Glycopeptide Vaccines

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Self-Adjuvanting Multicomponent Cancer Vaccine Candidates Combining Per-Glycosylated MUC1 Glycopeptides and the Toll-like Receptor 2 Agonist $Pam_3CysSer$**

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The aberrant glycosylation of cell surface mucin glycoproteins such as MUC1 and the overexpression of highly truncated, tumor-associated carbohydrate antigens (TACAs) are strongly correlated with tumor metastasis and poor prognosis.[1] Such TACAs, including the well-studied T_N and T antigens and their sialylated derivatives, are important targets for the development of antigen-specific carbohydrate vaccines.[2] However, these structures are self antigens that generally elicit T-cell independent immune responses which are tolerated by the immune system and lead to poor immunogenicity. One approach aimed at enhancing the immune recognition of TACAs utilizes conjugation of tumor-associated MUC1 glycopeptides or TACAs alone to foreign carrier proteins such as keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA).[3] Kunz and co-workers have described several impressive examples of MUC1 glycopeptide carrier protein vaccine candidates which have elicited significant antibody responses in murine models.[3d–h] While conjugate vaccines have shown promise in tumor vaccinology, the inherent immunogenicity of the carrier protein has been associated with suppression of the immune response towards the desired carbohydrate/glycopeptide epitope.[4]

Self-adjuvanting, multicomponent vaccines however, avoid anti-carrier immune responses while incorporating the necessary structural features for evoking an essential class switch from low-affinity and short lived immunoglobulin M (IgM) antibodies, to high-affinity immunoglobulin G (IgG) antibodies.[5–7] Recently, Boons and co-workers reported the synthesis and immunological activity of multicomponent vaccines incorporating the immunostimulating Toll-like receptor 2 (TLR2) ligand, $Pam_3CysSerK_4$[8] and a 12-amino acid peptide bearing a single copy of the T_N antigen as the B-cell epitope.[6] These multicomponent vaccines elicited high titers of IgG antibodies which were able to recognize epitopes on MCF7 cancer cells.[6a] It was reasoned that multicomponent vaccines incorporating the TLR2 ligand enhance local production of inflammatory mediators, leading to the upregulation of co-stimulatory proteins and the activation and regulation of the adaptive component of the immune system.[5,6,9]

Glycosylation of the MUC1 variable number tandem repeat (VNTR) sequence (GVTSAPDTRPAPGSTAPPAH) is an important feature for modulating the immunogenicity of MUC1 peptide epitopes with the aim of breaking self-tolerance and inducing humoral immunity.[3,10,11] Previous studies from the laboratories of Clausen and Livingston have shown that per-glycosylated MUC1 glycopeptides are able to elicit high IgG antibody titers when used in conjunction with a carrier protein and an external adjuvant.[10a,11] In addition, Kunz and co-workers have demonstrated that the peptide fragment (YSYFPSV) of the tetanus toxoid protein, when conjugated to MUC1 glycopeptide antigens, is capable of inducing proliferation of CD3+ and CD8+ cells.[13]

With these observations in mind, we were interested in exploring the use of synthetic multicomponent vaccines incorporating a per-glycosylated MUC1 peptide as the B-cell epitope covalently linked to an immunostimulating lipopeptide ($Pam_3CysSer$), with or without the tetanus toxin peptide as the helper T-cell epitope. We recently reported an efficient fragment condensation strategy for the construction of MUC1–lipopeptide conjugates employing pentafluorophenyl esters as the *N*-acyl donor.[12] Here, we exploit this strategy for the convergent assembly and immunological investigation of dicomponent vaccines **1a–c**, comprised of multiple copies of T_N or T antigens conjugated to the TLR2 ligand $Pam_3CysSer$, and tricomponent vaccines **2a–c** incorporating the tetanus toxin-derived helper T-cell epitope[13] between a per-glycosylated MUC1 B-cell epitope and $Pam_3CysSer$ (Scheme 1). We chose to install flexible, immunogenically silent ethylene glycol spacer units between each component to minimize conformational distortion and to allow for optimal display of each recognition element to the immune system.[7a,13]

The synthesis of the target vaccine candidates began with the preparation of the three requisite peptide fragments, namely the B-cell (glyco)peptide epitopes **3a–c**, tetanus toxin peptide **4**, and lipopeptide **5**. Synthesis of the completely deprotected MUC1 peptide **3a** and glycopeptides **3b** and **3c** bearing a per-glycosylated array of the T_N and T antigens,

