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# a Totally Synthetic, Self-Assembling, Adjuvant-Free MUC1 Glycopeptide Vaccine for Cancer Therapy

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Supporting Information

**ABSTRACT:** In the development of vaccines for epithelial tumors, the key targets are MUC1 proteins, which have a variable number of tandem repeats (VNTR) bearing tumor-associated carbohydrate antigens (TACAs), such as Tn and STn. A major obstacle in vaccine development is the low immunogenicity of the short MUC1 peptide. To overcome this obstacle, we designed, synthesized, and evaluated several totally synthetic self-adjuvanting vaccine candidates with self-assembly domains. These vaccine candidates aggregated into fibrils and displayed multivalent B-cell epitopes under mild conditions. Glycosylation of Tn antigen on the Thr residue of PDTRP sequence in MUC1 VNTR led to effective immune response. These vaccines elicited a high level antibody response without any adjuvant and induced antibodies that recognized human breast tumor cells. These vaccines appeared to act through a T-cell independent pathway and were associated with the activation of cytotoxic T cells. These fully synthetic, molecularly defined vaccine candidates had several features that hold promise for anticancer therapy.

Many epithelial tumor cells overexpress MUC1 glycoprotein with aberrant glycosylation patterns. This protein has served as an effective target for cancer immunotherapy. The "variable number of tandem repeats (VNTR)" of MUC1, which comprises a 20-amino acid extracellular domain, can be used as a B-cell epitope. This sequence, HGVTSAPDTRPAPGSTAPPA, has several potential glycosylation sites at Ser/Thr residues.[1] Currently, many groups are endeavoring to develop effective anticancer vaccines on the basis of glycosylated MUC1 peptides. Because these glycopeptides are only weakly immunogenic, they have been conjugated to carrier proteins, such as bovine serum albumin (BSA)[2,3] or keyhole limpet hemocyanin (KLH),[4] to enhance the immunogenicity. Unfortunately, most of carrier proteins are highly immunogenic, and this might cause the suppression of antiglycopeptide antibody production.[5]

To avoid undesired immune response, fully synthetic molecularly defined vaccine candidates have shown promise, because only the elements required for relevant immune responses are incorporated into the constructs. Kunz developed a two-component vaccine with a B-cell epitope and a T-cell epitope from the carrier protein, ovalbumin (OVA).[6] Kunz[7] also constructed a tumor vaccine that contained a B-cell epitope and Toll-like receptor 2 (TLR2) ligand, which could stimulate the innate immune system.[8] Boons[9] constructed a three-component vaccine with a synthetic triacylated lipopeptide ($Pam_3CSK_4$) group, a short MUC1 B-cell epitope, and a T-epitope from polio virus; this led to a robust immune response. Our group constructed several multicomponent tumor vaccine candidates with click chemistry.[10]

Recently, Payne[11] used a simplified TLR2 ligand $Pam_3CS$ to construct three-component MUC1 vaccines without any adjuvant. Nevertheless, there are only a limited number of glycopeptide vaccines capable of inducing an effective immune response without external adjuvant. This has created a demand for chemically defined self-adjuvanting vaccines that can elicit both antibody production and cellular antitumor immune responses, particularly those with a mode of delivery compatible with human systems.

Herein, we designed and synthesized several vaccine candidates **H1**, **H2**, **H3**, and **H4**, that contained full-length MUC1 VNTR domains (**M1**, **M2**, **M3**, **M4**, respectively) conjugated to a self-assembly peptide sequence (Q11 domain)[12] (Figure 1). The Q11 domain could aggregate into fibers under mild conditions, and it served as both adjuvant and a vaccine carrier.[13] It was reported that the peptide motifs, PDTRP and PGST, in the VNTR domain of MUC1 formed a $\beta$-turn structure,[14] and were highly immunogenic compared to the other parts of the sequence; in fact, the Thr residues of PDTRP and PGST were glycosylated with the Tn antigen.

To determine the self-assembly character of **H1**, **H2**, **H3**, and **H4**, they were allowed to aggregate for 8 h at concentration of 400 $\mu$M in 6:1 mixture of pure water and PBS solution at room temperature. The aggregates were then analyzed with transmission electron microscopy; the results showed that **H1**, **H2**, **H3**, and **H4** aggregated into fibers over 200 nm long. The aggregates also displayed B-cell epitopes on the fiber surface.[13] The Tn modification at different sites had little influence on the self-assembly structure (Figure 2). Furthermore, **H3** did not aggregate during 36 h at the concentration lower than 50 $\mu$M, and **H4** did not aggregate during 36 h at the concentration lower than 100 $\mu$M (Supporting Information Figures S1–S12).

