Fast Epitope Mapping for the Anti-MUC1 Monoclonal Antibody by Combining a One-Bead-One-Glycopeptide Library and a Microarray Platform

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Abstract : Anti-MUC1 monoclonal antibodies (mAbs) are powerful tools that can be used to recognize cancer-related MUC1 molecules, the O-glycosylation status of which is believed to affect binding affinity. We demonstrate the feasibility of using a rapid screening methodology to elucidate those effects. The approach involves i) “one-bead-one-compound”-based preparation of bilayer resins carrying glycopeptides on the shell and mass-tag tripeptides coding O- glycan patterns in the core, ii) on-resin screening with an anti-MUC1 mAb, iii)separating positive resins by utilizing secondary antibody conjugation with magnetic beads, and (iv) decoding the mass-tag that is detached from the positive resins pool by using mass spectrometric analysis. We tested a small library consisting of 27 MUC1 glycopeptides with different O-glycosylations against anti-MUC1 mAb clone VU-3C6. Qualitative mass-tag analysis showed that increasing the number of glycans leads to an increase in the binding affinity. Six glycopeptides selected from the library were validated by using a microarray-based assay. Our screening provides valuable information on O-glycosylations of epitopes leading to high affinity with mAb.

Introduction

Mucin 1 (MUC1) is a heavily O-glycosylated protein found at epithelial cells. MUC1 is one of the most studied and highly intriguing human mucins because it manifests different characteristics in normal and cancerous tissues.[1–3] In normal cells, MUC1 carries extended and highly branched O-glycans in a tandem repeat domain. MUC1 is overexpressed in the majority of breast carcinomas and it appears frequently in other types of cancer including lung, pancreatic, ovarian, and colon carcinomas. In tumoral cells, the MUC1 protein core is exposed due to expression of truncated O-glycans such as Tn (GalNAca1) and T (Galb1-3GalNAca1).[4] This is an immunologically and clinically important phenomenon because it may reveal novel epitopes and could act as a biological marker for disease and disease progression.[5–7]

Several studies have focused on the elucidation of the MUC1 antigenic elements that are essential for antibody (Ab) recognition. The peptide epitopes of many anti-MUC1 antibodies are concentrated in the PDTR region in a tandem repeat unit HGVTSAPDTRPAPGSTAPPA.[8,9] The specificity of the antibodies is additionally characterized by carbohydrate moieties linked to their peptide epitopes. Attaching short glycans to the Thr of this immunodominant motif improved Abs binding ;[10, 11] however, some of them showed no difference between Tn and T antigens.[10] Other antibodies showed different binding affinities towards MUC1 peptides with glycosylation sites that are occupied with Tn and T antigens.[12] Furthermore, in the case of anti-KL-6 monoclonal antibody (mAb), we found that the minimal MUC1 glycopeptide epitope is a heptapeptide PDTRPAP bearing sialyl T antigen (Neu5Aca2-3Galb1-3GalNAca) at the Thr.[13] This mAb did not bind with heptapeptide bearing other tumor-relevant carbohydrate antigens. In our previous

study using microarrays of monoglycosylated peptides library, we found different binding affinity of anti-MUC1 mAbs caused by the O-glycan structure on the PDTR region.[14]

mAbs that are sensitive to multiple O-glycans may be valuable for the detection of MUC1 under certain cancerous status. It was suggested that the multivalency of the glycan epitope is also a critical factor for binding affinity; this feature depends on mAb denoting the epitope pattern specificity. For example, secretory MUC1 produced by T47D breast cancer cells have a high degree of glycan substitution.[15] Assays with a chemoenzymatic 60 mer MUC1 library revealed that the mAb bindings were modulated by the density of glycans.[16] mAbs specific to multivalent O-glycosylations is considered to be one of the important factors for developing the next generation of mAbs.