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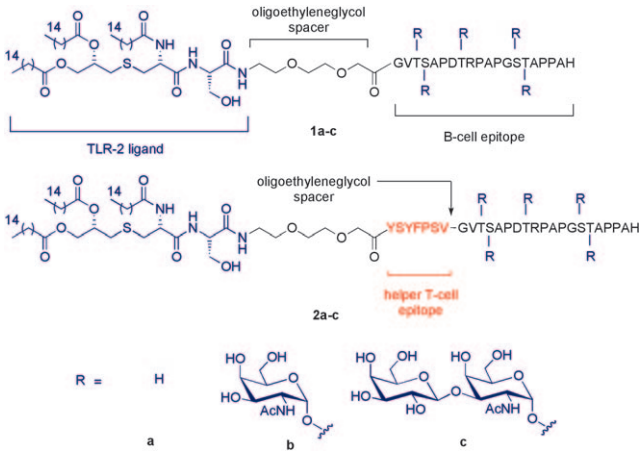
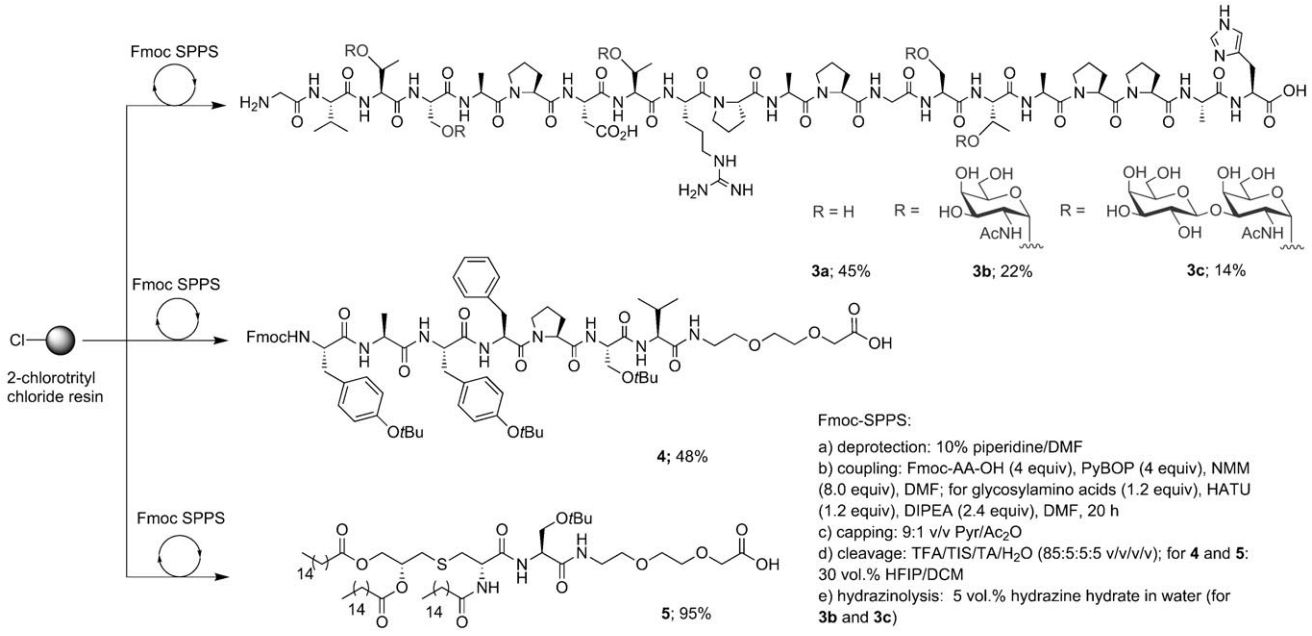


