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Full Paper

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Preparation and Immunological Activity of Self-Adjuvanting Vaccines

Synthesis and Immunological Evaluation of Self-Assembling and Self-Adjuvanting Tricomponent Glycopeptide Cancer-Vaccine Candidates

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<pict> Supporting information for this article is available on the WWW under <url>http://dx.doi.org/10.1002/chem.201202629</url>.

Just add water! The synthesis of a number of tricomponent glycopeptide cancer-vaccine candidates is described. These vaccines contain a tumor-associated peptide or glycopeptide antigen covalently linked to a universal T-cell helper peptide and an immunoadjuvant. These vaccines spontaneously self-assembled in aqueous media to form stable nanoparticles and elicited a strong humoral immune response in mice models without the addition of an external adjuvant (see figure).<?><?>text and figure were shortened for space reasons, ok?<?><?>

Cancer

cancer
carbohydrates

glycopeptides
nanoparticles
vaccines

Self-adjuvanting tricomponent vaccines were prepared and assessed for their self-assembly and immunological activity in mouse models. The vaccines each consisted of a peptide or glycopeptide antigen that corresponds to a complete copy of the variable-number tandem repeat (VNTR) of the tumor-associated mucin 1 (MUC1) glycoprotein, the universal T-cell helper peptide epitope PADRE, and the immunoadjuvant $Pam_3CysSer$. The vaccines were shown to spontaneously self-assemble in water to form isotropic particles varying in size from 17 to 25^^nm and elicited robust humoral responses in murine models without the addition of an external adjuvant. The serum antibodies could recognize tumor-associated MUC1 epitopes on the surface of MCF7 breast-cancer cells and B16 melanoma cells, which overexpress this tumor-associated glycoprotein.

Introduction

Training the immune system to recognize and eliminate tumor cells through vaccination strategies is considered an extremely promising approach for the safe control of metastases in cancer patients.[1] An important feature of an effective vaccine-induced immune response is that it is cancer specific, a task made difficult by the ubiquitous presentation of antigens on both normal and cancer cells. The key to vaccine design has therefore centered on exploiting differences in expression levels and/or specific modifications of proteins found on cancer cells to mount a selective immunological attack against tumors. Mucin 1 (MUC1) is a transmembrane glycoprotein expressed on the apical surface of a range of epithelial cells and has a specific tumor-associated profile.[2] The extracellular section of MUC1, which extends almost 200^^nm above the cell surface, contains a VNTR domain comprised of 20 amino acid residues (GV*T*S*A*PD*T*RPAPG*ST*APPAH) with five sites for potential *O*-glycosylation.[2a] Although present on normal cells, MUC1 has been shown to be highly overexpressed in over 90^% of solid and nonsolid tumors, including carcinomas of the breast, colon, pancreas, prostate, ovary, rectum, and stomach.[2] Another notable feature of MUC1 is the aberrant glycosylation of the MUC1 VNTR domain on tumorogenic cells.[3] This alteration in the

glycosylation profile arises from the dysregulation of glycosyltransferase enzymes that serve to elongate *O*-glycan chains on normal cells.[4] This behavior leads to the presentation of highly truncated tumor-associated carbohydrate antigens (TACAs) on the surface of numerous epithelial cancer cells, including the well-studied T_N and T antigens and their sialylated derivatives (sialyl T_N and sialyl T; Figure^^1<figr1>).[2--4] These TACAs serve as crucial biomarkers for disease progression and have emerged as promising targets for the development of antigen-specific cancer vaccines.[2a,^5] However, immunization using TACAs alone has failed to provide adequate immune protection due to the fact that they are self-antigens and are tolerated by the immune system. A key challenge underpinning the successful development of a MUC1-based vaccine is the need to break immune self-tolerance to provide a durable cellular and humoral immune response, characterized by the induction of immunoglobulin G (IgG) antibodies and tumor-specific cytotoxic T-lymphocytes (CTLs) against TACAs and/or MUC1 (glyco)peptide epitopes.[5]

One approach is aimed at enhancing the immune response and has focused on the conjugation of TACAs and tumor-associated MUC1 (glyco)peptide fragments to immunogenic carrier proteins, such as keyhole limpet hemocyanin, bovine serum albumin, and tetanus toxoid.[5,^6] A number of groups of have reported impressive examples of MUC1 glycopeptide-carrier protein vaccines bearing an array of TACAs, which have elicited high immunological responses in mice models.[6d,^6e,^7] Although vaccines in this class have been shown to provide robust cellular or humoral immunity in animal models, they suffer from the production of strong B-cell immune responses toward the protein carrier, thus resulting in immune suppression toward the TACA or (glyco)peptide epitope. Furthermore, vaccines of this type often require administration as heterogeneous mixtures after supplementation with an external immunoadjuvant to generate the desired immune response.