Evaluation of the influence of various O-glycosylation patterns on mAb recognition is crucial, but many pieces of the MUC1 puzzle are required to address this issue. Various entities are needed for the preparation of a library, but the limitation of the glycopeptide library is the parallel synthesis of so many compounds with defined structure, including valuable glycan amino acids. Thus, parallel synthesis of a chemical glycopeptide library is complex, time consuming, and costly. We therefore need an approach that generates a large number of entities on a sufficient scale for screening without wasting building blocks. So far, combinatorial tools allow for chemical synthesis and biological screening of compound libraries in a fast and efficient way. This is a powerful approach not only for basic research but also for drug discovery. Combinatorial chemistry has a huge diversity of applications and different techniques, and the "one-bead-one-compound" (OBOC) combinatorial library method is widely used. This method, developed by Lam etal., has been successfully used to obtain oligomer and small-molecule libraries by solid-phase synthesis.[17] Among other applications, it has successfully been applied for creation of glycopeptide libraries with random variations of amino acids and single glycoform,[18,19] for the discovery of selective O-glycopeptide-based galectin inhibitors[20] and to obtain Nglycopeptide libraries for the identification of lectin ligands.[21] Specifically, the glycopeptide library having a single glycoform generated by Kracun et al.[18] was evaluated by anti-MUC1 mAb 5E5 and patient sera. To our knowledge, OBOC has not yet been used for generating mucin-type O-glycopeptide libraries glycosylated at multiple sites and with variation of glycoforms. Such glycopeptides might mimic better the tandem repeat glycosylation of natural mucin-type glycoproteins.

Progress on the work requires another approach to validate the combinatorial method results and, if possible, to incorporate new relevant data to the study. One of the possibilities is the new age microarray platform. The microarray technique, when employed for glycans, peptides and glycopeptides, is a powerful tool for ligand–protein binding assays.[22,23] Due to the high sensitivity and requirement for only a small amount of sample, microarrays are becoming a major tool for the highthroughput screening of antibodies and the mapping analysis of their epitopes.[14,24,25]

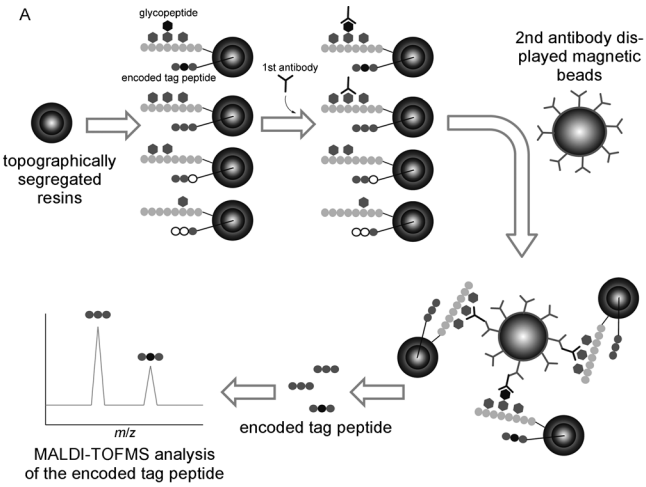
Recently, new insights into the glycan moiety, hyperglycosylation and position recognized by the anti-MUC1 mAb have emerged, but there remain some open questions concerning the specific epitopes that are recognized with high affinity by several anti-MUC1 mAbs. Specificity is crucial for diagnosis application, therefore it is necessary to identify the highest affinity epitope to avoid structural elements that provoke a false positive or false negative result. For this purpose, it is essential to develop a powerful platform that is able to produce a diversity library of glycopeptides and conduct rapid screening in combination with a highly sensitive analysis method. In this study, we combined combinatorial glycopeptide synthesis using the core–shell bifunctional resin,[26,27] a fast protocol for screening positive epitopes by mass tag, and validation by a microarray technique[14] to obtain a high number of candidates and to elucidate the critical elements of the epitope required for antibody recognition. The present results confirm the importance of site-directed glycosylation and demonstrate that multivalency in the glycosylation influences the binding profile of the antibody.