To evaluate the immune response of the vaccine candidates, **H1**, **H2**, **H3**, and **H4** were incubated in pure water overnight at 4 °C and then diluted in PBS solution for injection. Four mice

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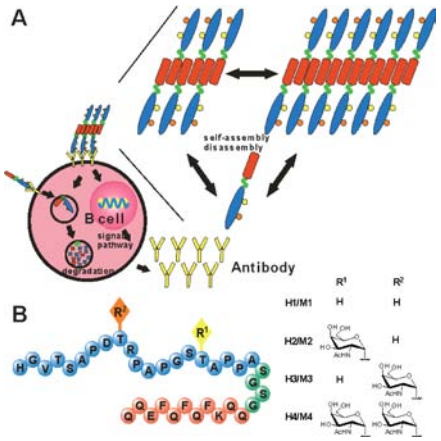


**Figure 1.** Design of self-adjuvanting vaccines, **H1**, **H2**, **H3**, and **H4**. (A)Vaccine candidates can self-aggregate into fibers and elicit activation of B cells. (B) The vaccine candidates **H1**, **H2**, **H3**, and **H4** include the 20-mer B-cell epitope **M1**, **M2**, **M3**, and **M4** from the MUC1 VNTR (peptide sequence in blue). Each vaccine has a different MUC1 glycosylation pattern (R, yellow and orange, R moieties are specified on the right), respectively, a self-assembly **Q11** domain (red), and a flexible spacer (green).

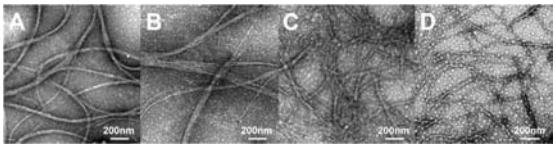


**Figure 2.** Vaccine candidates aggregated into fibrils. (A) **H1**, (B) **H2**, (C) **H3**, and (D) **H4** were prepared in a 400 $\mu$M solution, and allowed to aggregate for 8 h at room temperature. The aggregates were negatively stained with tungstophosphoric acid, and imaged with a Hitachi H-7650B transmission electron microscope.

per group were immunized with 100 $\mu$L of injection samples, which contained 100 nmol of self-assembly vaccine candidates. Injections were administered on days 0, 14, 28, 42, and 56. Sera were collected on day 63 and titers were measured by ELISA method. The results showed that **H3** elicited the highest immune response and the highest IgG titer was 6400 (Figure 3A). Compared to **H1** and **H2**, **H3** and **H4** were glycosylated with Tn antigen on the Thr residue of PDTRP, previously shown to be the most immunogenic domain. These results indicated that glycosylation on a specific site was necessary for an effective immune response, and confirmed the previous

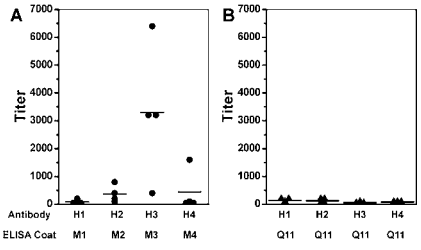


**Figure 3.** ELISA results of anti-MUC1 IgG titers elicited by different vaccines. Each spot represents the serum of one mouse after the fifth immunization. Black line represents the average value in each group. (A) **H1**, **H2**, **H3**, and **H4** elicited antibodies against the B-cell epitope, and (B) almost no antibody against **Q11**. Titers are defined as the greatest dilution that yielded an optical absorption of at least 0.1 above that of negative control sera.[9].

finding that the Thr glycosylation site of PDTRP showed the highest potential for development of a glycopeptide vaccine.[14,15] In addition, as expected, **H1**, **H2**, **H3**, and **H4** elicited almost no production of antibodies against the **Q11** peptide (Figure 3B).

Unlike the other adjuvants tested, such as TLR2 ligand and Monophosphoryl Lipid A,[16] **Q11** had a totally peptide backbone. Thus, it could be readily incorporated into vaccines during peptide synthesis. The linear T-cell epitope can also be incorporated into vaccines, but extra adjuvant is necessary to elicit an effective response and an emulsion delivery must be used to release antigens slowly.[17] In this study, the peptides were designed to aggregate into long fibrils, which presented multivalent B-cell epitopes on their surfaces, similar to antigen presentation when conjugated with carrier proteins[18] or virus-like particles.[19] A multivalent antigen may elicit a stronger B-cell response than a monovalent antigen, due to cluster effect.[20] Exclusion of a carrier protein or built-in adjuvant from the vaccine construct greatly simplifies the construct preparation and also eliminates undesired immune responses to the carrier proteins, which would dilute the immune focus on the target B epitope. Moreover, the balance between self-assembly and disassembly can achieve a slow release and long-term effect. In addition, a short peptide provides broad biocompatibility and biodegradability, with low toxicity. Interestingly, we found that when injected with Freund's adjuvant, these vaccines nearly lost the ability to elicit an immune response, with or without glycosylation (Figure S13 in Supporting Information). We hypothesized that an emulsion formed by Freund's adjuvant may have destroyed the long-fiber structure of self-assembled vaccine candidates, and this structure was most likely to be crucial in inducing the immune response. Thus, an external adjuvant may inhibit the immunological activity of a self-adjuvanting vaccine.[21] Moreover, a self-adjuvanting vaccine can effectively avoid adverse side effects caused by external adjuvants.