Fully synthetic, multicomponent vaccines that incorporate tumor-associated MUC1 glycopeptides covalently linked to small-molecule immunoadjuvants with or without the presence of a T-cell helper peptide epitope have recently emerged as an attractive alternative to traditional conjugate vaccines.[5] These constructs are designed with the necessary immunogenic components to evoke a robust immune response and are synthesized as a single-

molecular species with high reproducibility, thus enabling unambiguous dissection of structure--activity relationships. In 2007, Boons and co-workers reported on a MUC1-based tricomponent vaccine that posseses a decapeptide fragment of the MUC1 VNTR bearing one copy of the T_N antigen on the threonine residue of the well-studied PDTR epitope of the repeat linked to a promiscuous T-cell helper epitope and $Pam_3CysSer(Lys)_4$ as the immunoadjuvant.[8] This vaccine, which was prepared as a lysosomal formulation, was shown to elicit a robust humoral and cellular immune response and exhibited a therapeutic effect in a mouse model of breast cancer.

More recently, we and others have reported the synthesis and immunological evaluation of other fully synthetic tricomponent vaccines based around this design concept.[9] In a recent study, we have shown that tricomponent (glyco)peptide vaccines that incorporate the full-length MUC1 VNTR, an immunogenic peptide fragment of the tetanus toxoid protein as a T-cell helper epitope, and the immunoadjuvant $Pam_3CysSer$ could elicit strong antibody responses in murine models.[9a] Importantly, these vaccines were completely self-adjuvanting and did not require supplementation with commonly employed immune stimulators such as alum or the complete Freund adjuvant.

We were interested in extending this concept by designing self-adjuvanting vaccines that possess a universal T-cell helper epitope, which, unlike our previous constructs, would be directly translatable to human studies should they prove to be efficacious in an animal model. To this end, we now report the efficient synthesis and immunological evaluation of fully self-adjuvanting tricomponent conjugate vaccine candidates **1**--**3** that possess a MUC1 peptide or glycopeptide antigen, an immunoadjuvant, and a synthetically-derived universal T-cell helper epitope (Figure^^2<figr2>). We also report the spontaneous self-assembly of these synthetic vaccines in aqueous media to form discrete nanoparticles.

Results and Discussion

<+>**Vaccine design**: The target vaccines **1**--**3** were designed to incorporate the necessary structural features to elicit robust cellular and/or humoral immunity (Figure^^1<xfigr1>). Specifically, **1**--**3** were designed to comprise a complete copy of the 20-amino-acid VNTR domain of MUC1 bearing either no glycosylation or five copies of the T_N

and T antigens as the vaccine antigen, covalently tethered to the PADRE T-cell helper peptide epitope, and $Pam_3CysSer$ as a built-in immunoadjuvant. Installation of an immunogenically silent triethylene glycolate spacer unit between each vaccine component was proposed to minimize conformational distortion and to allow for optimal display of each recognition element to the immune system, as has been reported previously.[9b,^10] We chose to incorporate MUC1 glycopeptides with saturated occupancy with TACAs because this architecture is known to be present on tumor-associated MUC1.[11] Despite a report that suggests that antigen-presenting cells (APCs) have an impaired ability to process peptides bearing clustered presentation of TACAs,[12] vaccines that incorporate these features have been shown to elicit high IgG antibody titres when conjugated to a foreign carrier protein or an external adjuvant.[13] The inclusion of the full-length, per-glycosylated VNTR into the vaccine constructs enables simultaneous presentation of multiple immunogenic epitopes on major histocompatibility complex (MHC) molecules, including the antibody immunodominant sequences RPAPGS and PDTRP and the per-glycosylated HLA-A2 binding peptide SAPDTRPAPG (which includes the H-2Kb binding epitope SAPDTRPA).[14]

The PADRE T-cell helper peptide component was incorporated to facilitate the induction of a robust protective immune response against the MUC1 (glyco)peptide antigens. This synthetically engineered T-cell helper epitope was chosen because it has been demonstrated to elicit effective T-cell responses and bind multiple human leukocyte antigen (HLA) binding molecules, thereby overcoming strong HLA polymorphism within the human population.[15] The inclusion of this T-cell helper peptide would therefore enable promising candidates to be directly translated from models in vivo to human studies. $Pam_3CysSer$ is a well-studied agonist of toll-like receptor-1--toll-like receptor-2 (TLR1--TLR2) heterodimers, which activates nuclear factor κB (NF-κB) and stimulates the secretion of proinflammatory cytokines through signaling by the myeloid differentiation primary response gene 88 (MYD88).[16] This behavior leads to the maturation of dendritic cells and antibody-producing plasma cells upon vaccination.[16b] It should be noted that traditionally $Pam_3CysSer(Lys)_4$ is utilized as an immunoadjuvant, whereby the four lysine (Lys) residues are presumably present to aid in the aqueous solubility of the final constructs.[8a,c] We chose to omit these Lys

residues from our vaccine candidates to provide a molecule with defined hydrophobic and hydrophilic moieties to facilitate self-assembly in solution.