Results and Discussion

Design of a one-bead-one-glycopeptide (OBOGP) library containing a mass-reporting tag

MUC1 is expressed by tumor cells and exhibits a deficiency in glycosylation because it carries immature glycans such as the GalNAca1 (Tn) and Galb1,3GalNAca1 (T) antigens, which are recognized by the immune system.[4] Given that most anti- MUC1 Abs show a higher binding affinity for peptide epitopes that contain a carbohydrate moiety,[14] we decided to construct a MUC1 peptide library focusing on the glycan valency and heterogeneity on the basis of glycopeptides bearing Tn and/or T antigens.

Figure 1. A novel approach for the screening of the MUC1 epitope identified as a cluster of glycopeptides bearing a shared antigenic structure. a) A concept of the OBOGP and a protocol for the identification of glycopeptide epitopes by means of MALDI-TOFMS-based detection of designated peptide tags. b) Glycopeptides synthesized on the outer layer and the corresponding inner layer tripeptide tags (x-y-z) coding three individual glycosylation sites (R^1, R^2, and R^3).



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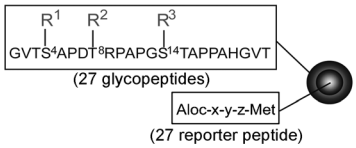


TAG Code	x	y	z
Glycosylation	R^1	R^2	R^3
None	Ala (A)	Phe (F)	Ala (A)
Tn Antigen	Lys (K)	Lys (K)	Lys (K)
T Antigen	Orn (O)	Orn (O)	Orn (O)

R may be substituted by none, GalNAcα1(Tn) or Galβ1,3GalNAcα1(T) antigen

The “one-bead-one-compound” concept is based on the fact that during solid-phase synthesis, each resin bead bears a single compound. The OBOC library method involves splitting of the resin at the diversity point and, after coupling, the beads are randomly combined and then split again. This iterative procedure allows for the synthesis of many compounds and, if biocompatible beads are used, further on-bead biological screening.[28] By employing the OBOC approach, after selecting the positive hits, direct ladder sequencing or other methods are not suitable for positive glycopeptide characterization because of the instability of the glycosidic bond under the acidic and basic conditions employed.[29] A novel version of the OBOC is the “one-bead-two-compounds (OBTC)” concept, with each bead bearing two compounds.[26, 30, 31] This approach involves synthesis of the compound of interest on the outer surface with its mass-reporting tag in the interior of the bead. Thus, the glycopeptide library was synthesized by using topographically segregated resins prepared with Tentagel resin, by following a modified protocol reported originally by the Lam group.[26, 27] Figure 1 A illustrates the concept of “one-bead-one- glycopeptide” (OBOGP), based on the core–shell bifunctional resin concurrently displaying the glycopeptide on the outer surface and the encoded tripeptide tag in the inner layer; the protocol is adapted from the OBTC method. The topographically segregated resins provide versatility and have many possible applications, as well as other merits.

In the chemical library of glycopeptides with an encoded tripeptide in the inner layer, the synthesis of glycopeptides on the outer surface has two additional advantages. The first is related to glycopeptide elongation: a free amino group is located on the external surface, and thereby increases the accessibility of the free amino group in the growing peptide to obtain a higher purity testing compound.[32] The second advantage benefits biological screening on the bead. Antibodies are huge biomolecules that presumably have problems diffusing into the core domain of a resin. The candidate epitopes are on the outer surface of the bead and are more easily accessible to the antibodies, thus avoiding the interference of coding tags with biological screening. Given that incorporation of a tag system into the OBOGP strategy that encodes the peptide on the same bead alleviates the problem associated with epitope elucidation,[21] we considered that each diverse glycosylation site in the MUC1 tandem repeat could be coded by

tagging the inner layer amino acids for each glycopeptide on the outer surface (Figure 1 B). Furthermore, the use of a tripeptide-tagging protocol might allow for mass-based rapid identification of the antigenic glycopeptides in clusters and enable handling of all the positive epitopes as a pool.

