Bioconjugate Chemistry

Communication

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Glycopeptide Nanoconjugates Based on Multilayer Self-Assembly as an Antitumor Vaccine

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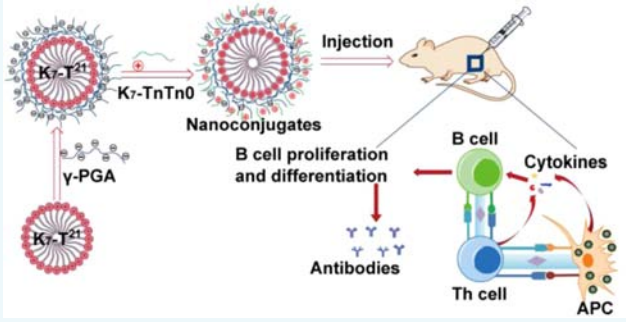
Supporting Information

ABSTRACT: Antitumor vaccine, which is promising for tumor therapy, has been extensively studied. Some encouraging results of chemically synthetic vaccine designs based on the tumor-associated antigen mucin 1 have been achieved. However, some shortcomings such as low efficiency and difficult purification restrict their clinical application. To overcome these difficulties, we designed a novel antitumor vaccine of glycopeptide nanoconjugates based on the multilayer self-assembly through the interaction of positive and negative charges. This vaccine formed the spherical structure and effectively activated the macrophage in vitro. Besides, it also induced high titer of antibodies against mucin 1 glycopeptide. The induced antibodies could highly bind to the tumor cells and effectively kill them by activation of the complement dependent cytotoxicity complex. This novel strategy provides a new way for the development of simple and effective antitumor vaccine.



Tumor immunotherapy, utilizing patients' own immune system to attack tumor cells with high selectivity and specificity, has become one of the most promising therapies.[1] The chemically synthetic vaccines are of vital importance in immunotherapy and have been studied extensively. These vaccines have confirmable structure and the homogeneous component. However, their immunogenicity is always weak. To solve this problem, some multicomponent vaccines were developed. For example, carrier protein,[2−8] helper T (Th) cell epitope peptide,[9,10] Toll-like receptor 2 ligand,[11−19] and monophosphoryl lipid A[20] were employed in the design of chemically synthetic vaccines to enhance the immunogenicity. Besides, the size effect of vaccines has been widely noted. Nanoscaled vaccines can be easily recognized and captured by antigen-presenting cells (APCs), and then can induce a robust immune response.[21] Several nanoscaled vaccine systems based on Q11 self-assembly peptide,[22] gold nanoparticle,[23] virus-like particle,[24] and water-soluble polymer[25] have been developed. Though these vaccines can elicit strong immune responses, their preparations are highly demanding. These have restricted the clinical application of these vaccines. So, the novel strategies with high efficiency and strong immune response are in high demand to address this problem.

Herein, we developed a new strategy for constructing an antitumor vaccine of glycopeptide nanoconjugates based on the multilayer self-assembly through the interaction of positive and negative charges (Figure 1a). We chose mucin MUC1, which is extensively overexpressed and aberrantly glycosylated on epithelial tumor cells,[26] as the specific antigen epitope, and MUC1 glycopeptide was extended to seven lysine residues at the N-terminal to offer the positive charge. Similarly, the Th cell epitope peptide **P1** derived from the tetanus toxoid[9] was used. Besides, we chose the immunostimulant γ-polyglutamic acid (γ-PGA)[27,28] to offer the negative charge.

For the preparation of the vaccine, the Th cell epitope peptide **P1** could first self-assemble to form spheres and the surface was positively charged, and the γ-PGA containing a large number of negative charges could be adsorbed on the spherical surface and the surface charge was negative. Then, B cell epitope MUC1 glycopeptide **P2** was added. These three components could self-assemble to form the nanovaccine through interaction between positive and negative charges. So the vaccine could be prepared facilely and efficiently by this strategy.

The Th cell epitope peptide **P1** was synthesized on solid phase on the basis of the established Fmoc protocol. Recent research demonstrated that the peptide motifs PDTRP and GSTAP of the MUC1 tandem repeat were highly immunogenic and the glycosylation at sites of Thr9 and Ser15 were very important.[3] Therefore, MUC1 glycopeptide **P2**, **P4**, and **P5** bearing Tn antigen at sites of Thr9 and Ser15 were synthesized (Figure 1b).