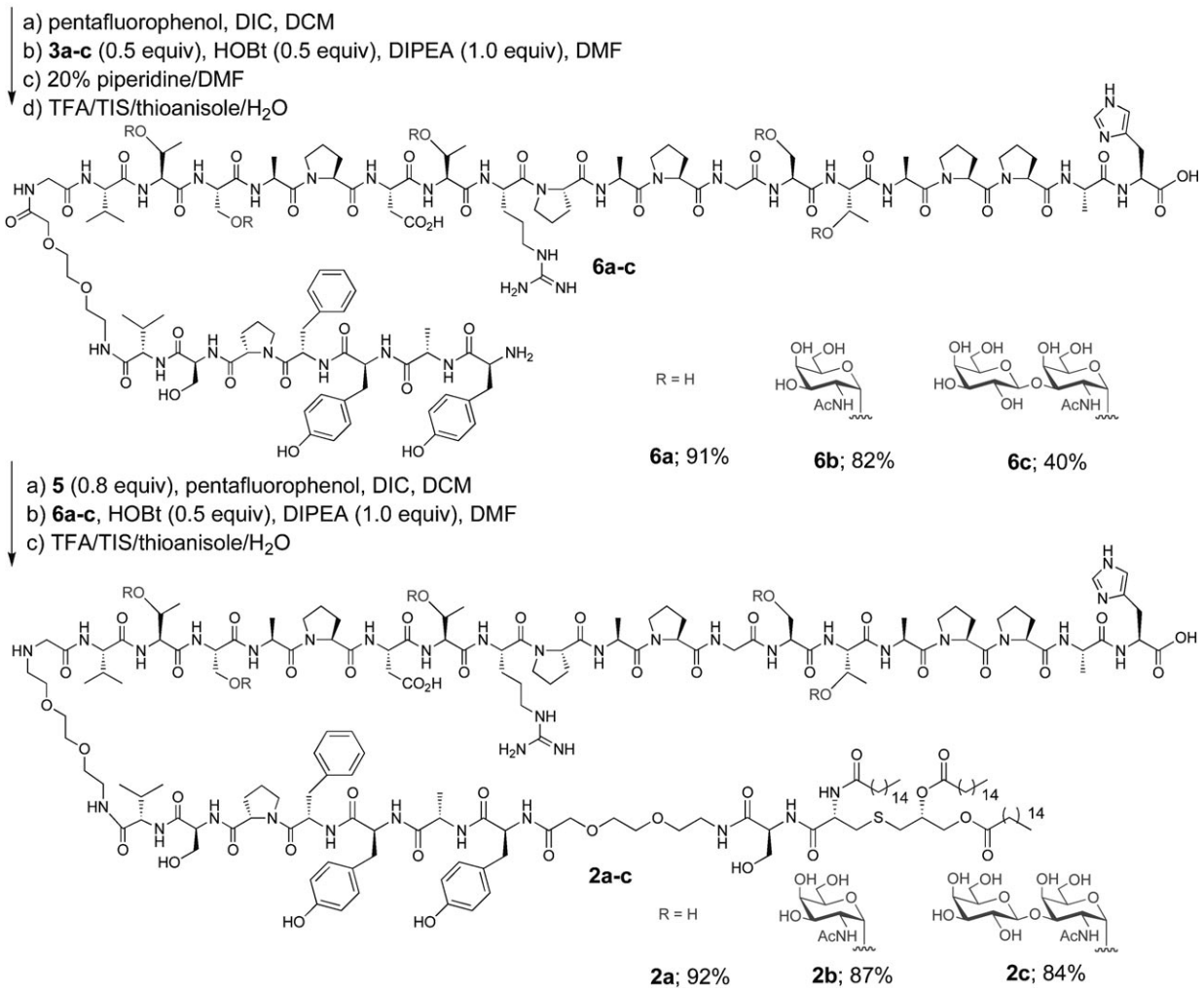
Scheme 1. Di- and tricomponent MUC1-based vaccine candidates **1a–c** and **2a–c**.

respectively, was achieved by Fmoc-strategy solid-phase peptide synthesis (SPPS) employing 2-chlorotrityl chloride resin preloaded with Fmoc-His(Trt)-OH (see Supporting Information and Scheme 2).[14] Cleavage from the resin and removal of the side-chain protecting groups was achieved using an acidic cocktail of TFA/TIS/thioanisole/water (85:5:5:5, v/v/v/v). The *O*-acetate protecting groups on the glycans of glycopeptides **3b** and **3c** were subsequently removed by hydrazinolysis. Purification by preparative reverse-phase HPLC afforded the target (glyco)peptides **3a–c** in 45%, 22%, and 14% yield, respectively, based on the original resin loading. Protected tetanus toxin T-cell epitope fragment **4** was also prepared by Fmoc-strategy SPPS. Following elongation, cleavage of the side-chain protected peptide from the resin using 30% hexafluoroisopropyl alcohol (HFIP) in DCM and subsequent precipitation afforded pure peptide **4** in 48% yield. Likewise, the lipopeptide fragment was assembled using Fmoc-strategy SPPS on 2-chlorotrityl chloride resin pre-loaded with Fmoc-protected triethylene glycolic acid. Mild acidic cleavage then furnished the protected lipopeptide **5** in 95% yield following purification by flash chromatography.