<+>**Synthesis**: The synthesis of **1**--**3** was proposed to proceed through the conjugation of the three vaccine components by using our recently reported pentafluorophenyl ester-mediated fragment condensation strategy.[9a,^17] Our synthetic strategy therefore commenced with the synthesis of the requisite fragments, namely the antigenic (glyco)peptides **4**--**6** (Scheme^^1<schr1>), the side-chain-protected PADRE T-cell helper peptide **7** bearing a C-terminal triethylene glycolate linker functionalized as a pentafluorophenyl ester, and the triethyleneglycolic acid-derived immunoadjuvant lipopeptide $Pam_3CysSer$ (**8**; Scheme^^2<schr2>). Synthesis of the completely deprotected MUC1 VNTR peptide **4** and glycopeptides **5** and **6** bearing multiple copies of the T_N or T antigens, respectively, was achieved by using a linear Fmoc-strategy solid-phase peptide synthesis (Fmoc SPPS) starting from 2-chlorotrityl chloride resin preloaded with Fmoc-His(Trt)-OH (**9**; Scheme^^1<xschr1>; see the Supporting Information for the synthetic details). Coupling of standard *N*-Fmoc- and side-chain-protected amino acids was achieved with PyBOP (4^^equiv) as the coupling reagent and NMM (8^^equiv) as the base in DMF. The glycosylserine and glycosylthreonine building blocks **10**--**13** (synthesized as reported previously)[18] were coupled in slight excess (1.2^^equiv) with HATU (1.2^^equiv) as the coupling reagent and DIPEA (2.4^^equiv) in DMF. Following elongation, the peptides were deprotected and cleaved from the resin by using an acidic cocktail of TFA/triisopropylsilane/water (90:5:5, v/v/v). The *O*-acetate protecting groups on the glycan units were subsequently removed by treatment with hydrazine hydrate.[19] The target MUC1 (glyco)peptides **4**--**6** were purified by reversed-phase HPLC and isolated in 14--45^% yield, based on the original resin loading.

Synthesis of the *N*-Fmoc- and side-chain-protected PADRE peptide was achieved by using linear Fmoc SPPS and employing 2-chlorotrityl chloride resin **14** preloaded with an Fmoc-protected triethylene glycolic acid unit (see Scheme^^2<xschr2>, the Experimental section, and the Supporting Information for the synthetic details). The synthesis was optimized by installing the pseudoproline--dipeptide Fmoc-Trp(Boc)- Thr($\psi^{Me,Me}$Pro)-OH (Pro=proline, Thr=threonine, Trp=tryptophan) in the presence of HATU/DIPEA at the Trp-7

and Thr-8 positions to prevent aggregation during peptide elongation as described by Delmas and co-workers previously.[20] Following cleavage from the resin by using 30^% HFIP in dichloromethane, the side-chain-protected peptide acid was isolated (in a purity of approximately 85^% by analytical HPLC). The crude protected peptide acid was treated with an excess of pentafluorophenyl trifluoroacetate and pyridine,[21] thus resulting in a smooth conversion into the corresponding pentafluorophenyl ester within 4^^hours (as judged by LC-MS and TLC analysis). The C-terminal pentafluorophenyl ester **7** was isolated in 17^% yield (based on the resin loading of **14**) following purification by preparative normal-phase HPLC.

The protected Pam_3CysSer lipodipeptide fragment **8** was also assembled by using linear Fmoc SPPS from the resin-bound intermediate **14** (see Scheme^^2<xschr2> and the Supporting Information).[9a] Following elongation, the side-chain-protected lipopeptide **8** was cleaved from the resin by using 30^% HFIP in dichloromethane and isolated in near quantitative yield (95^%) following purification by flash chromatography on silica gel (see the Supporting Information for the synthetic details).

With the fully unprotected MUC1 (glyco)peptides **4**--**6**, peptide pentafluorophenyl ester fragment **7**, and lipopeptide **8** in hand, we sought to assemble the proposed tricomponent vaccines **1**--**3** by a convergent fragment-condensation approach (Scheme^^3<schr3>). Unprotected MUC1 (glyco)peptides **4**--**6** were treated with PADRE pentafluorophenyl ester **7** in the presence of HOBt and DIPEA to afford the desired conjugate. These reactions were complete within 16^^hours (as judged by LC-MS), at which point the Fmoc carbamate was removed in situ with 10 vol.^% piperidine in DMF. Purification by reversed-phase HPLC provided the partially protected MUC1--PADRE conjugates **15**--**17** in good yields (41--59^%). Lipopeptide adjuvant fragment **8** was preactivated as the pentafluorophenyl ester **18** by treating with stoichiometric DIC and pentafluorophenol in dichloromethane (see Scheme^^3<xschr3> and the Supporting Information) before reacting with the PADRE--MUC1 conjugates **15**--**17** in the presence of HOBt and DIPEA. The conjugation reactions were followed by LC-MS and were completed after 16^^hours. Acidolytic cleavage of the *tert*-butyl ether and Boc carbamate side-chain-protective groups provided the target

tricomponent vaccines **1**--**3** in excellent yields (79--86^%) following purification by preparative C-4 HPLC.