Received: March 22, 2015
Revised: June 5, 2015
Published: June 24, 2015

DOI: 10.1021/acs.bioconjchem.5b00150
Bioconjugate Chem. 2015, 26, 1439−1442

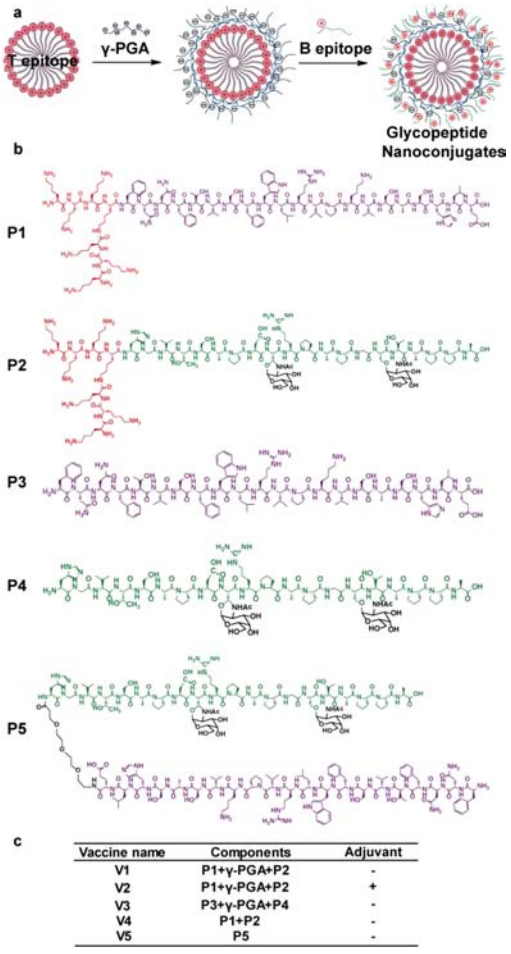


Figure 1. Multilayer self-assembly antitumor vaccine candidates. (a) Process of the vaccine assembly based on the interaction of positive and negative charges. (b) Structure of peptides **P1**−**P5**. **P1** represented the Th cell epitope peptide extended by seven lysine residues at the N-terminal. **P2** represented B cell epitope MUC1 glycopeptide extended by seven lysine residues at the N-terminal. **P3** represented the Th cell epitope peptide. **P4** represented the B cell epitope MUC1 glycopeptide. **P5** represented covalent two-component peptide. **P3** and **P4**, which lack seven lysine residues, were used for the negative control. Covalent peptide **P5** was used for the positive control. (c) Vaccine name and its components used in this study.

To obtain the nanovaccine **V1**, **P1** was first dissolved in water. Subsequently, γ-PGA (molecular weight 50−100 kD) and MUC1 glycopeptide **P2** were successively added. After being shaken for 5 min, the mixture was diluted to 1:10 with the phosphate buffer solution (PBS), and the 54 μM nanovaccine **V1** in terms of the B epitope MUC1 glycopeptide **P2** was formed. The concentration ratio of **P1**, γ-PGA, and **P2** was 1:7:1, and other vaccines **V2**−**V5** were prepared by the same method (Figure 1b,c). **V2** containing Freund's adjuvant was prepared to investigate the effect of adjuvants. **V3** lacking lysine residues and **V4** lacking γ-PGA were prepared as negative control groups. Covalent vaccine **V5** was prepared as positive control.

To evaluate the characteristics of vaccine candidates, transmission electron microscopy (TEM) experiments were performed. The result showed that **V1** formed a spherical assembly (Figure 2a). The peptide **P1** could form the sphere as well (Figure S1a). The TEM images of **V2**, **V3**, **V4**, and **V5** were also shown in Figure S1 and Figure S2. Then, the sizes of

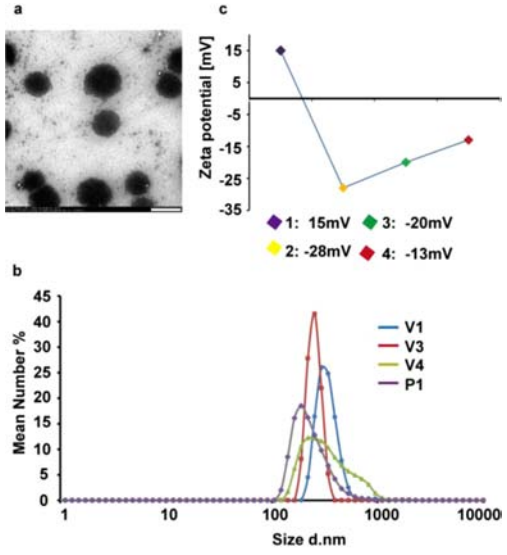


Figure 2. Characterization of the self-assembly nanovaccine. (a) TEM image of **V1** at concentration of 54 μM in terms of the B epitope MUC1 glycopeptide **P2** in PBS (scale bar: 500 nm). (b) DLS of **V1**, **V3**, **V4**, **P1**. Diameter size of **V1**, **V3**, **V4**, **P1** was, respectively, about 342 nm, 255 nm, 255 nm, 190 nm. (c) Zeta potential of different vaccine candidates. Purple 1 stands for **P1**. Yellow 2 stands for **P1**+γ-**PGA**. Green 3 stands for **P1**+γ-**PGA**+**P2**. Red 4 stands for **P1**+γ-**PGA** +2*P2.

