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A High-throughput O-Glycopeptide Discovery Platform for Seromic Profiling

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Abstract

Biomarker microarrays are becoming valuable tools for serological screening of disease-associated autoantibodies. Post-translational modifications (PTMs) such as glycosylation extend the range of protein function, and a variety of glycosylated proteins are known to be altered in disease progression. Here, we have developed a synthetic screening microarray platform for facile display of O-glycosylated peptides (O-PTMs). By introducing a capping step during chemical solid-phase glycopeptide synthesis, selective enrichment of *N*-terminal glycopeptide end products were achieved on an amine-reactive hydrogel coated microarray glass surface, allowing high-throughput display of large numbers of glycopeptides. Utilizing a repertoire of recombinant glycosyltransferases enabled further diversification of the array libraries *in-situ* and display of a new level of potential biomarker candidates for serological screening. As proof-of-concept we have demonstrated that MUC1 glycopeptides could be assembled and used to detect autoantibodies in vaccine induced disease free breast cancer patients and in patients, with confirmed disease at time of diagnosis.

Keywords

Glycopeptide; synthesis; chemoenzymatic; enzyme; microarray; glycan array; autoantibodies; post-translational modification (PTM)

Introduction

Autoantibody signatures are appealing biomarkers for detection of cancer with potential for early diagnosis with high sensitivity and specificity.1·2 Autoantibodies to proteins may be induced by mutations and altered expression that overcome self-tolerance.1 Perhaps more frequently, cancer-associated changes in post-translational modifications (PTMs) may generate aberrantly modified proteins that renders these recognized as non-self, and thus may lead to induction of autoantibodies. Although considerable efforts have been devoted in the past to the identification of protein targets for autoantibodies of cancer patients, a fairly

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small number of antigens have been determined. Most of these are cytosolic proteins and only a few cell membrane or secreted proteins have been found.[1] One common limitation of the leading strategies employed to identify autoantibodies, including screening expressed cDNA libraries (SEREX), protein and peptide arrays, is that these do not take into consideration antigen epitopes affected by PTMs.[3–6] Relatively few examples of disease-associated autoantibodies to PTM-protein epitopes have been reported so far[7] and these have been found in cancer[8,9], in autoimmune diseases such as citrullination associated with rheumatoid arthritis[10], or aberrant O-glycosylation of IgA1 associated with IgA nephropathy.[11]

Glycosylation is one of the most abundant PTMs of proteins, and aberrant glycosylation is a hallmark of malignant transformation.[12] Mucin-type O-glycosylation is particularly important in relation to cancer-associated autoantibodies, because cancer cells generally form short truncated O-glycans such as Tn (GalNAcα1-O-Ser/Thr), T (Galβ1-3GalNAcα1-O-Ser/Thr), and STn (NeuAcα2-6GalNAcα1-O-Ser/Thr).[13] These carbohydrates are recognized by natural IgM antibodies present in all individuals,[14,15] and they can generate novel cancer-specific O-glycopeptide epitopes on proteins comprised of both the peptide sequence and the specific O-glycan.[8,16–18] We recently identified autoantibodies to distinct O-glycopeptide epitopes on MUC1 in cancer patients[8], and given the abundance of this type of O-glycosylation on proteins it is likely that many other similar epitopes can be found.

In the present study, we have developed a high throughput chemoenzymatic synthesis and microarray display platform that enables production and screening of large O-glycopeptide libraries for disease-associated autoantibodies (Fig. 1). Chemical and chemoenzymatic methods for synthesis of O-glycopeptides are available and smaller libraries of O-glycopeptides have been synthesized.[19–22] Methods for display on microarrays have also been developed,[21,23,24] but a method for robust high through-put synthesis and display of O-glycopeptide libraries on microarray has not.

We have therefore used a chemical 96-well block parallel synthesis approach to GalNAc O-glycopeptides that allows direct immobilization of crude glycopeptides on NHS-activated arrays, resulting in on-slide purification of desired glycopeptide products with free N-terminal amines. This approach allowed complete control of the site(s) of O-GalNAc occupancy which, combined with on-slide enzymatic glycosylation with different polypeptide GalNAc-transferases and other elongating glycosyltransferases, provided further diversity in O-glycan occupancy and structures. The platform was used to map unique glycopeptide epitopes of cancer-specific mouse monoclonal antibodies to MUC1 glycopeptides, human sera from a MUC1 glycopeptide vaccine study, and cancer induced human autoantibodies. This synthesis and display approach will enable further discovery of disease-associated immunity to aberrant glycopeptide epitopes for development of diagnostic biomarker assays.

Experimentals

Materials

The Fmoc-protected amino acids, MeOH, NMP, DMF, piperidine, DIEA, TFA, HBTU, HOBt, were purchased from Iris Biotech GmbH (Marktredwitz, Germany). Fmoc-protected Tn (GalNAcα)-Ser/Thr, Core1 (Galβ1-3GalNAcα)-Ser/Thr, Core3 (GlcNAcβ1-3GalNAcα)-Thr, and Core4 (GlcNAcβ1-3[GlcNAcβ1-6]GalNAcα)-Thr were purchased from Sussex Research (OT, Canada). TentaGel S Rink amide resin from Rapp Polymere GmbH (Tübingen, Germany). DCM, Et_2O, acetic anhydride, THF, formic acid, 0.5M solution of NaOMe in MeOH, Goat anti-mouse-IgG (Fc-specific) alkaline phosphatase conjugate, goat anti-mouse-IgG (Fc-specific) Cy-3 conjugate, goat anti-human-IgG (Fc-specific) Cy-3

conjugate, the biotinylated HPA lectin, CHAPS, BSA, $NaBH_4$ and UDP-GlcNAc were from Sigma (MO, USA). The Zymax Streptavidin-Cy-3 conjugate was from Invitrogen (Carlsbad, CA, USA). Multi-well filter plates and filter plates vacuum manifold were purchased from Pall (Ann Arbor, MI, USA). Stratosphere PL-HCO_3 MP SPE resin was purchased from Varian (Palo Alto, CA, USA). All salts for all the buffers, including TES, Triton-X-100 Tween 20, iodine and ethanolamine were from Merck (NJ, USA). Peptides MUC2.1, MUC7, OSM-fragment, GCSF, hCG-beta, CD59 used for substrate specificity analysis of GalNAc-transferases were custom synthesized by Schafer-N (Copenhagen, Denmark) and Carlbiotech (Copenhagen, Denmark). The monoclonal antibodies 5E5, 2D9[25] to Tn/STn-MUC1, and 3F126 to STn were prepared as described, whereas 1E10, a core3-MUC1 specific mAb, were generated following similar protocols (supporting info S4). The VU3C6 mAb were from Chemicon, Millipore, MA[27]. Antibodies and serum were diluted in the staining buffer (SB) (0.5M NaCl, 3mM KCl, 1.5mM KH_2PO_4, 6.5mM Na_2HPO_4, 1% BSA, 1% Triton-X-100, pH=7.4). Printing was performed on Schott Nexterion® Slide H or Schott Nexterion® Slide H MPX 16 (Schott AG, Mainz, Germany). Printing of the 16-well microarray slides was performed using a BioRobotics MicroGrid II spotter (Genomics Solution) with a 0.21 mm pitch using Stealth 3B Micro Spotting Pins (Telechem International ArrayIt Division) with deposit volume of approx. 6 nL of glycopeptide in print buffer (150 mM phosphate, 0.005% CHAPS pH 8.5). The compounds were distributed (20 μL per well) in 384-well source plates (BD Falcon MicrotestTM 384-well 30 μL assay plates from BD Biosciences, Le Pont De Claix, France) and printed in 3 replicates using an 8-pin (2×4) configuration within a 28×28 subgrid at a 0.21 mm pitch between each spot. The pin dwell time in the wells was of 4 seconds and the pins underwent 3 wash cycles in between source plate visits. The complete 4×2 array pattern was printed on a 16 well slide in duplicate, distributed in two columns and eight rows. Immediately after printing the slides were incubated at 80% humidity for 60 min. Remaining NHS groups on the slides were blocked by immersion in the blocking buffer (50 mM ethanolamine in 50 mM borate buffer, pH 9.2) for 1 h. Slides were rinsed in Millipore water, dried by centrifuging and probed as described below. Slide that were not to be probed immediately were stored at −18 °C before the blocking step. Scanning of the slides was performed on ProScanArray HT Microarray Scanner (PerkinElmer) followed by image analysis with ProScanArray Express 4.0 software (PerkinElmer). Data were analyzed and plotted using Microsoft® Excel or GraphPad Prism software. Commercially human cancer taken at time of diagnosis, from breast cancer (n = 10), ovarian cancer (n = 10) and rectal cancer (n = 10) as normal control sera (n=16) were available from Asterand plc. (UK).