<+>**Self-assembly**: We envisaged that the unique structural features within the proposed vaccines, specifically a hydrophilic MUC1 peptide or glycopeptide antigen and a hydrophobic Pam_3Cys moiety at opposing ends of the construct, would lead to amphiphilic molecules that may self-assemble in aqueous media into micelles or particles, thus providing access to discretely sized nanoparticle vaccines. Such vaccines have been shown to possess numerous advantages over conventional approaches, including improved stability in vivo, stabilization of the native peptide antigen conformation,[22] and multivalent antigen presentation, which provides enhanced B-cell responses owing to the clustering of the antigens on the particle surface.[23] Furthermore, several self-assembled vaccines have demonstrated adjuvanting activity in the absence of any other immunostimulatory agents.[24] Nanoparticle vaccines are also powerful vehicles for vaccine delivery because they have been shown to display improved delivery to the lymph nodes and enhanced uptake by antigen-presenting cells (APCs).[25] To test the self-assembly properties of the vaccines under the proposed vaccination conditions, **1**--**3** were initially dissolved in DMSO and diluted with water (final ratio of DMSO/water=1:9, v/v) to provide completely dissolved solutions. Dynamic light scattering (DLS) revealed the presence of nanoparticles with hydrodynamic diameters of 17--25^^nm (>95^% of the population based on the number distribution) in all three vaccines, although a small and broad distribution of larger aggregates made more accurate characterization of the main population difficult (see the Supporting Information for the experimental details). Given that the length of a fully elongated vaccine is estimated to be 12^^nm, the size of the particles from the DLS experiments are consistent with what would be expected from spherical micellar aggregates with the hydrophilic MUC1 B-cell epitopes presented on the surface. Remarkably, identical results were obtained when the vaccines were dissolved in pure water (see the Supporting Information). TEM confirmed the presence of particles. In this case, the particles were 12--20^^nm in diameter for the three vaccines, slightly smaller than their measured diameter in solution, as might be expected from the drying process. Although the particles could be imaged on unstained carbon-coated grids (Figure^^3^A<figr3>), we found a better contrast when we used lacey formvar grids with

graphene oxide supports, which have recently been shown to facilitate high-contrast imaging without the need for staining (Figure^^3^B<xfigr3>).[26]

To assess if the particles would dissociate upon dilution (which would occur upon vaccination), we tested the stability of the aggregations by further diluting the vaccines in water. The particle sizes (as measured by DLS) were independent of concentration (1--0.05^^mg^mL$^{<M->1}$), and the surface tension was measured to be that of pure water in all the cases. If the aggregates were dynamic micelles, we would expect the surface tension to be measurably lower than that of water for any sample that contains a significant proportion of assembled aggregates. We observed both aggregates by DLS and no change in surface tension from that of water at low concentrations was found, thus suggesting that the aggregates are frozen rather than dynamic micelles in aqueous solution and are likely to remain in the assembled form down to very low concentrations in vivo. This spontaneous self-assembly of **1--3** in aqueous solution to form nanoparticles of discrete sizes may well have implications for stimulation and increased uptake of the vaccines by antigen-presenting cells and, as such, may contribute (together with activation through TLR1--TLR2 pathways) to the immunostimulatory properties of the vaccines.[25b]

<+>**Immunology**: To evaluate the immunogenicity of tricomponent vaccines **1--3**, C57BL/6 mice (*n*=4) were injected intradermally with 20^^μg of each vaccine (diluted in 10 vol.^% DMSO in phosphate-buffered saline (PBS)) three times on days 0, 10, and 17. Two weeks following the final immunization, serum-antibody levels were assessed by enzyme-linked immunosorbent assay (ELISA) to identify vaccine-induced antibodies. For the determination of antibody titres, ELISA plates were coated with unconjugated versions of MUC1 (glyco)peptides **4--6** dissolved in carbonate/bicarbonate coating buffer (0.05^M $NaHCO_3/Na_2CO_3$; pH^^9.6). Mouse sera (serially diluted in 0.5^% skim milk/PBS) was added to the plates to assess antibody levels against each corresponding (glyco)peptide antigen (see the Supporting Information). Robust antibody responses were observed for all the vaccines, in the absence of an external adjuvant, with the total IgG endpoint titres ranging from 1775 to 8400 (Figure^^4<figr4>). These antibodies were selective for the MUC1

peptide and glycopeptide antigens to which they were raised, as determined by cross-reactivity ELISA assays (see the Supporting Information).