vaccine candidates were determined by dynamic light scattering (DLS) assay. The result revealed that the hydrodynamic diameter of **V1** was about 355 nm, which was larger than the control groups **V3**, **V4**, and **P1** (Figure 2b). We speculated that it was caused by the self-assembly of **V1**. Furthermore, to observe the assembly process, the surface zeta potentials of particles were measured. We observed the surface zeta potential of **P1** changed from around 15 mV to around −28 mV by adding the γ-PGA, and the surface zeta potential of particles changed to −20 mV and −13 mV by adding equivalent and 2-fold MUC1 glycopeptide **P2** (Figure 2c). Through these results, we presumed that the nanovaccine **V1** was generated through multilayer self-assembly based on the interaction between positive and negative charges.

To determine the cellular uptake ability of **V1**, we first manufactured the fluorescence labeling nanovaccine **CF-V1** containing 5(6)-carboxylfluorescein labeled MUC1 glycopeptide **CF-P2** (Figure S3). Next, RAW264.7 cells were incubated with different concentration of **CF-V1** for 90 min. Flow cytometry analysis (FACS) showed that the cellular uptake ability of **CF-V1** was enhanced with the increase of the concentration (Figure 3a). Besides, we also incubated RAW264.7 cells with 54 μM **CF-V1** for various time. The result showed that the amount of intracellular **CF-V1** increased with increasing incubation time (Figure 3b).

The γ-PGA, which is an immune stimulant, can stimulate APCs to produce cytokines and activate the immune system.[27,28] To evaluate the immune effect of **V1** in vitro, we incubated RAW264.7 cells with 54 μM **V1** for 48 h. The production of cytokines was examined by ELISA. The result indicated that the production of IL-6 (Figure 3c) and IL-12 (Figure 3d) was greatly increased when RAW264.7 cells were stimulated by **V1** compared with the PBS group. The generation of IL-6 and IL-12 is of vital importance for inducing the effective humoral immunity and cellular immunity.[29,30]

DOI: 10.1021/acs.bioconjchem.5b00150
Bioconjugate Chem. 2015, 26, 1439−1442

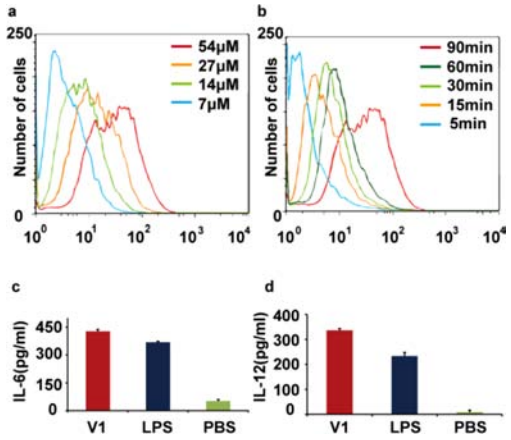


Figure 3. In vitro studies of nanovaccine **V1** based on RAW264.7 cells. Flow cytometry analysis of cellular uptake. (a) RAW264.7 cells were cultured with different concentration of **V1** for 90 min at 37 °C. The concentration was in terms of the B epitope MUC1 glycopeptide **P2**. (b) RAW264.7 cells were cultured with 54 μM **V1** for different time at 37 °C. (c) IL-6 and (d) IL-12 release from RAW264.7 cells cultured with 54 μM **V1** or 10 μg/mL lipopolysaccharide (LPS, Sigma-Aldrich). LPS was used as the positive control and PBS was used as the negative control. Data are reported as mean ± SD. The results are a representation of three separate experiments.