Glycopeptide libraries with a focus on glycan moieties have a smaller size in comparison with sequential peptide libraries because posttranslational modifications occur only at specific residues. Furthermore, posttranslational modifications can lead to large differences in peptide reactivity, so a slight difference of the glycan moiety may provoke an entirely different result. Here, we propose a novel screening method, and tested the approach with a small library of compounds, with future prospects of synthesizing larger glycopeptide libraries that can be challenged with other antibodies.

In the present study, we selected a 23-amino acid sequence from the variable tandem repeat region (G^{1}VTSAPDTRPAPGSTAPPAHGVT^{23}) with six possible positions to be O-glycosylated. Our aim was to obtain a high affinity epitope of anti-MUC1 antibody, thus we selected it from a pool of different epitopes. In terms of the carbohydrate motif, we selected MUC1 pancarcinoma antigens Tn (GalNAca1) and T (Galb1-3GalNAca1).[4] Concerning the position of glycosylation, to obtain sufficient data on the multivalent effect and the limitations of the synthesis in the OBOGP platform with multiple glycosylated amino acids, we decided to keep the balance in three amino acid residues Ser4, Thr8, and Ser14 that are candidates for glycosylation. A rational study of the three potential glycosylation sites was conducted based on the following criteria: i) at the N-terminal region, Thr3 and Ser4 could be selected, but due to the complications of linking two neighboring glycosylated amino acids, we decided to use Ser4 as the glycosylation candidate; ii) the same consideration arises in relation to Ser14 and Thr15, so Ser14 was chosen as the glycosylation candidate; iii) in the central area, Thr8 was selected as the glycosylation candidate, because previous studies highlighted the importance of this residue and flanking amino acids for Ab binding;[9,14] iv)finally, it was not desirable for Thr23 to be glycosylated because it is the C-terminal residue. Thus, the three chosen positions can be nonglycosylated or glycosylated with T and Tn antigens. Taking into account the three positions and the three possibilities for their glycosylation, from eicosapeptide (without a glycan group) to multiple glycosylations in the three positions with different glycans, the library was composed of 27 epitope candidates, as listed in Table 1.

Table 1. Glycopeptides library constructed on the core–shell bifunctional resin.

Glycopeptide cluster	TAG	Glycan profile[a]		
		R^1	R^2	R^3
Non-glycosylated peptide	AFA			
Monoglycosylated peptide				
Glycosylated at Thr8	AKA		Tn	
	AOA		T	
Non-glycosylated at Thr8	KFA	Tn		
	AFK			Tn
	OFA	T		
	AFO			T
Diglycosylated peptide				
2×Tn antigens	tKKA	Tn	Tn	
	AKK		Tn	Tn
	KFK	Tn		Tn
1×Tn antigen and 1×T antigen	KOA	Tn	T	
	OKA	T	Tn	
	AKO		Tn	T
	AOK		T	Tn
	KFO	Tn		T
	OFK	T		Tn
2×T antigens	OOA	T	T	
	AOO		T	T
	OFO	T		T
Triglycosylated peptide				
3×Tn antigens	KKK	Tn	Tn	Tn
2×Tn antigens and 1×T antigen	OKK	T	Tn	Tn
	KOK	Tn	T	Tn
	KKO	Tn	Tn	T
1×Tn antigen and 2×T antigens	OOK	T	T	Tn
	OKO	T	Tn	T
	KOO	Tn	T	T
3×T antigens	OOO	T	T	T

[a] Blank refers to a nonglycosylated position. Tn (Tn antigen), GalNAcα1; T (T antigen), Galβ1-3GalNAcα1.