The breakdown in antibody isotypes was also determined for **1**--**3** for each animal by using specific secondary antibodies (Figure^^5^A<figr5>--C). In comparison to the IgG total titres, relatively low levels of IgM antibodies were produced by the vaccines following the third immunization, thus suggesting effective antibody-class switching, an important factor for the production of an effective vaccine. A predominance toward the type-2 helper T-cell (Th2) phenotype was observed, with higher levels of circulating IgG1 antibodies detected relative to the Th1 indicator, that is, IgG2c, in C57BL/6 mice.[27] Interestingly, high levels of IgG3 antibodies were also induced by all the vaccines. The principal specificity of this isotype is against carbohydrates, and it elicits a powerful effector function in early immune responses. The strong titres elicited by these vaccines may indicate a capacity to generate anti-glycopeptide humoral immunity, deemed an important consideration in the generation of targeted antitumor responses. However, this outcome is contradicted by the generation of IgG3 antibodies from mice immunized with unglycosylated vaccine **1**. In this case, the IgG3 antibodies could recognizing both unglycosylated 20-mer MUC1 peptide **4** and a recombinant unglycosylated MUC1 protein that possesses five copies of the VNTR (see the Supporting Information). Interestingly, high IgG3 antibodies have also been observed in humoral responses to other peptide-based nanoparticle vaccines[22d] and vaccines in which protein antigens are conjugated to the surface of liposomes.[28] The IgG3 antibody responses against **1**--**3** may therefore result in part from the multivalent presentation of the peptide and glycopeptide MUC1 antigens on the surface of the nanoparticles and will be a subject of future investigations.

Finally, to investigate whether sera antibodies could recognize MUC1 epitopes expressed on cancer cells, we investigated the binding of antisera from **1**--**3** to two different MUC1 positive tumor cell lines, namely, MCF7 breast-cancer cells and B16 melanoma cells stably transfected with the MUC1 gene (B16.MUC1). Sera antibodies from all three vaccines could bind to MUC1 epitopes on the surface of MCF7 cells, as determined by fluorescence-activated cell sorting (FACS) analysis (see the Supporting Information). The antisera could

also bind to MUC1 epitopes on B16.MUC1 cells; however, the binding was weaker than that observed toward MCF7 cells.

Conclusion

We have successfully synthesized a small library of tricomponent self-adjuvanting MUC1 (glyco)peptide vaccines by using a convergent-fragment condensation approach. These vaccines, which possessed a MUC1 (glyco)peptide antigen, PADRE as a universal T-cell helper epitope and $Pam_3CysSer$ as an immunoadjuvant, were shown to self-assemble in aqueous media, thus providing isotropic particles of uniform size (average size=20^^nm), as confirmed by DLS and TEM imaging. All three vaccines induced strong humoral responses in murine models. These antibodies were selective for the antigen to which they were raised and analysis of antibody isotypes showed significant levels of IgG1 antibodies suggestive of a Th2 skewed response. Sera antibodies could also recognize and bind to two types of tumor cell that overexpress MUC1 on their surface. The results from this study provide significant insight into the design features required for the effective self-assembly of molecularly defined vaccines and for the generation of strong antibody responses without the need for additional adjuvants. Future work in our laboratories will involve the investigation of the self-assembly and immunological activity of other multicomponent self-adjuvanting vaccines, the results of which will be reported in due course.

Experimental Section

Solid-phase synthesis of peptide pentafluorophenyl ester (7; scale: 100^^µmol):

Resin loading: 2-Chlorotrityl chloride resin (Novabiochem) was swollen in dry dichloromethane (5^^mL) for 30^^min. A solution of Fmoc-PEG(9^^atoms)-OH (77^^mg, 200^^µmol, 2.0^^equiv) and DIPEA (70^^µL, 0.4^^mmol) in DMF/dichloromethane (1:1 v/v, 1^^mL) was added, and the resin shaken at room temperature for 16^^h. The resin was filtered and washed with DMF (5×3^^mL), dichloromethane (5×3^^mL), and DMF (5×3^^mL). The resin was treated with a solution of dichloromethane/CH_3OH/DIPEA (17:1:1 v/v/v, 3^^mL) for 1^^h, filtered, and washed with DMF (5×3^^mL), dichloromethane (5×3^^mL), and DMF (5×3^^mL).

Fmoc deprotection: The preloaded 2-chlorotrityl chloride resin (100^^μmol) was initially swollen in DMF (5^^mL) for 30^^min. A solution of piperidine/DMF (1:9 v/v, 5^^mL) was added to the resin, which was shaken for 3^^min and the procedure was repeated. The resin was subsequently washed with DMF (5×3^^mL), dichloromethane (5×3^^mL), and DMF (5×3^^mL).