General solid-phase synthesis of peptides and glycopeptides

Peptides and glycopeptides were prepared by automated peptide synthesis on a Syro II peptide synthesizer (MultiSynTech, Witten, Germany) by standard SPPS on TentaGel S Rink Amide resin (loading 0.24 mmol/g) with Fmoc for protection of *N*α-amino groups. Side-chain protecting groups were *tert*-butyl (Ser, Thr), 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl (Pbf, for Arg), Boc (Lys) and trityl (Trt, for Asn, Gln, His). *N*α-Fmoc amino acids (4.0 equiv) were coupled using *N*-[(1*H*-benzotriazol-1-yl) (dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide HBTU (3.8 equiv), 1-HOBt (4.0 equiv) and *N*,*N*-diethylisopropylamine DIEA (8.0 equiv) in DMF for 120 min. Capping of the unreacted *N*-terminal amines was performed following each coupling step using three consecutive treatments with acetetic anhydride-DCM solution (1:4) of 15 min each and thorough NMP washing were undertaken in between treatments. *N*α –Fmoc de-protection was performed using piperidine-DMF (2:3) for 3 min followed by piperidine-DMF (1:4) for 12 min. The peptides and glycopeptides were released from the solid support, and all acid-labile protecting groups were removed by treatment with trifluoroacetic acid (TFA)/triethylsilane (TES)/H_2O (95:2:3) for 2 h. The TFA solutions

were concentrated by nitrogen flow and the compounds were precipitated with Et_2O and crude materials were collected as white pellets after centrifugation.

De-acetylation of glycopeptides

Prior to deacetylation, the crude glycopeptides were mixed with Stratosphere PL-HCO_3 MP SPE resin in order to remove the excess of ion-paired TFA, as follows. Typically, a 96-member batch of crude glycopetide pellets (1–4 mg each) were taken up in 500 μL each of water-MeOH (4:1) solution and loaded into a 96-well filter plate (one glycopeptides per well) containing Stratosphere PL-HCO_3 MP SPE resin (2.0 g, evenly distributed in the 96 wells) preconditioned in MeOH. After 5 min incubation the filter plate was drained under vacuum and thereby the filtrate from each well collected separately in a 2mL/well, 96-well receiving plate. The Stratosphere PL-HCO_3 MP SPE resins in the filter plate were then washed with 100 μL fresh MeOH per well and likewise collected in the receiving plate under vacuum suction. MeOH was removed from the receiving plate whereupon the remaining water phase was lyophilized. The base-free solid crude glycopeptides were then dissolved in 300 μl of MeOH and the pH adjusted at 9 (as judged from pre-wetted pH strips) with a 0.015M solution of MeONa in MeOH. After 10h of incubation at RT, 250 μL of NaOAc 0.1M solution pH 6 was added in order to quench the basicity and thereby giving rise to glycopeptides stock solutions. The stock solutions were stored at −20°C until needed for on-slide glycopeptide capture microarray printing.

General procedure for glycopeptide capture and release with activated Sepharose beads

Typically, 96 wells of a 384-well filter plate were filled with 30 μL each of freshly activated Sepharose beads water slurry while the remaining empty wells were sealed with adhesive paper. The filter plate was loaded onto the vacuum manifold and the beads drained. 15 μL aliquots of stock solution for each glycopeptide to be captured (*i.e.* 96 samples) were added to 15 μL of $NaHCO_3$ 0.1M and the resulting solutions loaded each into a unique well containing the activated beads so as to fill all wells. The wells were sealed with adhesive paper and the plate kept under vigorous agitation for a minimum of 1h whereupon the wells were drained by suction on the manifold and the beads washed extensively with NaCl 0.5M, acetic acid 50 mM, ethanol 50% in water and water. The wells containing the drained beads were added 20 μL each of DL-dithiothreitol (DTT) 50 mM solution in PBS and the resulting slurries incubated for 30 min at RT. The wells were finally drained by vacuum suction into a receiving plate, washed with 20 μL of water and drained again. The solutions thus collected into the receiving plate contain the captured glycopeptides released from the beads and were subjected to MALDI-TOF analysis for mass characterization.

General procedure for Sepharose 4B on-bead enzymatic glycosylation

Beads with captured glycopeptides (50 uL wet drained beads, 0.5umol) were consecutively treated with one or several recombinant glycosyltransferases.[18] Briefly, GalNAc-T2, core3 β3GlcNAc-T6 and/or ST6GalNAc-I (50–100 μg/mL) in sodium cacodylate buffer (2.0 mL, 0.5 M, pH 7.2) containing $MnCl_2$ (20 mM) and corresponding sugar nucleotide (5 mM) respectively and the reaction mixture was shaken for 18 h at 37C. After extensive washings with NaCl 0.5M, acetic acid 50 mM, ethanol 50% in water. The glycopeptides were released by the addition of DDT (50 mM) for 30 min and the supernatant was analyzed by MALDI-TOF-MS.

General procedure for on-slide enzymatic glycosylation

Slides with immobilized peptides were blocked with ethanolamine (1hr, RT), rinsed thoroughly with milli-Q purified water, and then spun dry on a Galaxy mini-array tabletop slide centrifuge (VWR, West Chester, PA, USA). 16-well superstructures (Schott AG,

Mainz, Germany) were applied and slides were treated for 2 hrs at 37°C with either of the 50ul glycosylation mixtures for Tn glycosylation: 200uM UDP-GalNAc, 25μg/ml GalNAc-transferases, 2mM $MnCl_2$, 1%BSA, 0.5% NP40, 25mM Mes (pH 7.4); core3 glycosylation: 200uM UDP-GlcNAc, 25μg/ml β1-3GlcNAc-T6 enzyme, 2mM $MnCl_2$, 1%BSA, 0.5% NP40, 25mM Mes (pH 7.4); STn glycosylation: 500uM CMP-Neu5Ac, 25μg/ml ST6GalNAc-I enzyme, 25mM Mes (pH 7.0). Immediately following glycosylation, slides were washed with PBST (5 min, shaking), PBS (5 min, shaking) and then treated with citrate buffer (pH 2.5) for 15 min, rocking. Following acid wash, slides were again washed with PBST and PBS as before and then blocked with 1% BSA, 0.5% NP40, PBS 20min, shaking. Slides were again washed with PBST, PBS, rinsed thoroughly with MQ, and dried and used in the next step.

Lectin, mAb and serum microarray analysis

Incubation volumes for each MPX16 well were performed with adhesive superstructures at 50μL/well. Lectins were diluted to 1–10μg/mL, mAbs to 1μg/mL and human serum were analyzed at 1:20 dilution. All samples were incubated on the slide for 1 h, followed by 1 h incubation with appropriate secondary antibodies. All dilutions were made in SB buffer pH 7.4. Murine monoclonal antibodies were detected with Cy3-conjugated goat anti-mouse IgM (μ chain specific) and goat anti-mouse IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc.) diluted 1:1000. Human IgG antibodies were detected with Cy3-conjugated goat anti-human IgG (Fc-specific)(1:1000) and biotinlyated lectin detected with Streptavidin-Alexa Fluor®488 (Invitrogen)(1:5000). All incubation steps were separated by two wash steps in PBS with 0.05% Tween-20 (PBS-T) and one in PBS. After the final wash, slides were rinsed in H_2O, dried by centrifugation (200×g) and scanned followed by image analysis and quantification.

Results and Discussion

Automated solid-phase GalNAc-glycopeptide synthesis with on-slide microarray isolation

In the first part of our study we established methods for automated solid-phase synthesis of GalNAc-glycopeptide libraries followed by direct printing on *N*-hydroxysuccinimide (NHS) slides without need for prior time-consuming purification of glycopepties. Synthesis was carried out using standard Fmoc-protected solid-phase peptide synthesis (SPPS) with HBTU/HOBt as coupling reagents.[28] The solid support Tentagel resin, preloaded with Rink amide handle, was applied in a 96-well block automated HT solid-phase parallel synthesizer using glyco-amino acid building blocks for introduction of GalNAcα1-O-Ser and GalNAcα1-O-Thr at defined positions (Fig 1A).[29–31] Introduction of a capping step (*N*-acetylation with acetic anhydride) after each amino acid coupling ensured that deletion peptide products were *N*-capped, and thus devoid of *N*-terminal reactive amines, which would otherwise result in the immobilization of these by products onto the amine-reactive slide upon printing.[32] Glycopeptides were cleaved (TFA:H_2O:TES; 95%:3%:2% v/v) from TentaGel resin, the cleavage cocktail was concentrated and the crude peptides collected by precipitation in diethyl ether. At this stage all acid labile side-chain protecting groups were removed as well, and only the base sensitive O-acetyl moieties on the sugar hapten were left in place, which could be swiftly removed under Zemplén conditions. In order to easily control the basicity of the required methanolic NaOMe solution, we found it helpful to de-TFA salt the crude glycopeptides, by mean of solid supported hydrogen carbonate, prior to exposure to the methoxide solution. In such fashion base-induced β-elimination of the sugar moiety could be prevented. Finally, selective immobilization of desired glycopeptide product onto amine-reactive NHS glass surfaces (Fig 1B) was undertaken to form the glycopeptides chips. In order to compensate for variability in yields during synthesis, and enhance the probability of maximizing print density on slides (50 μM recommended by

manufacturer), a twenty-fold excess print concentration (1mM) of crude products was used. Hydrogel coated glass slides have been shown to be very useful with human serum assays[8,33–35] and they are also suitable for enzymatic modifications.[36] Preliminary experiments with uncoated epoxy slides (Corning) produced background issues with human serum, especially in combination with on-slide enzymatic treatments (not shown). Available immobilization chemistries on hydrogel-coated slides are limited and we have continued to use the NHS-activated amino-reactive slide H to covalently display biomolecules. The simplicity with which glycopeptides are attached to the amine-reactive microarary glass surface along with selective enrichment of full synthetic targets, facilitates high through-put fabrication of glycopeptide microarrays suitable for serological assays.