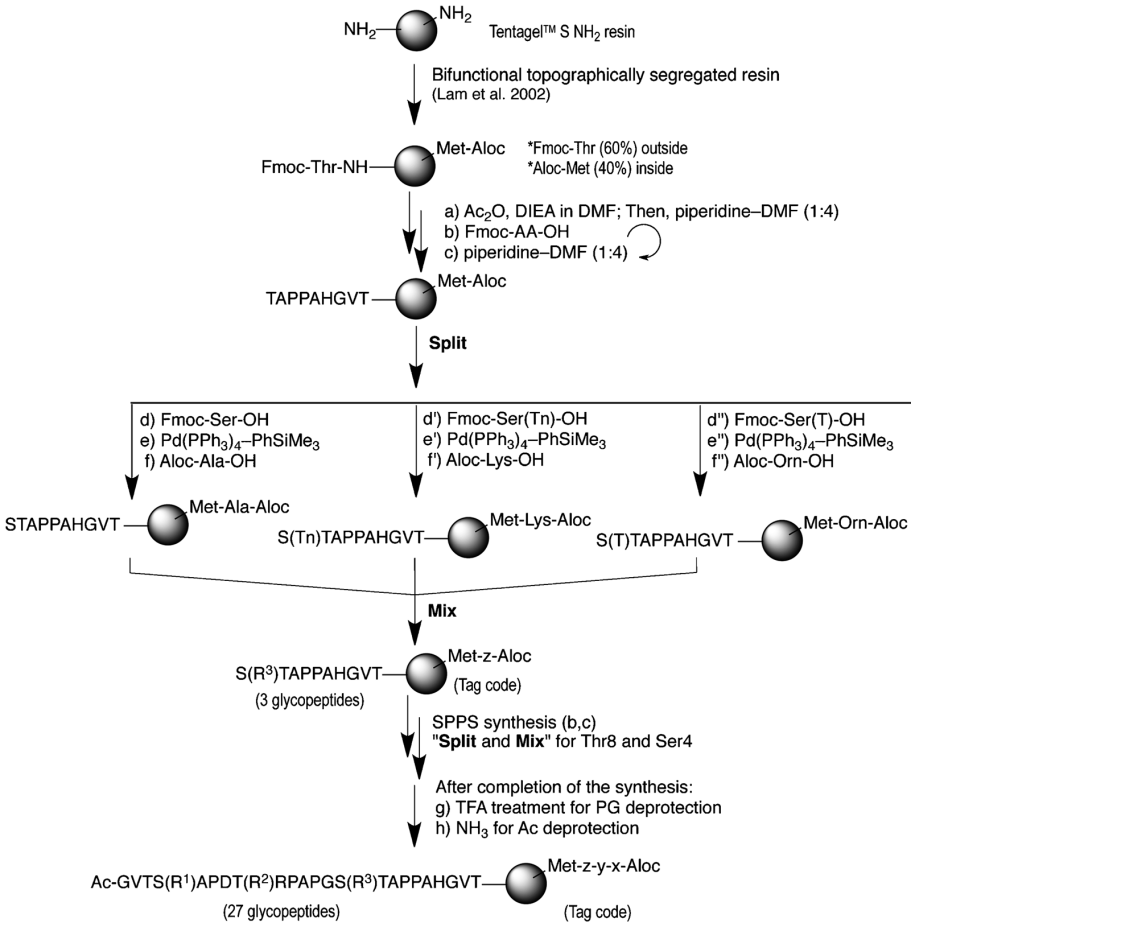
Combinatorial synthesis of OBOGP on core–shell bifunctional resin platform

The process of synthesizing the 27 MUC1 glycopeptides in the outer layer with the encoded tag tripeptide in the inner layer of the resin is illustrated in Scheme 1. The strategic chemical synthesis requires compatibility of the temporal Na-protecting groups, permanent protecting groups of the amino acids (acid sensitive) and acetyl protecting group for the glycan moieties (mild basic conditions). The outer glycopeptides were synthesized with the Fmoc Na-protecting group and Aloc was chosen as the temporal protecting group of the encoded peptide in the inner layer. The Fmoc group is not removed under the cleavage conditions of Aloc, but is removed by bases, mainly secondary amines. Aloc is stable under basic and acidic conditions and is removed under neutral conditions by a palladium-catalyzed transfer in the presence of acceptor nucleophiles.[35–37]