To examine the immune response of these vaccine candidates, Balb/c mice were immunized with different vaccines as shown in Figure 1c. Four mice per group were intraperitoneally injected with 100 μL vaccine candidate, which contained 25 μg MUC1 glycopeptide. Five-time injections were administered biweekly. One week after the last immunization, sera were collected and were used for immunological evaluation. The titers of the corresponding MUC1 glycopeptide antibodies were measured by ELISA. As shown in Figure 4a, the titer of IgG antibodies induced by **V1** was 5200, which was similar to the covalent vaccines **V5**, and the nonassembly vaccines **V3** and **V4** elicited very weak immune responses. Interestingly, we found that **V2**, which was immunized with Freund's adjuvant, induced weaker immune response compared with **V1**. We hypothesized that the introduction of Freund's adjuvant may destroy the multilayer self-assembly of the nanovaccine. Furthermore, we compared **V1** with our previously reported MUC1 vaccines[9,14,16,17,22] and we found that **V1** was more effective than others except the three-component vaccine (Figure S4 and Figure S5).

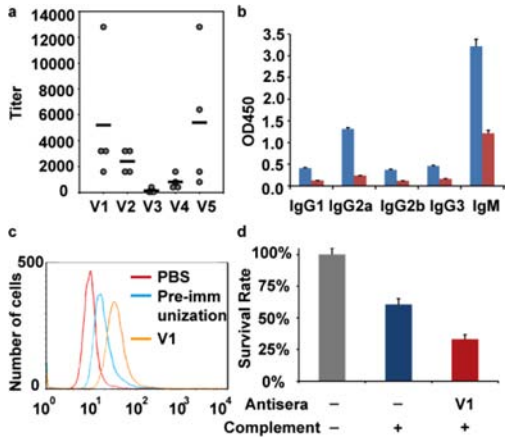


Figure 4. Immunological evaluation of nanovaccine **V1**. (a) Anti-MUC1 IgG titers of vaccines. Each spot stands for the antisera induced by one mouse after the last immunization. Black line stands for the average of each group. (b) Analysis of antibody isotypes. Blue histogram represents the antisera induced by **V1**; red histogram represents the preimmunization sera. (c) FACS analysis of the binding of antisera to MCF-7 cells. Red line means the PBS solution, blue line means the preimmunization sera, and orange line means antisera induced by **V1**. (d) Complement-dependent cytotoxicity of antisera induced by **V1** measured by MTT assay. Data are reported as mean ± SD. The results are a representative of four separate experiments.

To further evaluate the immune effect of these vaccines, isotypes of antibodies were analyzed by ELISA. The results showed that **V1** induced predominantly high levels of IgG2a and IgM (Figure 4b). IgG2a has a strong affinity for the FcγRI and has the effective killing effect. After injection, the nanovaccine V1 can be captured by APCs, which can secrete the cytokines such as IL-6 and IL-12 (Figure 3c,d). IL-12 can sequentially activate the type 1 T-helper cell (Th1) to produce IFN-γ, which can stimulate B cells to differentiate and generate IgG2a antibody.[30]

In order to observe the binding of antibodies to tumor cells, human breast-tumor cells (MCF-7) were incubated with the induced antibodies and measured by FACS. Antibodies elicited by **V1** showed obvious binding to the tumor cells (Figure 4c), while almost no binding was observed for control groups **V3** and **V4** (Figure S6). After binding to the MCF-7 cells, antibodies are presumed to mediate the killing of tumor cells through complement dependent cytotoxicity (CDC). The results demonstrated that the antibodies induced by **V1** successfully killed MCF-7 cells (Figure 4d).

In conclusion, a novel multilayer self-assembly vaccine was well designed and synthesized, based on the interaction of positive and negative charges. T-helper cell epitope peptide was employed as the core of the assembly. Immunostimulant γ-PGA was applied as the inner layer. MUC1 glycopeptide bearing Tn antigen at sites of Thr9 and Ser15 was used as the outer layer. This nanovaccine prepared by the aforementioned method can similarly elicit a robust immune response in wild type mice, and the induced antibody can specifically recognize MCF-7 cells and effectively initiate CDC to kill MCF-7 cells. This novel strategy may provide a new way for development of simple and effective antitumor vaccines.

■ ASSOCIATED CONTENT

Supporting Information

Detailed experimental methods, supplementary figures, synthesis, and identification of carbohydrate and peptides. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00150.

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Author Contributions

#Yan-Fang Liu and Zhan-Yi Sun contributed equally.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Prof. Host Kunz for useful discussions. We thank Prof. Xi Zhang for providing the Malvern ZEN3690 Zetasizer

DOI: 10.1021/acs.bioconjchem.5b00150
Bioconjugate Chem. 2015, 26, 1439−1442

apparatus and beneficial discussions. This work was supported by the Major State Basic Research Development Program of China (2013CB910700) and (2012CB821600), the National Natural Science Foundation of China (21332006 and 91313301), the Research Project of Chinese Ministry of Education (113005A) and the Open Project Program of National Engineering Research Center for Carbohydrate Synthesis.

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DOI: 10.1021/acs.bioconjchem.5b00150
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