Throughout the project several libraries of more than 1,700 peptides and glycopeptides all together derived from human mucins and other tumor-related glycoproteins were synthesized in a multi-block peptide synthesizer. To assure optimal print concentration, all glycosylated compounds were test-printed at multiple concentrations and assessed by reactivity with monoclonal antibodies (when available) to O-glycans and peptides as well as with Tn-specific VVA lectin (Fig 1B and supporting info S2 and S7). As exemplified in Fig. 1B, VVA lectin reacted specifically with GalNAc-glycopeptides, indicating sufficient quality and quantity of 2 μmole-scale synthesized glycopeptides printed at 2-fold dilutions starting at 1 mg/mL concentration. A current limitation is that glycopeptides with internal lysine residues also have the capability to covalently bind to the array surface via side chain amino groups and these may compete with the preferred N-terminal site. However, this may not influence detection of autoantibodies as it is likely that at least part of the glycopeptides will be in the correct orientation.

To further confirm the synthesis and validate the on-slide surface isolation approach we prepared Sepharose 4B beads containing an amine-reactive cleavable disulfide linker for selective capture of amine containing N-terminal glycopeptides.[37] Briefly, Amino-Sepharose 4B was activated with diethyl squarate and reacted with cysteamine in excess (>50 eq) (see supplementary info S1). The beads were reactivated with diethyl squarate and this followed by binding of selected GalNAc-glycopeptides via the *N*-terminal amine (Fig 1C). Following washing of unbound material the beads containing immobilized Tn-peptides were cleaved with dithiothreitol (DTT) and analyzed with MALDI-TOF-MS spectroscopy. Using this protocol a representative selection of glycopeptides containing naked, single, and up to five Tn glycosylation sites were analyzed to confirm identity and purity of 147 out of 160 tested 20mer glycopeptides (92%) (supplementary info S2). The bead capture and cleavage procedure can be handled in high-throughput by using 96- or 384-well filterplates.

To validate the practical utility of the approach we synthesized a complete set of immobilized Tn-MUC1 glycopeptides covering all possible Tn-permutations of one tandem repeat isoform of the MUC1 protein and evaluated the exact binding epitopes of our previously established mAbs 5E5, 2D9[18, 25] and VU3C6[27] (Fig 2 compounds **1–31**). The binding epitope of 5E5 was confined to glycopeptides containing at least a single GalNAc residue at the Thr in the -GS**T**A- sequence, which is in agreement with our previous studies (eg. compound **1** and **9**). Interestingly, the other mAb, 2D9, reactive with the same GalNAc-glycopeptide epitope in the MUC1 tandem repeat, only reacted with glycopeptides having GalNAc residues in both the Thr and Ser of the -GSTA- sequence epitope (compare compound **1** to compounds **15**, **23–28** and **31**). An Ala mutation walk over the GSTAPP region of MUC1 confined the minimum epitope to -T(GalNAc)AP- sequence (supplementary info S3). The third MUC1 mAb tested, VU3C6, had specific preferences for glycosylation at the PDTR motif[27], a common target for murine and human anti-MUC1 MAbs.[17,38,39]

Epitope mapping of Tn-MUC1 glycopeptide vaccine induced human IgG antibodies

Next, we demonstrated that our 20mer glycopeptide platform is sensitive and can be used to detect glycoform specific autoantibodies in human sera. We have previously shown that vaccination with a fully glycosylated 106-mer glycopeptide Tn-MUC1-KLH vaccine induced Tn-MUC1 IgG antibodies in disease-free breast cancer patients,[40] and we recently confirmed this by microarray analysis using chemoenzymatically formed 60mer glycopeptides.[8] In order to further validate the utility of the current high-throughput 20mer microarray platform and fine-tune the vaccine induced IgG antibodies, we tested the same sera before and after vaccination on the scanning Tn-MUC1 glycopeptide library. Interestingly, the 20mer array, now expanded to a complete set of immobilized Tn-MUC1 glycopeptides (comp. **1–62**), could further discern distinct glycopeptide epitopes as exemplified with three different positive serum samples (Fig. 3A). For example, patient 1 has mixed reactivity to -PDT(GalNAc)R- and mono-glycosylated epitopes -GS(GalNAc)TAP- (eg. compounds **2** and **3** in Fig 3) epitopes, whereas patient 2 has a strict reactivity to bis-glycosylated -GS(GalNAc)T(GalNAc)AP-glycopeptides. Patient 3, on the other hand, has a predominant reactivity to -PDT(GalNAc)R- glycopeptides (eg. compounds **3** and **34** in Fig 3). By selecting compound **25** and **47** covering glycoepitopes of the tandem repeat we identified 18 out of 18 tested patient sera with Tn-MUC1 reactive antibodies post-immunization while no antibodies were found before vaccination collection (Fig. 3B).

Rapid expansion of the GalNAc-glycopeptide array library by on-slide enzymatic glycosylation

On-slide enzymatic glycosylation of peptides and GalNAc-glycopeptides offer a simple and cost-effective expansion of O-glycan sites and structures. We first showed that non-glycosylated Ser or Thr residues on either naked or GalNAc-glycopeptides printed on NHS slides, serve as substrates for recombinant GalNAc-transferases. Figure 4A demonstrates that on-slide treatment with GalNAc-T2 readily glycosylates unglycosylated (compound **1**) or partially glycosylated MUC1a peptides (compounds **2** and **3**). This was evaluated with the MUC1 glycopeptide specific mAbs 5E5 and 2D9 (Fig 4A). Since GalNAc-T2 is known to glycosylate both Ser and Thr residues in the -GSTA- sequence of this MUC1 tandem repeat peptide in solution,[41] the induction of 2D9 signal following this treatment suggests this site-specificity is maintained on-slide. The outcome of the enzymatic GalNAc-transferase glycosylation of immobilized peptides was further investigated by on-bead glycosylation of the MUC1a peptide, as followed by MALDI-TOF-MS analysis. The crude glycopeptides were captured on Sepharose beads functionalized with cleavable linker as described above, then subjected to enzymatic glycosylation with the same conditions applied on slide. Upon release of the glycopeptides, MALDI-TOF-MS analysis enabled a readout of the relative efficiency of GalNAc units enzymatically added. As is the case for solution glycosylation of this peptide, three of the five potential Ser or Thr residues are glycosylated by on-bead GalNAc-T2 treatment (Fig. 4A, 4B), further highlighting the similarity between enzymatic glycosylation of immobilized peptides and those in solution.

GalNAc O-glycosylation is directed by a large family of up to 20 GalNAc-transferase isoforms with partially overlapping peptide substrate specificity.[42–45] Three isoforms, GalNAc-T1, -T2, and -T3, have been studied in greatest detail and common as well as unique acceptor substrate peptides have been identified in the past.[41,46,47] To evaluate the potential use of different polypeptide GalNAc-T isoforms for site-directed GalNAc glycosylation of immobilized peptides, a selection of purified peptides was treated on array or in solution with each of these three enzymes and glycosylation was analyzed by lectin reactivity (array reactions) or MALDI-TOF mass spectrometry (solution reactions). As shown in Figure 4C, which the conditions used, all three isoforms incorporated GalNAc residues into the recombinant MUC in tandem repeat peptides from MUC2 and MUC7 with

multiple acceptor sites. Other peptides from previous reports were also tested such as CD59, hCG-b, (MUC2, MUC7);[48] (MUC2), hCG-b, OSM-fragment;41 G-CSF49 (Fig4C). The OSM-fragment peptide was glycosylated by GalNAc-T1 and to a lesser extent GalNAc-T3, the gCSF and hCG-β peptides almost exclusively by GalNAc-T2, and CD59 only by T3, all in agreement with results of solution reaction studies. Thus, the selected peptide substrates included in the analysis demonstrate that it is possible to develop conditions for a high degree of isoform specific on-slide GalNAc incorporation into peptides. Taken together, these data show that use of different GalNAc-transferase isoforms in combination with chemically synthesized GalNAc glycopeptide array libraries allow for a high degree of site selection for the O-glycan, as well as rapid and efficient on-slide enzymatic expansion of libraries to present different densities and patterns of GalNAc-glycopeptides. Thus, a simple approach to cover as many permutations as possible of a given O-glycoprotein with complete control, would be to synthesize glycopeptide libraries with single a walking GalNAc residue per peptide covering all O-glycosylation sites, and then use on-slide glycosylation with GalNAc-T isoforms to add GalNAc residues to adjacent sites in all single GalNAc glycopeptides.