With the desired peptide fragments in hand, we next assembled the proposed vaccine candidates by pentafluorophenyl ester-mediated fragment condensations.[12,15] We have previously employed such an approach in the preparation of dicomponent vaccine candidates (**1a–c**) lacking the helper T-cell epitope. We now sought to examine the utility of this approach for the construction of tricomponent vaccine candidates **2a–c** employing two consecutive pentafluorophenyl ester-mediated fragment condensations (Scheme 3). To this end, treatment of the tetanus toxin fragment **4** with equimolar amounts of pentafluorophenol and *N*,*N*′-diisopropyl carbodiimide (DIC) afforded the crude pentafluorophenyl ester after 1 h (Scheme 3). Treatment of the crude ester with a solution of the respective MUC1 (glyco)peptides **3a–c** (0.5 equiv), HOBt (0.5 equiv), and DIPEA (1.0 equiv) in DMF furnished the partially protected conjugates. Subsequent deprotection and purification by semi-preparative reverse-phase HPLC furnished the MUC1–tetanus toxin conjugates **6a–c** in 40-91% yields. The homogeneous, completely deprotected fragments **6a–c** were subsequently conjugated to the lipopeptide **5** which was pre-activated as the pentafluorophenyl ester to afford tricomponent vaccine candidates **2a–c** in excellent yields (84–92%) after HPLC purification.



Scheme 2. Solid-phase peptide synthesis (SPPS) of peptide, glycopeptide, and lipopeptide fragments. Fmoc = 9-fluorenylmethoxycarbonyl; DMF = dimethylformamide; PyBOP = benzotriazolyl-1-oxy-tripyrrolidinophosphonium hexafluorophosphate; NMM = *N*-methylmorpholine; HATU = *O*-(7-azabenzotriazol-1-yl)-tetramethyluronium hexafluorophosphate; DIPEA = *N*,*N*-diisopropylethylamine; TFA = trifluoroacetic acid; TIS = triisopropylsilane; TA = thioanisole; HFIP = hexafluoroisopropyl alcohol; DCM = dichloromethane.



Scheme 3. Synthesis of MUC1 vaccine candidates **2a–c** through a fragment condensation approach. HOBt = 1-hydroxybenzotriazole.

Having successfully prepared di- and tricomponent vaccines **1a–c** and **2a–c**, respectively, we next evaluated their capacity to induce humoral immunity in murine models.[16] To this end, four C57BL/6 mice were immunized with 20 μg of each vaccine candidate in the absence of an external adjuvant. Booster immunizations were administered after days 10 and 17 before the mice were euthanized on day 31. Serum was taken after the second and third boosters for determination of IgG antibody titers, measured by their ability to bind immobilized MUC1 (glyco)peptides **3a–c** in an ELISA assay (see Supporting Information). Low-level antibody titers were obtained for the dicomponent vaccines **1a–c** following the second immunization. In the case of the unglycosylated construct **1a**, IgG titers were considerably increased after the third immunization (Figure 1). The analogous glycosylated vaccine constructs, **1b** and **1c**, also elicited significant IgG levels after the third immunization (reciprocal endpoint IgG titers were 1250 for **1b** and 937 for **1c**). However, IgG titers for **1b** and **1c** were significantly lower than the unglycosylated counterpart **1a**, highlighting the relative importance of exposed peptide epitopes combined with the TLR2 ligand for generating an immune response. It is proposed that the clustered arrangement of the T_N and T antigens on constructs **1b** and **1c**, respectively, may impede epitope recognition, leading to reduced immunoge-

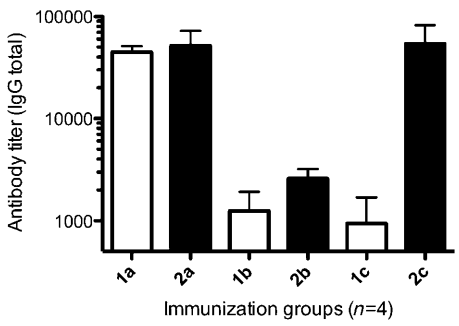


Figure 1. ELISA anti-MUC1 IgG reciprocal antibody titers after three immunizations for constructs **1a–c** and **2a–c.** Histograms represent the mean endpoint titers of the sera from four C57BL/6 mice.