Amino acid coupling: A solution of the protected amino acid (400^^μmol, 4.0^^equiv), PyBOP (208^^mg, 400^μmol, 4.0^^equiv), and NMM (88^^μL, 800^^μmol, 4.0^^equiv) in DMF (1^^mL) was added to the resin and shaken. After 1^^h, the resin was washed with DMF (5×3^^mL), dichloromethane (5×3^^mL), and DMF (5×3^^mL). Coupling of Trp-7 and Thr-8 was achieved by adding a solution of Fmoc-Trp(Boc)-Thr($\psi^{Me,Me}$pro)-OH (150 μmol, 1.5^^equiv), HATU (150^^μmol, 1.5^^equiv), and DIPEA (300^^μmol, 3.0^^equiv) in DMF (1^^mL) to the resin, which was shaken for 16^^h. The resin was washed as described above.

Resin cleavage: The resin was washed thoroughly with dichloromethane (10×3^^mL) and treated with a solution of hexafluoroisopropanol in dichloromethane (30^^vol.^%) and shaken for 2^^h at room temperature. The resin was filtered and washed with dichloromethane (5×2^^mL), and the filtrate and washings evaporated to dryness. The residue was coevaporated with toluene (3×5^^mL) and dried under high vacuum overnight.

Activation: The crude peptide acid was dissolved in dry DMF (0.1^M) under argon. Pyridine (5.0^^equiv) was added followed by the dropwise addition of pentafluorophenyl trifluoroacetate (5.0^^equiv). The reaction was stirred at room temperature for 4^^h and the solvent was removed in vacuo. The crude residue was purified by preparative, normal-phase HPLC to afford pentafluorophenyl ester **7** following lyophilization from *tert*-butanol/acetonitrile (1:1 v/v).

7: Yield: 17^% (based on resin loading of 100^^μmol). R_t=34.8^^min (0→100^% B over 40^^min; A=1^% acetic acid in dichloromethane, B=1^% acetic acid in methanol); HRMS (ESI^+): *m*/*z* calcd for $C_{107}H_{151}F_5N_{16}O_{25}$: 1101.0386 $[M+2\text{^}Na]^{2+}$; found: 1101.0369 $[M+2\text{^}Na]^{2+}$; elemental analysis calcd (%) for $C_{107}H_{151}F_5N_{16}O_{25}$: <?><?>Do you have the results?<?><?>.

General procedure for the synthesis of (glyco)peptides 15--17 (scale: 2.0^^μmol): DIPEA (10^^μL, 4.8^^μmol, 2.4^^equiv) in DMF (48^^μL^mL$^{<M->1}$) and 1-hydroxybenzotriazole (10^^μL, 2.4^^μmol, 1.2^^equiv) in DMF (24^^mg^mL$^{<M->1}$) were added to a solution of (glyco)peptides **4**--**6** (2.0^^μmol) in dry DMF (80^^μL). This solution was added to the peptide pentafluorophenyl ester

(4.0^^μmol), and the reaction mixture was gently agitated at room temperature for 16^^h. Piperidine (20^^μL) was added to the reaction mixture, which was agitated for a further 30^^min at room temperature. The solvent was removed in vacuo and the residue was purified by preparative reverse-phase HPLC followed by lyophilization to afford the desired (glyco)peptides **15--17**.

15: Yield: 3.0^^mg, 41^%. R_t=34.7^^min (0→75^% B over 40^^min; A=0.1^% TFA in H_2O, B=0.1^% TFA in acetonitrile); HRMS (ESI^+): *m*/*z* calcd for $C_{166}H_{267}N_{41}O_{50}$: 1227.9846 $[M+2Na+H]^{3+}$; found: 1227.9842 $[M+2^Na+H]^{3+}$; 926.7354 $[M+3^Na+H]^{4+}$; found: 926.7356 $[M+3^Na+H]^{4+}$; elemental analysis calcd (%) for $C_{166}H_{267}N_{41}O_{50}$: <?><?>Do you have the results?<?><?>.

16: Yield: 5.4^^mg, 59^%. R_t =33.2^^min (0→75^% B over 40^^min; A=0.1^% TFA in H_2O, B=0.1^% TFA in acetonitrile); HRMS (ESI^+): *m*/*z* calcd for $C_{206}H_{332}N_{46}O_{75}$: 1180.5847 $[M+H+3^Na]^{4+}$; found: 1180.5852 $[M+H+3^Na]^{4+}$; 949.0656 $[M+H+4^Na]^{5+}$; found: 949.0660 $[M+H+4^Na]^{5+}$; elemental analysis calcd (%) for $C_{206}H_{332}N_{46}O_{75}$; <?><?>Do you have the results?<?><?>.