Enzymatic on-slide expansion of Tn-glycopeptide library reveals cancer-associated IgG autoantibodies to distinct MUC1 glycopeptide epitopes in cancer patients

Tumor cells form a variety of aberrantly glycosylated proteins, often with truncated O-glycan structures such as GlcNAcβ1-3GalNAc (Core3), Galβ1-3GalNAc (Core1) and NeuAcα2-6GalNAc (STn).[13] To rapidly transform the Tn-library to extended glycoforms, we could further diversify the GalNAc glycopeptide arrays by applying corresponding elongating glycosyltransferases. In Figure 5A, Panels VII and IX illustrate that the glycosylation reactions effectively convert Tn-glycopeptides to Core3 and STn glycoforms as detected with core3 and STn specific mAbs, respectively. It is evident from these data that both these mAbs have strong preferences for specific glycoepitopes as they do not detect all available glycopeptides on the array. For example the Core3 mAb recognizes the mono-glycosylated MUC1 -GST(core3)APP- sequence whereas the bis-core3 glycoform -GS(core3)T(core3)APP- is not recognized (Supplementary info S4). The STn reactive mAb 3F1 is hapten specific and only reacts with bis-STn-glycopeptides without any evident preferences for the peptide backbone. To confirm the efficiency of enzymatic on-slide glycosylation reactions on our Tn-library we performed the same reaction on the Tn-MUC1a loaded Sepharose beads. The core3 β1-3GlcNAcT reaction went to completion and glycosylated all three available Tn sites, whereas the hST6Gal-I generated underglycosylated products (supplementary info S5).

Using the enzymatically diversified MUC1 array library, we next evaluated a selection of cancer sera with known MUC1 autoantibody reactivity to Core3 and STn glycoforms as demonstrated in our previous study.[8] For example, cancer serum 1 showed increased reactivity to various MUC1 epitopes after core3 β1-3GlcNAcT6 treatment (Fig. 5A-II) whereas cancer serum 2 did the same only after ST6GalNAc-I treatment (Fig. 5A-VI). We could also visualize in more detail a rather complex poly-reactive autoantibody response to different MUC1 glycoforms that varied between patient sera. The data in Figure 5B demonstrate that autoantibodies from patients have reactivity similar to the Tn-MUC1 vaccinated patient antibodies. Reactivities to mono-glycosylated -GS(GalNAc)TAP- or -PDT(GalNAc)R- glycopeptide epitopes and bis-glycosylated - GS(GalNAc)T(GalNAc)AP- peptides were detected. As our MUC1 array also contains additional Tn-MUC1 glycopeptides, such as mutations and scan-sequences, the complete autoantibody epitope could in some cases be completely mapped (sequence inserts in *bold* Fig. 5B). Furthermore, we could block the autoantibody binding by competitive inhibition with purified

MUC1-20mer glycopeptides, thus confirming the interaction was specific (supplementary info S6).

Next we expanded our serum analysis to a panel of cancer sera taken at time of diagnosis (breast n=10, ovarian n=10, rectal n=10) and healthy sera n=16. We also included Core3 and STn MUC1-60mer glycopeptides and a chemically synthesized Core3 MUC1-20mer control (C3-1, comp. 131 supporting info S2) for comparison with our previous study, where some of these sera have been characterized.[8] After performing on-slide glycosylation reactions (2hrs) and extensive washings, the un-treated Tn-library, Core3- and STn-extended libraries were incubated with the cancer sera. Autoantibody reactivity to Tn-, STn- and Core3-MUC1 glycoforms were essentially absent in healthy sera, but a substantial number of cancer patient sera demonstrated the same high reactivity to the control peptides C3-1-MUC1a-20mer, core3- and STn-MUC1-60mer glycopeptides after on-slide glycosylations (Fig. 5C). This confirms that enzymatic on-slide reactions are effective and generate sufficient amount of extended glycoforms to detect glycoform specific autoantibodies. The MUC1 reactivity was not specific for a cancer type, but about 40–70% cumulative glycoform hit rate for each cancer group was found. The healthy control sera (n=16) were all negative except that one individual seemed to have a poly-Tn-hapten reactivity. We have validated the autoantibody reactivities to re-synthesized and purified 20mer glycopeptides, and are currently evaluating a large clinical cohort of early stage breast cancer sera and controls (n=700) that will be published elsewhere.

Conclusion

Strategies to assemble large peptide arrays for large-scale multiplex characterization of biological samples have matured to the point that they are now broadly applicable to researchers, and major advantages include limited consumption of target compounds as well as the binding probe, e.g. serum antibodies.[50] Each immobilization approach has its own advantages or limitations and is generally custom made for a specific use. Typically chemo-selective immobilizations for covalent binding or avidin-biotin and His6-tagged proteins for con-covalent adsorptive immobilization are utilized.[51] We have shown that 20mer glycopeptides can be rapidly synthesized and presented in a high through-put manner on a microarray format. A combination of chemical and enzymatic methods allows site-specific synthesis of up to five glycosylations per 20mer peptides, including complex glycans such as Core1, Core2, Core3 and Core4 glycoforms. The glycopeptide array platform can be extended based on both increased glycosylation sites, by using GalNAc transferases, and diversification, by using elongating glycosyltransferases. These arrayed libraries were shown to serve as a biomarker platform for disease-associated autoantibodies directed to aberrant O-glycopeptide epitopes, allowing for broader discovery screening for human autoantibodies to such epitopes, as well as further elucidation of multiple epitopes within the MUC1 tandem repeat. Thus, not only the previously identified immunodominant -GSTA-epitope,[18, 25] but also epitopes in the -PDTR- and -VTSA- regions, were found, albeit at lower frequency.

Epitope mapping of antibodies with overlapping peptide “walks” have been widely used, and the current platform now allows for detailed epitope mapping of antibodies to O-glycopeptides. An extension of this approach may eventually consist of combinations of appropriate protein arrays with on-slide enzymatic O-glycosylation to build the O-glycoproteome on folded proteins. For this latter purpose proteins would have to be expressed in prokaryotes without capacity for O-glycosylation, by in vitro translation such as with the on-slide DNA translation in the NAPPA technology,[52] or by use of glycoengineered eukaryotic cells.[25]

The study furthermore provided a simple method for structural confirmation of any targets identified using parallel on-bead isolation, on-bead enzymatic glycosylation and complete mass spectrometric characterization of released glycopeptide. The methods are in principle applicable to other PTM modified epitopes that can be synthesized with modified Fmoc building blocks and/or where modification can be induced with selectivity in short immobilized peptides by recombinant enzymes. The overriding aim is to enable the display of appropriate epitomes including PTM modified epitopes for discovery of disease-associated immunity. The combined approach described here provides: (1) multi-well synthesis at low scale of peptides and GalNAc-glycopeptides combined with direct printing without prior purification for a facile and economical approach to display 100s of glycopeptides; (2) further amplification by sequential enzymatic on-slide expansion of GalNAc incorporation and O-glycan elongation to achieve diverse O-glycopeptide libraries with thousands of glycopeptides displaying different O-glycan patterns and structures; and (3) a highly sensitive and low background array platform by use of the hydrogel microarray glass surface.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Reuschenbach M, von Knebel Doeberitz M, Wentzensen N. A systematic review of humoral immune responses against tumor antigens. Cancer Immunol Immunother 2009;58(10):1535–1544. [PubMed: 19562338]
2. Tan EM, Zhang J. Autoantibodies to tumor-associated antigens: reporters from the immune system. Immunol Rev 2008;222:328–340. [PubMed: 18364012]
3. Krueger KE, Srivastava S. Posttranslational protein modifications: current implications for cancer detection, prevention, and therapeutics. Mol Cell Proteomics 2006;5(10):1799–1810. [PubMed: 16844681]
4. Robinson WH, DiGennaro C, Hueber W, Haab BB, Kamachi M, Dean EJ, Fournel S, Fong D, Genovese MC, de Vegvar HE, Skriner K, Hirschberg DL, Morris RI, Muller S, Pruijn GJ, van Venrooij WJ, Smolen JS, Brown PO, Steinman L, Utz PJ. Autoantigen microarrays for multiplex characterization of autoantibody responses. Nat Med 2002;8(3):295–301. [PubMed: 11875502]
5. Desmetz C, Cortijo C, Mange A, Solassol J. Humoral response to cancer as a tool for biomarker discovery. J Proteomics 2009;72(6):982–988. [PubMed: 19539066]
6. Tainsky MA. Genomic and proteomic biomarkers for cancer: a multitude of opportunities. Biochim Biophys Acta 2009;1796(2):176–193. [PubMed: 19406210]
7. Desmetz C, Maudelonde T, Mange A, Solassol J. Identifying autoantibody signatures in cancer: a promising challenge. Expert Rev Proteomics 2009;6(4):377–386. [PubMed: 19681673]
8. Wandall HH, Blixt O, Tarp MA, Pedersen JW, Bennett EP, Mandel U, Ragupathi G, Livingston PO, Hollingsworth MA, Taylor-Papadimitriou J, Burchell J, Clausen H. Cancer biomarkers defined by autoantibody signatures to aberrant O-glycopeptide epitopes. Cancer Res 2010;70(4):1306–1313. [PubMed: 20124478]
9. Schietinger A, Philip M, Yoshida BA, Azadi P, Liu H, Meredith SC, Schreiber H. A mutant chaperone converts a wild-type protein into a tumor-specific antigen. Science 2006;314(5797):304–308. [PubMed: 17038624]