nicity. Nonetheless, these constructs provided comparable titers to previously reported MUC1-based glycopeptide vaccine candidates using carrier proteins or immunoadjuvants, without the use of a liposomal formulation or an external adjuvant such as complete Freund's adjuvant (CFA).[3f,g,7a] This indicates that the $Pam_3CysSer$ adjuvant within these multicomponent vaccines is capable of inducing strong humoral immunity, resulting in significant IgG antibody titers. We were surprised to find that no IgM antibodies could be detected in the sera for vaccines **1a**–**c**. This suggests the establishment of immunological memory, consistent with a recent report from Kunz and co-workers with tetanus toxoid–MUC1 vaccines.[3h]

The tricomponent vaccine constructs **2a** and **2b**, incorporating the tetanus toxin helper T-cell epitope, proved to be significantly more immunogenic than the dicomponent vaccines following the second immunization and maintained higher IgG levels after the third immunization (reciprocal endpoint IgG titers were 52000 for **2a** and 2600 for **2b**). Tricomponent vaccine **2c**, bearing multiple copies of the T antigen, elicited impressive levels of IgG antibodies (reciprocal endpoint IgG titer 54600), higher than **2a** containing a completely exposed peptide epitope. Compared with the endpoint titer for the corresponding dicomponent vaccine **1c**, this result clearly demonstrates that, in the presence of a TLR2 ligand and a helper T-cell epitope, tricomponent MUC1-based glycopeptide vaccines bearing clustered glycosylation with the T-antigen can elicit high IgG antibody titers.

The serum antibodies induced by vaccines **2a**–**c** also exhibited selectivity for the (glyco)peptides to which they were raised (see Supporting Information). Unglycosylated MUC1 vaccine **2a** did not cross-react with the per-glycosylated glycopeptide epitopes **3b** and **3c**, suggesting a peptide-specific antibody response. In addition, antibodies generated from per-glycosylated tricomponent T_N antigen-containing vaccine **2b** did not bind to glycopeptide **3c**, bearing the T antigen. Antibodies elicited from vaccine **2c**, presenting multiple copies of the T antigen, exhibited weak binding to the unglycosylated peptide **3a** (reciprocal endpoint IgG titer 5400 as compared to 52000 for **3c**). Interestingly, anti-sera did show appreciable binding to glycopeptide **3b** (reciprocal endpoint IgG titer 18400), presenting multiple copies of the T_N antigen, suggesting that IgG antibodies can recognize the GalNAc moiety of these two TACAs, an observation also made by Franco and co-workers.[17] Finally, to ensure that the antibodies generated from tricomponent vaccines **2a**–**c** were capable of recognizing MUC1 epitopes expressed on tumor cells, binding of the anti-sera to the MUC1 expressing human breast cancer cell line MCF7 was investigated. Gratifyingly, sera from all three constructs exhibited significant binding to MCF7 (22–48% of cells bound) as determined by fluorescence-activated cell sorting (FACS) analysis (see Supporting Information). In contrast, anti-sera from **2a**–**c** did not bind to native MUC1 isolated from human breast milk,[18] suggesting that antibodies raised from these vaccines are tumor-selective and do not recognize the more heavily glycosylated MUC1 epitopes displayed on normal cells.

In summary, we have utilized a fragment condensation strategy for the high yielding assembly of a number of self-adjuvanting, multicomponent MUC1-based vaccine candidates. Immunological evaluation in murine models identified a number of key structural features required to invoke strong humoral immune responses which should greatly assist in the rational design of synthetic MUC1-based cancer vaccines in the future. Specifically, vaccines incorporating per-glycosylated MUC1 VNTR glycopeptides in combination with a T-cell helper peptide and the TLR2 agonist $Pam_3CysSer$ were able to elicit high levels of IgG antibodies in murine models in the absence of an external adjuvant, liposomal preparation or carrier protein. The anti-sera from tricomponent vaccines **2a**–**c** were shown to possess some selectivity for the (glyco)peptides to which they were raised and were capable of binding to epitopes presented on the surface of the MCF7 breast cancer cell line. Future work in our laboratories is focused on the evaluation of these vaccine constructs in MUC1-transgenic mice. In addition, the preparation of MUC1-based tricomponent cancer vaccine candidates bearing other TACAs, T-cell helper peptides, and synthetic immunoadjuvants is currently underway.

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