17: Yield: 6.0^^mg, 55^%. R_t =33.7^^min (0→75^% B over 40^^min; A=0.1^% TFA in H_2O, B=0.1^% TFA in acetonitrile); HRMS (ESI^+): *m*/*z* calcd for $C_{238}H_{386}N_{46}O_{101}$: 1383.4170 $[M+3^H+Na]^{4+}$, found: 1383.3998 $[M+3^H+Na]^{4+}$; 1111.3315 $[M+3^H+2^Na]^{5+}$; found: 1111.3195 $[M+3^H+2^Na]^{5+}$; 929.9411 $[M+3^H+3^Na]^{6+}$; found: 929.9297 $[M+3^H+3^Na]^{6+}$; elemental analysis calcd (%) for $C_{238}H_{386}N_{46}O_{101}$: <?><?>Do you have the results?<?><?>.

General procedure for the synthesis of vaccine candidates 1--3 (scale: 1.0^^μmol): Pentafluorophenol (10^^μL, 1.0^^μmol, 1.0^^equiv) in dichloromethane (20^^mg^$mL^{<M->1}$) and DIC (10^^μL, 1.0^^μmol, 1.0^^equiv) in dichloromethane (20^^μL^$mL^{<M->1}$) was added to a solution of lipopeptide **8** (1.2^^mg, 1.0^^μmol) in dry dichloromethane (50^^μL). The solution was placed in an atmosphere of argon and gently agitated for 1^^h at room temperature. TLC analysis (5^% methanol/dichloromethane) showed consumption of starting material (R_f=0.2) and formation of the product (R_f=0.5). The solvent was gently evaporated under a stream of argon. A second solution of (glyco)peptide **15--17** (1.2^^μmol, 1.2^^equiv), *N*,*N*-diisopropylethylamine (10^^μL, 2.4^^μmol, 2.4^^equiv) in DMF (24^^μL^$mL^{<M->1}$) and 1-hydroxybenzotriazole (10^^μL, 1.2^^μmol, 1.2^^equiv) in dry DMF (30^^μL, 12^^mg^$mL^{<M->1}$) were added to the reaction mixture, which was gently agitated under argon for 16^^h at room temperature. The solvent was removed in vacuo and

TFA/TIS/water (90:5:5 v/v/v, 2^^mL) was added. The mixture was agitated for 2^^h at room temperature. Evaporation and purification by semipreparative reverse-phase HPLC (C-4) afforded the desired vaccine candidates **1**--**3** following lyophilization.

1: Yield: 3.8^^mg, 86^%. R_t=28.4^^min (0→100^% B over 40^^min; A=0.1^% TFA in H_2O/acetonitrile/isopropanol, 8:1:1 v/v/v; B=0.1^% TFA in H_2O/acetonitrile, 1:1 v/v); HRMS (ESI^+): *m/z* calcd for $C_{211}H_{356}N_{44}O_{55}S$: 1106.1621 $[M+4^H]^{4+}$; found: 1106.1629 $[M+4^H]^{4+}$; elemental analysis calcd (%) for $C_{211}H_{356}N_{44}O_{55}S$: <?><?>Do you have the results?<?><?>

2: Yield: 2.2^^mg, 82^%. R_t=29.0^^min (0→100^% B over 40^^min; A=0.1^% TFA in H_2O/acetonitrile/isopropanol, 8:1:1 v/v/v; B=0.1^% TFA in H_2O/acetonitrile, 1:1 v/v); MS (ESI^+): *m/z* calcd for <?><?>formula?<?><?>: 1372.5 $[M+2^H+2^Na]^{4+}$, 1092.0 $[M+3^H+2^Na]^{5+}$; found: <?><?>?<?><?>.

3: Yield: 2.5^^mg, 79^%. R_t=28.7^^min (0→100^% B over 40^^min; A=0.1^% TFA in H_2O/acetonitrile/isopropanol, 8:1:1 v/v/v; B=0.1^% TFA in H_2O/acetonitrile, 1:1 v/v); MS (ESI^+): *m/z* calcd for <?><?>formula?<?><?>: 1575.1 $[M+4^H]^{4+}$, 1260.1 $[M+4^H+Na]^{5+}$, 1050.1 $[M+6^H]^{6+}$; found: <?><?>?<?><?>.

Immunology

Antibody ELISA: MUC1 unglycosylated and glycopeptide-specific serum antibodies were detected by ELISA. Ninety-six-well ELISA plates (Nunc Maxisorp) were coated with either MUC1 unglycosylated peptide **4** or MUC1 glycopeptides **5**, **6** (10^^μg^$mL^{<M->1}$) diluted in carbonate/bicarbonate coating buffer (0.05^M, pH^^9.6) and incubated overnight at 4^°C. The plates were washed with PBS 0.05^% Tween (PBST) and blocked with PBST containing 5^% skim milk for 2^^h at 37^°C. The mouse serum diluted in PBST and 5^% skim milk was added to the plates at appropriate dilutions and incubated for 90^^min at 37^°C. The plates were washed and incubated with 0.5^^μg^$mL^{<M->1}$ horseradish peroxidase conjugated goat antimouse IgG Ab for 2^^h at 37^°C. The serum antibodies were detected by developing with 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution (Sigma--Aldrich) for 20^^min, reactions were stopped using 1^M HCl, and the absorbance was measured at <Gl>=450^^nm on an ELISA plate reader. The endpoint titre was defined as the highest dilution of serum at which a positive antibody response is detectable, and a positive response was calculated as the mean absorbance of naïve serum with two standard deviations.

