. *Angew. Chem. Int. Ed.* **52**, 11686 (2013).
[37] P. Thompson, V. Lakshminarayanan, N. T. Supekar, J. M. Bradley, Peter. A. Cohen, M. A. Wolfert, S. J. Gendler, G. Boons. *Chem. Commun*. **51**, 10214 (2015).
[38] N. Martınez-Saez, N. T. Supekar, M. A. Wolfert, I. A. Bermejo, R. Hurtado-Guerrero, J. L. Asensio, J. Jimenez-Barbero, J. H. Busto, A. Avenoza, G. Boons, J. M. Peregrina, F. Corzana. *Chem. Sci.* **7**, 2294 (2016).
[39] L. Shi, H. Cai, Z. H. Huang, Z. Y. Sun, Y. X. Chen, Y. F. Zhao, H. Kunz, Y. M. Li. *ChemBioChem*. **17**, 1412 (2016).
[40] Y. H. Liu, W. P. Zhang, Q. Q. He, F. Yu, T. B. Song, Z. Q. Zhang, P. G. Wang, W. Zhao. *Chem. Commun.* **52**, 10886 (2016).
[41] B. S. Zolnik, A. Gonzalez-Fernandez, N. Sadrieh, M. A. Dobrovolskaia. *Endocrinology*. **151**, 458 (2010).
[42] P. D. Dwivedi, A. Tripathi, K. M. Ansari, R. Shanker, M. Das. J. Biomed. *Nanotechnol.* **7**, 193 (2011).
[43] M. Zaman, M. F. Good, I. Toth. *Methods*. **60**, 226 (2013).
[44] Y. Tao, Y. Zhang, E. G. Ju, H. Ren, J. S. Ren. *Nanoscale*. **7**, 12419 (2015).
[45] Y. Wen, J. H. Collier. *Curr. Opin. Immunol.* **35**, 73 (2015).
[46] C. K. S. Pillai, W. Paul, C. P. Sharma. *Prog. Polym. Sci.* **34**, 641 (2009).
[47] M. Dash, F. Chiellini, R. M. Ottenbrite, E. Chiellini. *Prog. Polym. Sci.* **36**, 981 (2011).
[48] S. Kumar, P. Garg, S. Pandey, M. Kumari, S. Hoon, R. Kapavarapu, P. H. Choung, A. J. F. N. Sobral, J. H. Chung. *J. Mater. Chem. B* **3**, 3465 (2015).
[49] I. M. van der Lubben, J. C. Verhoef, G. Borchard, H. E. Junginger. *Adv. Drug. Deliver. Rev.* **52**, 139 (2001).
[50] I. Behrens, A. I. V. Pena, M. J. Alonso, T. Kissel. *Pharmaceut. Res.* **19**, 1185 (2002).
[51] A. A. Zubareva, T. S. Shcherbinina, V. P. Varlamov, E. V. Svirshchevskaya. *Nanoscale*. **7**, 7942 (2015).
[52] P. Dokurno, P. A. Bates, H. A. Band, L. M. D. Stewart, J. M. Lally, J. M. Burchell, J. Taylor-Papadimitriou, D. Snary, M. J. E. Sternberg, P. S. Freemont. *J. Mol. Biol.* **284**, 713 (1998).
[53] H. Cai, Z. H. Huang, L. Shi, P. Zou, Y. F. Zhao, H. Kunz, Y. M. Li. Eur. *J. Org. Chem*. 3685 (2011).
[54] K. A. Janes, M. P. Fresneau, A. Marazuela, A. Fabra, M. J. Alonso. *J. Control. Release.* **73**, 255 (2001).
[55] Y. Hu, X. Q. Jiang, Y. Ding, H. X. Ge, Y. Y. Yuan, C. Z. Yang. *Biomaterials*. **23**, 3193 (2002).
[56] Y. Kang, C. Wang, K. Liu, Z. Wang, X. Zhang. *Langmuir*. **28**, 14562 (2012).
[57] A. A. H. A. Fuaad, Z. F. Jia, M. Zaman, J. Hartas, Z. M. Ziora, I. C. Lin, P. M. Moyle, M. R. Batzloff, M. F. Good, M. J. Monteiro, M. Skwarczynski, I. Toth. *Nanomedicine-Uk*. **9**, 35 (2014).
[58] T. Uto, T. Akagi, K. Yoshinaga, M. Toyama, M. Akashi, M. Baba. *Biomaterials*. **32**, 5206 (2011).
[59] T. Uto, X. Wang, K. Sato, M. Haraguchi, T. Akagi, M. Akashi, M. Baba. *J. Immunol.* **178**, 2979 (2007).
[60] F. Nimmerjahn, J. V. Ravetch. *Science*. **310**, 1510 (2005).

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