10. Coenen D, Verschueren P, Westhovens R, Bossuyt X. Technical and diagnostic performance of 6 assays for the measurement of citrullinated protein/peptide antibodies in the diagnosis of rheumatoid arthritis. Clin Chem 2007;53(3):498–504. [PubMed: 17259232]
11. Hiki Y. O-linked oligosaccharides of the IgA1 hinge region: roles of its aberrant structure in the occurrence and/or progression of IgA nephropathy. Clin Exp Nephrol 2009;13(5):415–423. [PubMed: 19365705]
12. Hakomori S. Glycosylation defining cancer malignancy: new wine in an old bottle. Proc Natl Acad Sci U S A 2002;99(16):10231–10233. [PubMed: 12149519]
13. Brockhausen I. Pathways of O-glycan biosynthesis in cancer cells. Biochim Biophys Acta 1999;1473(1):67–95. [PubMed: 10580130]
14. Springer GF. Immunoreactive T and Tn epitopes in cancer diagnosis, prognosis, and immunotherapy. J Mol Med 1997;75(8):594–602. [PubMed: 9297627]
15. Smorodin EP, Kurtenkov OA, Sergeyev BL, Lilleorg AL, Chuzmarov VI. Antibodies to tumor-associated carbohydrate epitopes in sera of cancer patients and blood donors. Experimental Oncology 2001;23(2):109–113.
16. Reis CA, Sorensen T, Mandel U, David L, Mirgorodskaya E, Roepstorff P, Kihlberg J, Hansen JE, Clausen H. Development and characterization of an antibody directed to an alpha-N-acetyl-D-galactosamine glycosylated MUC2 peptide. Glycoconj J 1998;15(1):51–62. [PubMed: 9530956]
17. von Mensdorff-Pouilly S, Petrakou E, Kenemans P, van Uffelen K, Verstraeten AA, Snijdewint FG, van Kamp GJ, Schol DJ, Reis CA, Price MR, Livingston PO, Hilgers J. Reactivity of natural and induced human antibodies to MUC1 mucin with MUC1 peptides and n-acetylgalactosamine (GalNAc) peptides. Int J Cancer 2000;86(5):702–712. [PubMed: 10797294]
18. Tarp MA, Sorensen AL, Mandel U, Paulsen H, Burchell J, Taylor-Papadimitriou J, Clausen H. Identification of a novel cancer-specific immunodominant glycopeptide epitope in the MUC1 tandem repeat. Glycobiology 2007;17(2):197–209. [PubMed: 17050588]
19. Paulsen H, Peters S, Bielfeldt T, Meldal M, Bock K. Synthesis of the Glycosyl Amino-Acids N-α-Fmoc-Ser[Ac-4-β-D-Galp-(1-]3)-Ac-2-α-D-Galn(3)P]-Opfp and N-α-Fmoc-Thr[Ac-4-β-D-Galp-(1-]3)-Ac-2-α-D-Galn(3)P]-Opfp and the Application in the Solid-Phase Peptide-Synthesis of Multiply Glycosylated Mucin Peptides with Tn and T-Antigenic Structures. Carbohydrate Research 1995;268(1):17–34. [PubMed: 7736464]
20. Brocke C, Kunz H. Synthesis of tumor-associated glycopeptide antigens. Bioorganic & Medicinal Chemistry 2002;10(10):3085–3112. [PubMed: 12150854]
21. Westerlind U, Schroder H, Hobel A, Gaidzik N, Kaiser A, Niemeyer CM, Schmitt E, Waldmann H, Kunz H. Tumor-Associated MUC1 Tandem-Repeat Glycopeptide Microarrays To Evaluate Serum- and Monoclonal-Antibody Specificity. Angew Chem Int Ed Engl 2009;48(44):8263–8267. [PubMed: 19718733]
22. Ohyabu N, Hinou H, Matsushita T, Izumi R, Shimizu H, Kawamoto K, Numata Y, Togame H, Takemoto H, Kondo H, Nishimura S. An essential epitope of anti-MUC1 monoclonal antibody KL-6 revealed by focused glycopeptide library. J Am Chem Soc 2009;131(47):17102–17109. [PubMed: 19899793]
23. Laurent N, Haddoub R, Voglmeir J, Wong SCC, Gaskell SJ, Flitsch SL. SPOT Synthesis of Peptide Arrays on Self-Assembled Monolayers and their Evaluation as Enzyme Substrates. Chembiochem 2008;9(16):2592–2596. [PubMed: 18821537]
24. Oyelaran O, Li Q, Farnsworth D, Gildersleeve JC. Microarrays with Varying Carbohydrate Density Reveal Distinct Subpopulations of Serum Antibodies. J Prot Res 2009;8(7):3529–3538.
25. Sorensen AL, Reis CA, Tarp MA, Mandel U, Ramachandran K, Sankaranarayanan V, Schwientek T, Graham R, Taylor-Papadimitriou J, Hollingsworth MA, Burchell J, Clausen H. Chemoenzymatically synthesized multimeric Tn/STn MUC1 glycopeptides elicit cancer-specific anti-MUC1 antibody responses and override tolerance. Glycobiology 2006;16(2):96–107. [PubMed: 16207894]
26. Kjeldsen T, Clausen H, Hirohashi S, Ogawa T, Iijima H, Hakomori S. Preparation and characterization of monoclonal antibodies directed to the tumor-associated O-linked sialosyl-2----6 alpha-N-acetylgalactosaminyl (sialosyl-Tn) epitope. Cancer Res 1988;48(8):2214–2220. [PubMed: 2450649]

27. Price MR, Rye PD, Petrakou E, Murray A, Brady K, Imai S, Haga S, Kiyozuka Y, Schol D, Meulenbroek MF, Snijdewint FG, von Mensdorff-Pouilly S, Verstraeten RA, Kenemans P, Blockzjil A, Nilsson K, Nilsson O, Reddish M, Suresh MR, Koganty RR, Fortier S, Baronic L, Berg A, Longenecker MB, Hilgers J, et al. Summary report on the ISOBM TD-4 Workshop: analysis of 56 monoclonal antibodies against the MUC1 mucin. San Diego, Calif., November 17–23, 1996. Tumour Biol 1998;19 Suppl 1:1–20. [PubMed: 9422084]

28. Chan, W.; White, P. Fmoc solid phase synthesis: A practical Approach. Oxford University Press; 2000.

29. Paulsen H, Holck JP. [Synthesis of the glycopeptide O-beta-D-galactopyranosyl-(1 to 3)-O-(2-acetamido-2-desoxy-alpha-D-galactopyranosyl)-(1 to 3)-L-serine and -L-threonine]. Carbohydr Res 1982;109:89–107. [PubMed: 6817915]

30. Klich G, Paulsen H, Meyer B, Meldal M, Bock K. Synthesis and characterisation of highly glycosylated glycopeptides with Tn-antigenic structures corresponding to human glycophorin AN. Carbohydr Res 1997;299(1–2):33–48. [PubMed: 9129295]

31. Kihlberg J, Elofsson M, Salvador LA. Direct synthesis of glycosylated amino acids from carbohydrate peracetates and Fmoc amino acids: solid-phase synthesis of biomedicinally interesting glycopeptides. Methods Enzymol 1997;289:221–245. [PubMed: 9353724]

32. Rychlewski L, Kschischo M, Dong L, Schutkowski M, Reimer U. Target specificity analysis of the Abl kinase using peptide microarray data. J Mol Biol 2004;336(2):307–311. [PubMed: 14757045]

33. Blixt O, Head S, Mondala T, Scanlan C, Huflejt ME, Alvarez R, Bryan MC, Fazio F, Calarese D, Stevens J, Razi N, Stevens DJ, Skehel JJ, van Die I, Burton DR, Wilson IA, Cummings R, Bovin N, Wong CH, Paulson JC. Printed covalent glycan array for ligand profiling of diverse glycan binding proteins. Proc Natl Acad Sci U S A 2004;101(49):17033–17038. [PubMed: 15563589]

34. Blixt O, Allin K, Bohorov O, Liu X, Andersson-Sand H, Hoffmann J, Razi N. Glycan microarrays for screening sialyltransferase specificities. Glycoconj J 2008;25(1):59–68. [PubMed: 17914671]

35. Blixt O, Kumagai-Braesch M, Tibell A, Groth CG, Holgersson J. Anticarbohydrate Antibody Repertoires in Patients Transplanted with Fetal Pig Islets Revealed by Glycan Arrays. American Journal of Transplantation 2009;9(1):83–90.