Flow cytometry: MCF7 cells (2×105) were blocked with 2^% fetal-calf serum (FCS)/PBS and sera (100^^μL) from mice immunized with **1--3** were added and incubated for 45^^min at 4^°C. After washing with 2% FCS/PBS, a dilution of fluorescein-5-isothiocyanate (FITC)-conjugated sheep (Fab)2 antimouse immunoglobulin (secondary conjugate; 1:1000, 100^^mL) was added and incubated for 45^^min at 4^°C. After further washing, the cells were analyzed by flow cytometry with a FACScan flow cytometer (Becton Dickenson).

Vaccine self-assembly and characterization: DLS experiments were performed (<Gl>=633^^nm, spot size=400^^μm) at 6, 90, and 120° in a decalin bath at 25^°C. Correlation functions were fit using Brookhaven software with a CONTIN model. All the solvents were filtered through a polytetrafluoroethylene (PTFE) filter (20^^nm) prior to use to eliminate dust. The viscosity and refractive index of the mixture in DMSO/water was calculated by using data from previous reports.<?><?>Do you have the reference(s) used?<?><?> TEM images were taken on a JEOL JEM-1400 microscope at 120^^kV. Sample grids were prepared by coating 400^^mesh copper grids with a formvar film and sputter coating with carbon (~10^^nm). The samples were drop cast onto the grids and stained by placing the grids face-side down on a drop of urynal acetate solution (0.5^^mg^mL$^{<M->1}$, filter=20^^nm) for 30^^s. Excess solution was removed by blotting and the grids were dried overnight prior to imaging. Graphene oxide (GO) grids were prepared by stirring GO (obtained from R. O'Reilly, Warwick University, U.K.) in water (0.1^^mg^mL$^{<M->1}$) overnight at room temperature to create a clear dispersion. The solution was sonicated for 30^^s and dropped onto lacey formvar copper grids.

Surface-tension measurements were made on a goniometer by using a pendant-drop method and the Young/Laplace equation.<?><?>Do you have a reference?<?><?> A drop size of 10±1^^μL was used in all cases, and results were averaged for the first 20^^s of the measurement.

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Scheme^^1 Fmoc SPPS of MUC1 VNTR (glyco)peptide antigens. DIPEA=*N*,*N*-diisopropylethylamine, Fmoc=9-fluorenylmethoxycarbonyl, HATU=(7-azabenzotriazol-1-yl)tetramethyluronium hexafluorophosphate, MUC1= mucin^^1, NMM=*N*-methylmorpholine, PyBOP=benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate, Pyr=pyridine, TFA=trifluoroacetic acid, Trt=trityl, VNTR=variable-number tandem repeat.

Scheme^^2 Fmoc SPPS of side-chain-protected PADRE pentafluorophenyl ester (T-cell helper peptide) fragment **7** and $Pam_3CysSer$ (immunoadjuvant) fragment **8**. Boc=*tert*-butoxycarbonyl, Cys=cysteine, HFIP=hexafluoroisopropyl alcohol, PADRE=pan-allelic DR epitope, Pam=<?><?>please define<?><?> Ser=serine.

Scheme^^3 Convergent synthesis of self-adjuvanting vaccine candidates **1**--**3** by using pentafluorophenyl ester fragment condensation. DCM=dichloromethane, DIC=*N*,*N*'-diisopropylcarbodiimide, D-Gal=galactose, HOBt=1-hydroxybenzotriazole, TIS=<?><?>triisopropylsilyl…?<?><?>.

Figure^^1 Tumor-associated carbohydrate antigens (TACAs).

Figure^^2 Proposed synthetic tricomponent MUC1 (glyco)peptide cancer vaccine candidates. <?><?>Please define each letter given<?><?> a=D-alanine, X=L-cyclohexylalanine.

Figure^^3 TEM images of vaccine **1** assembled in water deposited on a)^^an unstained carbon-coated grid and b)^^a graphene oxide grid.

Figure^^4 Anti-MUC1 IgG total reciprocal antibody titres elicited by self-adjuvanting vaccines **1**--**3** after three immunizations.

Figure^^5 Anti-MUC1 IgG isotype reciprocal antibody titres elicited by self-adjuvanting vaccines A)^^**1**, B)^^**2**, and C)^^**3** after three immunizations