To characterize immune response further, the isotypes of antisera were evaluated by ELISA (Figure 4A–E) and binding of antibodies to MUC1-expressing MCF-7 human tumor cells was examined by flow cytometry (Figure 4F,G). The results showed that **H3** and **H4** elicited the higher levels of all antibody isotypes compared to **H1** and **H2**, and the antibodies elicited by **H3** and **H4** reacted strongly with MCF-7 cell line. In particular, **H3** and **H4** elicited significantly high levels of IgG2a and IgM, compared with the other isotypes. In the absence of a classical T-cell epitope, the **H3** and **H4** vaccine may have activated the immune system via a T-cell independent pathway.[22] Significant IgG2a and IgM levels corroborated this hypothesis. In this hypothetical pathway, T cells must be activated by B cell in another pathway that does not require T-cell epitope.[22] It has been shown that higher levels of IgG2a compared to IgG1 illustrated a preferential activation of the type 1 T-helper cell ($T_H1$) over the type 2 T-helper cell ($T_H2$). IgG2a production can be activated by the cytokine, IFN-$\gamma$,[23] which is secreted by type 1 helper T cells.[24] Thus, we hypothesized that **H3** and **H4** may stimulate $T_H1$ activation and, consequently, may mainly elicit IgG2a and activate cellular immunity through cytotoxic T lymphocytes. Cytotoxic T lympocytes are believed to play an important role in destroying tumor cells.[25] Moreover, IgG2a and IgM are better able to fix complement than other antibody isotypes in mouse,[26] and IgG2a has a high affinity for Fc$\gamma$RI on monocytes and macrophages, which may facilitate tumor depletion.[27] As a


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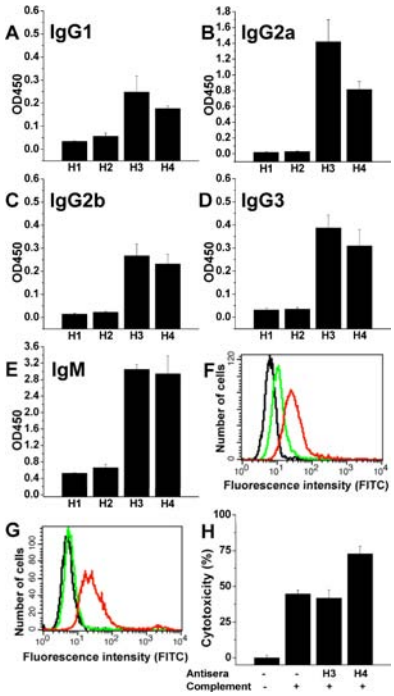


**Figure 4.** Determination of antibody isotypes elicited by vaccine candidates, and FACS analysis of the anti-MUC1 antibody. (A) IgG1, (B) IgG2a, (C) IgG2b, (D) IgG3, and (E) IgM elicited after immunizations with different vaccines were analyzed by ELISA. The negative control of antisera collected before immunization was cut off in the results. FACS analysis on MCF-7 cell was performed to evaluate the antibody elicited by (F) **H3** and (G) **H4** (red). PBS solution (black) and serum collected before immunization (green) as negative control. (H) Complement-dependent cytotoxicity measured by MTT method.

result, **H4** antisera effectively mediated complement-dependent cytotoxicity to kill MCF-7 cells (Figure 4H).

It was previously reported that aggregate-forming polyglutamine domains had the potential to act as an adjuvant for the immune system.[28] Thus, the self-assembling, glutamine-rich **H3** and **H4** may act in a similar way. Consequently, the **H3** and **H4** are expected to be safer, more biocompatible, and more biodegradable than other kinds of self-assembly vaccine delivery systems.

In conclusion, we designed and synthesized well-defined, self-adjuvanting MUC1 glycopeptide vaccine candidates. These vaccines comprised a B-cell epitope with different glycosylation patterns and a nonimmunogenic self-assembly domain. Immunological evaluations showed that the vaccines with Tn glycosylation in the PDTRP domain elicited significant immune response, and the induced antibody recognized human MUC1-expressing tumor cell, one of which mediated complement-dependent cytotoxicity against MCF-7 cells. Also, external adjuvant, like Freund's adjuvant, had an inhibitory effect on the vaccine. These totally synthetic MUC1 glycopeptide–**Q11** conjugate represents a novel vaccine candidate with simple and well-define formulation that should deserve further studies.

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

Detailed experimental procedures; immunization, ELISA and FACS method; and synthesis and identification of carbohydrate and peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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