36. Blixt O, Hoffmann J, Svenson S, Norberg T. Pathogen specific carbohydrate antigen microarrays: a chip for detection of Salmonella O-antigen specific antibodies. Glycoconj J 2008;25(1):27–36. [PubMed: 17558551]

37. Blixt O, Norberg T. Solid-Phase Enzymatic Synthesis of a Sialyl Lewis X Tetrasaccharide on a Sepharose Matrix. J Org Chem 1998;63(8):2705–2710. [PubMed: 11672140]

38. Taylor-Papadimitriou J, Burchell JM, Plunkett T, Graham R, Correa I, Miles D, Smith M. MUC1 and the immunobiology of cancer. J Mammary Gland Biol Neoplasia 2002;7(2):209–221. [PubMed: 12463741]

39. Danielczyk A, Stahn R, Faulstich D, Loffler A, Marten A, Karsten U, Goletz S. PankoMab: a potent new generation anti-tumour MUC1 antibody. Cancer Immunol Immunother 2006;55(11): 1337–1347. [PubMed: 16485130]

40. Sabbatini PJ, Ragupathi G, Hood C, Aghajanian CA, Juretzka M, Iasonos A, Hensley ML, Spassova MK, Ouerfelli O, Spriggs DR, Tew WP, Konner J, Clausen H, Abu Rustum N, Dansihefsky SJ, Livingston PO. Pilot study of a heptavalent vaccine-keyhole limpet hemocyanin conjugate plus QS21 in patients with epithelial ovarian, fallopian tube, or peritoneal cancer. Clin Cancer Res 2007;13(14):4170–4177. [PubMed: 17634545]

41. Wandall HH, Hassan H, Mirgorodskaya E, Kristensen AK, Roepstorff P, Bennett EP, Nielsen PA, Hollingsworth MA, Burchell J, Taylor-Papadimitriou J, Clausen H. Substrate specificities of three members of the human UDP-N-acetyl-alpha-D-galactosamine:Polypeptide N-acetylgalactosaminyltransferase family, GalNAc-T1, -T2, and -T3. J Biol Chem 1997;272(38): 23503–23514. [PubMed: 9295285]

42. Clausen H, Bennett EP. A family of UDP-GalNAc: polypeptide N-acetylgalactosaminyl-transferases control the initiation of mucin-type O-linked glycosylation. Glycobiology 1996;6(6): 635–646. [PubMed: 8922959]

43. Brockhausen I, Toki D, Brockhausen J, Peters S, Bielfeldt T, Kleen A, Paulsen H, Meldal M, Hagen F, Tabak LA. Specificity of O-glycosylation by bovine colostrum UDP-GalNAc:

polypeptide alpha-N-acetylgalactosaminyltransferase using synthetic glycopeptide substrates. Glycoconj J 1996;13(5):849–856. [PubMed: 8910012]

44. Hassan H, Reis CA, Bennett EP, Mirgorodskaya E, Roepstorff P, Hollingsworth MA, Burchell J, Taylor-Papadimitriou J, Clausen H. The lectin domain of UDP-N-acetyl-D-galactosamine: polypeptide N-acetylgalactosaminyltransferase-T4 directs its glycopeptide specificities. J Biol Chem 2000;275(49):38197–38205. [PubMed: 10984485]
45. Miwa HE, Gerken TA, Jamison O, Tabak LA. Isoform-specific O-glycosylation of osteopontin and bone sialoprotein by polypeptide N-acetylgalactosaminyltransferase-1. J Biol Chem 2010;285(2): 1208–1219. [PubMed: 19880513]
46. Bennett EP, Hassan H, Mandel U, Mirgorodskaya E, Roepstorff P, Burchell J, Taylor-Papadimitriou J, Hollingsworth MA, Merkx G, van Kessel AG, Eiberg H, Steffensen R, Clausen H. Cloning of a human UDP-N-acetyl-alpha-D-Galactosamine:polypeptide N-acetylgalactosaminyltransferase that complements other GalNAc-transferases in complete O-glycosylation of the MUC1 tandem repeat. J Biol Chem 1998;273(46):30472–30481. [PubMed: 9804815]
47. Schwientek T, Bennett EP, Flores C, Thacker J, Hollmann M, Reis CA, Behrens J, Mandel U, Keck B, Schafer MA, Haselmann K, Zubarev R, Roepstorff P, Burchell JM, Taylor-Papadimitriou J, Hollingsworth MA, Clausen H. Functional conservation of subfamilies of putative UDP-N-acetylgalactosamine:polypeptide N-acetylgalactosaminyltransferases in Drosophila, Caenorhabditis elegans, and mammals. One subfamily composed of l(2)35Aa is essential in Drosophila. J Biol Chem 2002;277(25):22623–22638. [PubMed: 11925450]
48. Bennett EP, Hassan H, Mandel U, Hollingsworth MA, Akisawa N, Ikematsu Y, Merkx G, van Kessel AG, Olofsson S, Clausen H. Cloning and characterization of a close homologue of human UDP-N-acetyl-alpha-D-galactosamine:Polypeptide N-acetylgalactosaminyltransferase-T3, designated GalNAc-T6. Evidence for genetic but not functional redundancy. J Biol Chem 1999;274(36):25362–25370. [PubMed: 10464263]
49. DeFrees S, Wang ZG, Xing R, Scott AE, Wang J, Zopf D, Gouty DL, Sjoberg ER, Panneerselvam K, Brinkman-Van der Linden EC, Bayer RJ, Tarp MA, Clausen H. GlycoPEGylation of recombinant therapeutic proteins produced in Escherichia coli. Glycobiology 2006;16(9):833–843. [PubMed: 16717104]
50. Kohn M. Immobilization strategies for small molecule, peptide and protein microarrays. J Pept Sci 2009;15(6):393–397. [PubMed: 19308932]
51. Uttamchandani M, Yao SQ. Peptide microarrays: next generation biochips for detection, diagnostics and high-throughput screening. Curr Pharm Des 2008;14(24):2428–2438. [PubMed: 18781992]
52. Ramachandran N, Hainsworth E, Demirkan G, LaBaer J. On-chip protein synthesis for making microarrays. Methods Mol Biol 2006;328:1–14. [PubMed: 16785638]

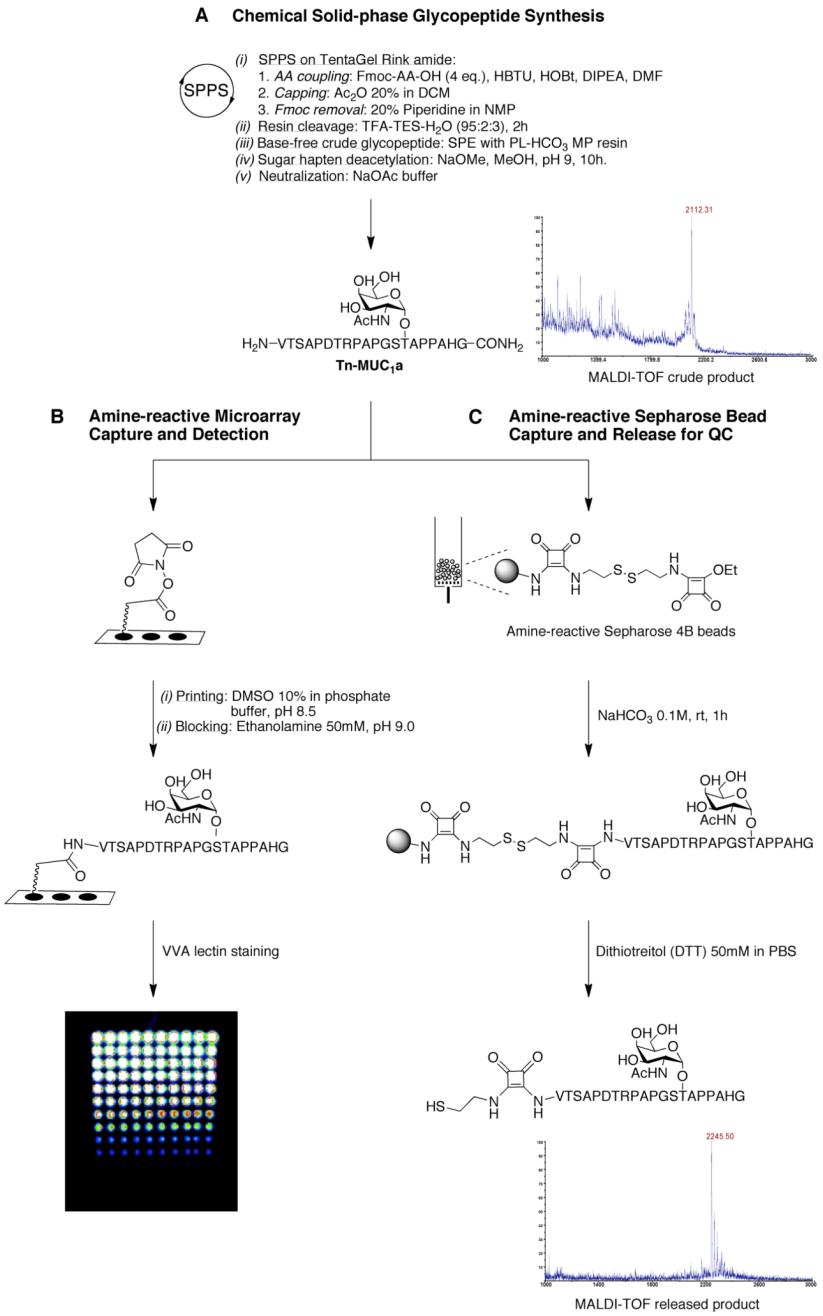


Figure 1.

Chemical synthesis and on-slide / on-bead capture of glycopeptides. (**A**) Solid-phase synthesis of 20mer glycopeptides with *N*-acetylation capping steps. (**B**) On-slide enrichment of crude product via amine-reactive microarray glass surface. Exemplified Tn-MUC1a glycopeptide printed in ten replicates at two-fold dilutions were detected with biotinylated lectin (VVA) followed by fluorescent labeled Streptavidin-AlexaFluor488 (**C**) On-bead capture, release (DTT) and MALDI-TOF spectroscopy analysis.

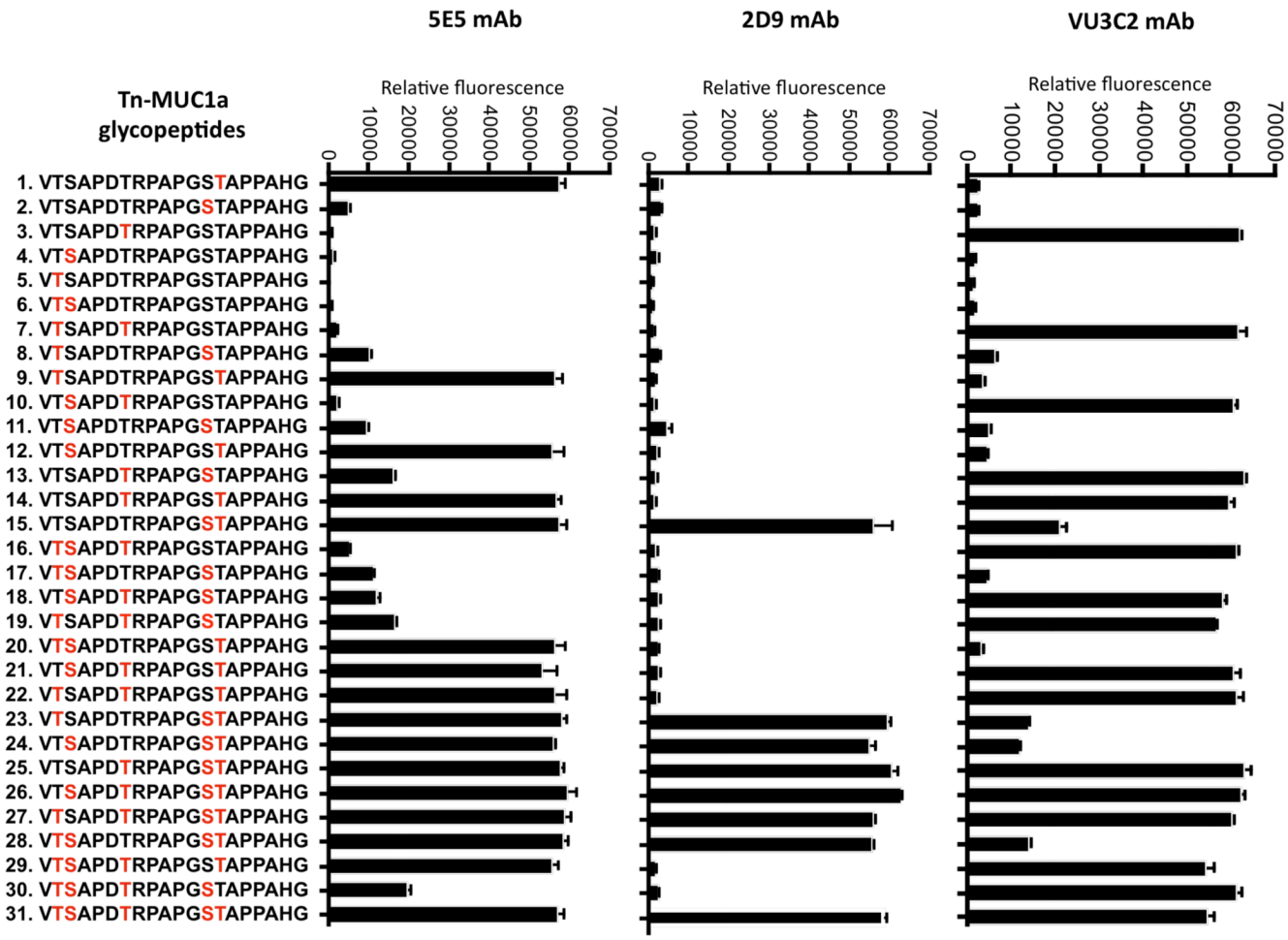


Figure 2.

Fine specificities of Tn-MUC1 reactive monoclonal tumor antibodies. Binding of mouse monoclonal antibodies 5E5, 2D9 and VU3C6 followed by detection with Cy3-labeled anti-mouse-IgG. Tn-glycosylated amino acids are displayed in *red*. Spot-to-spot variations for three replicates of each compound are represented by the error bars.

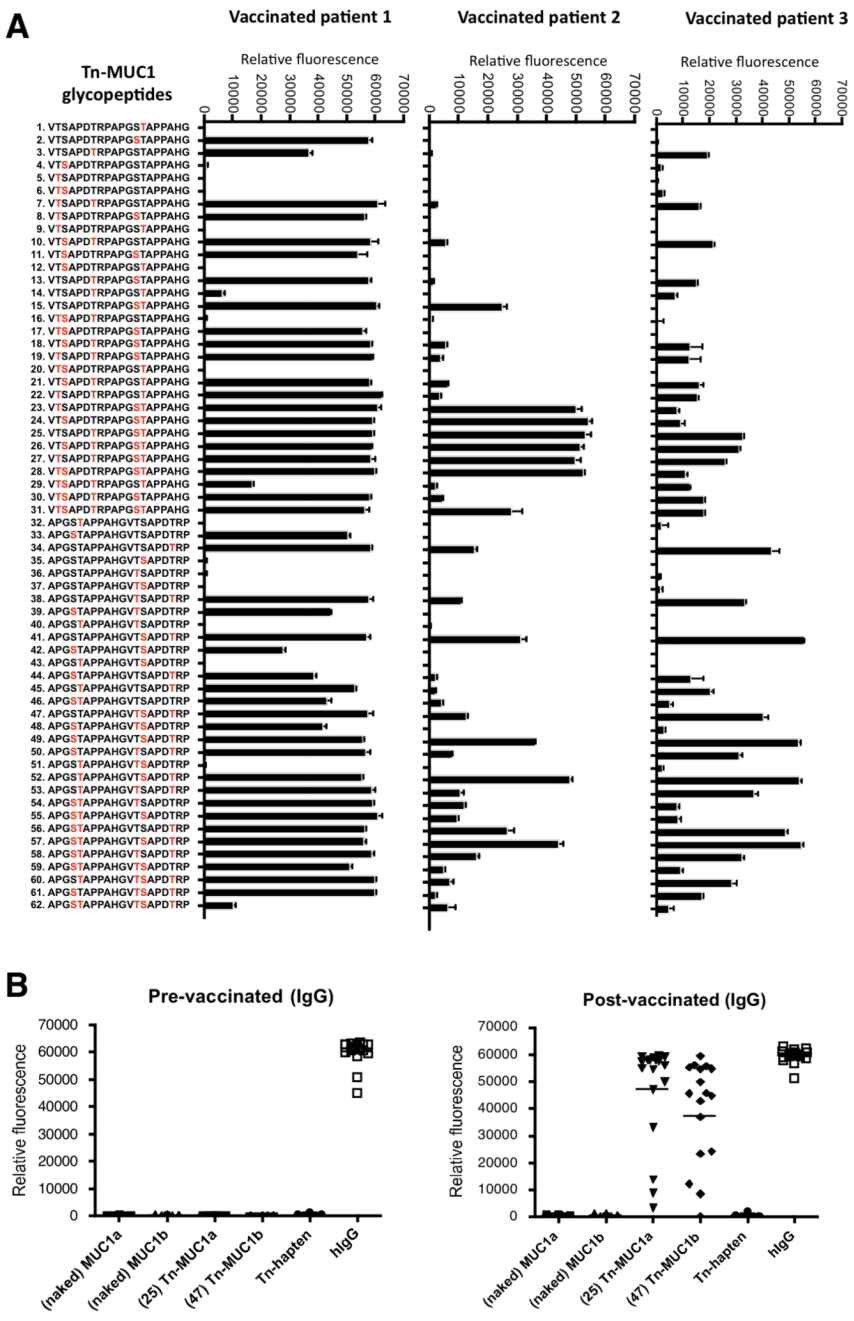
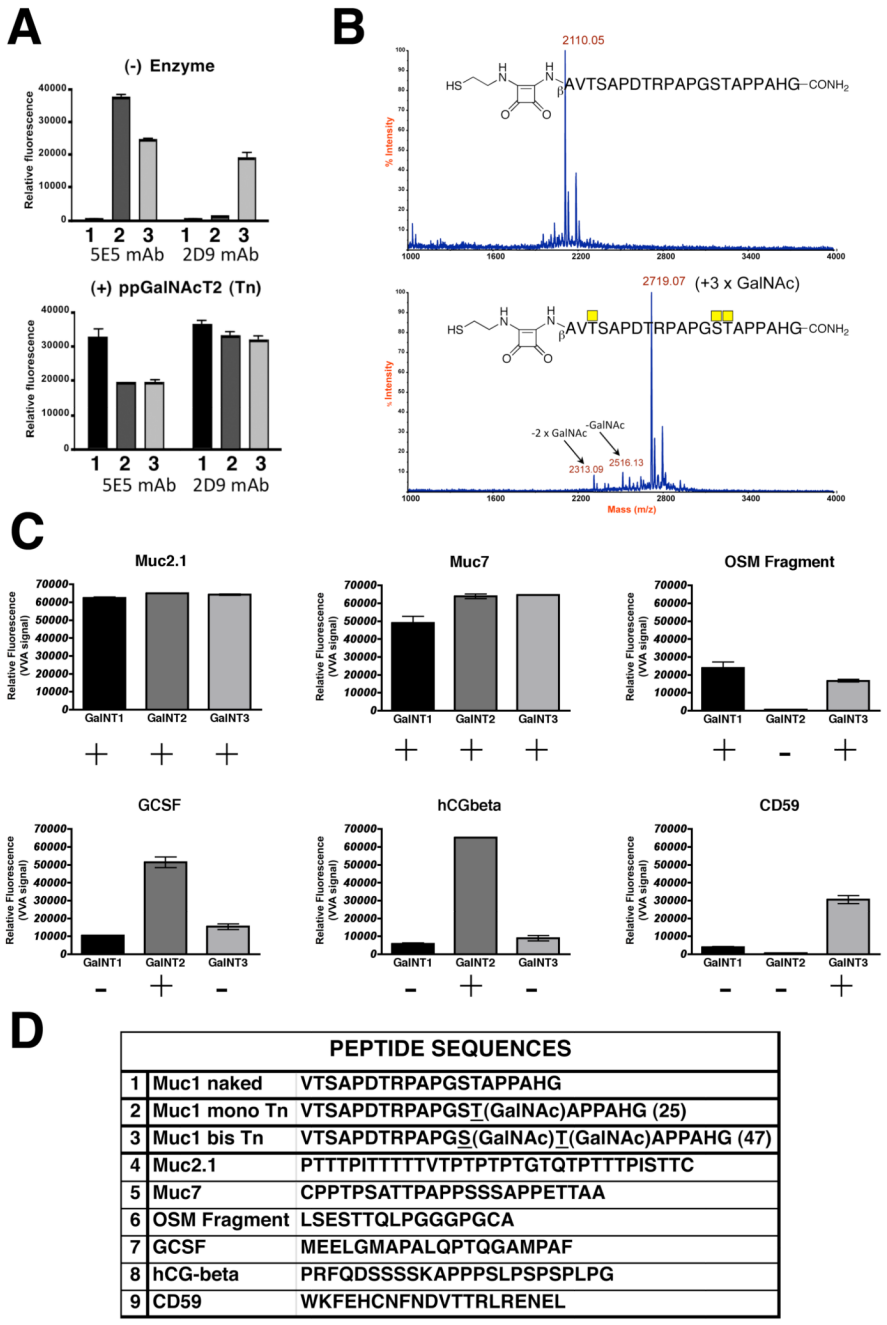


Figure 3.

Epitope mapping of vaccine induced human MUC1 specific antibodies. All sera were diluted 1:20 followed by detection with Cy3-labeled anti-human-IgG (1:1000). Spot-to-spot variations for three replicates of each compound are represented by the error bars. (**A**) Fine-specificities of selected post-vaccinated patients 1–3. Tn-glycosylated amino acids are displayed in *red*. (**B**) Pre-vaccinated patients. (**C**) Post-vaccinated patients.



PEPTIDE SEQUENCES		
1	Muc1 naked	VTSAPDTRPAPGSTAPPAHG
2	Muc1 mono Tn	VTSAPDTRPAPGST(GalNAc)APPAHG (25)
3	Muc1 bis Tn	VTSAPDTRPAPGS(GalNAc)T(GalNAc)APPAHG (47)
4	Muc2.1	PTTTPITTTTTVTPTPTPTGTQTPTTTPISTTC
5	Muc7	CPPTPSATTPAPPSSSAPPETTAA
6	OSM Fragment	LSESTTQLPGGGPGCA
7	GCSF	MEELGMAPALQPTQGAMPAF
8	hCG-beta	PRFQDSSSSKAPPPSLPSPSPLPG
9	CD59	WKFEHCNFNDVTTRLRENEL

Figure 4.

Rapid expansion of array and bead glycopeptide libraries by enzymatic glycosylation. (**A**) Analysis of substrate site-specificity by on-slide glycosylations of immobilized MUC1a glycopeptides (**1**) unglycosylated MUC1a, (**2**) mono-Tn-MUC1a and (**3**) bis-Tn-MUC1a with GalNAc-T2. Sites of incorporation were verified with Tn specific mAbs 5E5 and 2D9. (**B**) The extent of the glycosylation of peptide (**1**) from panel (A) was monitored by on-bead glycosylation, and analyzed by MALDI-TOF spectroscopy after DTT cleavage. (**C**) Comparison of GalNAc-T1, -T2, and -T3 substrate specificities in solution and on array. (**D**) Sequences of the peptides analysed in Panels A–C.

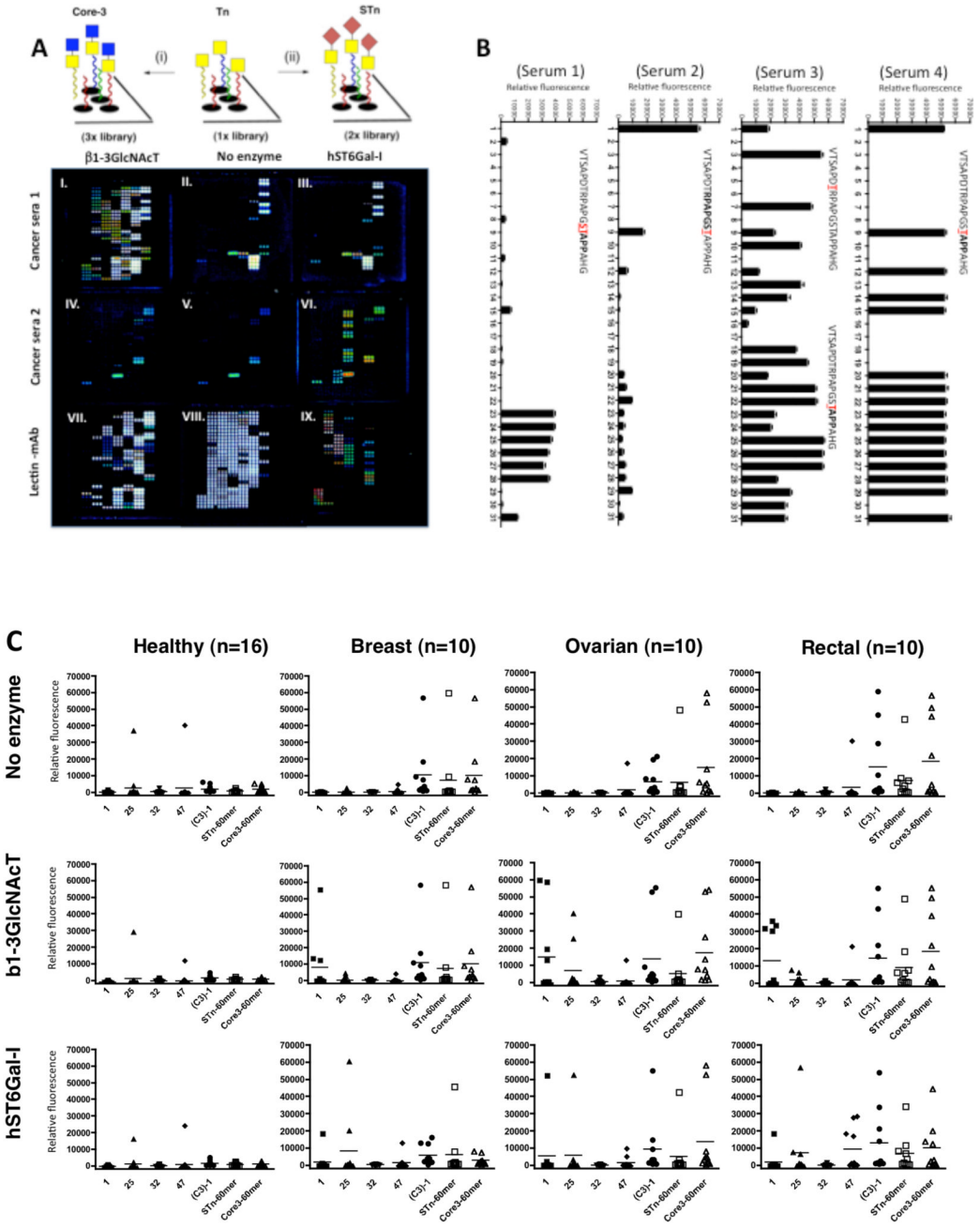


Figure 5.

On-slide enzymatic glycosylation and detection of serum autoantibodies. (**A**) Image display after on-slide enzymatic glycosylation using core3 β3GlcNAc-T6 (UDP-GlcNAc) (i) and ST6GalNAc-I (CMP-NeuAc) (ii) followed by subsequent staining with sera (1:20) and Cy3 labeled anti-human-IgG. (**B**) Glycoform specific MUC1 epitope autoantibody reactivity in four different cancer sera. Predominant MUC1 epitope for each sera is depicted as a bold sequence in each serum panel. (**C**) Detection of cancer sera and healthy sera on a panel of selected MUC1 20mers and MUC1 60mer controls. Spot-to-spot variations for three replicates of each compound are represented by the error bars.