The topographically segregated resin allowed the incorporation of Fmoc-Thr(tBu)-OH as the first amino acid of the glycopeptide library on the outer layer, whereas Aloc-Met-OH was incorporated in the interior of the bead. A methionine residue linking the mass tag to the resin can survive during the synthesis as well as mAb assays. The tag peptides were finally cleaved by cyanogen bromide (CNBr).

Peptide elongation was performed by following common solid-phase peptide synthesis (SPPS) until the first diversity point at Ser14, then the resin was split into three reactors. In the first reactor, nonglycosylated Ser residue was incorporated, and in the other two, Ser residues containing glycosylated moieties were coupled by the "double-activation method". Briefly, this novel protocol involves incorporating glycosylated

Scheme 1. Protocol for the synthesis of the MUC1 glycopeptide library by the "split and mix" method by means of a core–shell bifunctional resin platform.



amino acids using only 1.2 equiv under microwave irradiation, after half of the coupling time, 1.2 equiv more coupling reagent is added to reactivate the free carboxylic acid. Thus, after 20 min of coupling time under microwave energy, the hindered and valuable glycan amino acids could be incorporated in high yield.[38] Afterwards, the code tag is synthesized in the inner layer. In the interior of the resin, the Aloc group was removed by using palladium catalyst,

and Aloc-AA-OH was incorporated at each reactor: Ala-encoded Ser, Lys for Ser(Tn), and Orn corresponding to Ser(T), as shown in Figure 1 B.

The three batches of resins were then joined and the peptide synthesis was continued until the second diversity point at Thr8. The same split synthetic procedure as above was followed, but, in this case, Phe encoded the nonglycosylated Thr. Considering that previous studies highlighted the importance of glycosylation of this residue, this position was marked with a different code. The batches were joined again in one reactor, the glycopeptide library was kept elongated and before Ser4, the resin was again split in three and the same procedure was performed with the encoded amino acid tags as for Ser14. The batches were pooled and the synthesis was continued until the N-terminus. Finally, the N-termini of the glycopeptides in the library were modified by acetylation and the encoded tag peptide was kept protected with the Aloc group. Upon completion of the synthesis, the protecting groups of the lateral chains of amino acids were removed under trifluoroacetic acid (TFA) treatment for 90 min. After several washings of the resin, the acetyl groups protecting the glycan moieties were removed under NH_3 in MeOH for 2 h.

Finally, 27 glycopeptides with their respective encoded tags were simultaneously synthesized by the "split and mix" method. The coding tag-tripeptide on the interior of the beads was linked through a methionine amino acid that can be digested specifically by exposure to CNBr. This treatment is orthogonal and compatible with the chemical elongation and removal of the protecting groups, permitting the specific cleavage of the inner encoded peptide to be identified by mass- based analysis.

Positive hits identified by direct on-bead screening of 27 MUC1 glycopeptides

The high-throughput screening of a glycopeptide library will accelerate the identification of potential positive candidates and provide essential elements required for antibody binding. In our library, each bead has the same glycopeptide entity on the outer surface, with the corresponding encoded tag in the interior of the bead as illustrated in Figure 1. It was considered that mass-based detection of the tag peptides released from the hit beads would greatly facilitate the selection of the hit beads without requiring individual separation and identification of the outer surface glycopeptides; in contrast, the classical method involves individual separation of each hit bead and subsequent characterization of the compounds on the positive hits. The present protocol can be used to indicate a cluster of beads carrying "positive-hit glycopeptides" among the 27 MUC1 glycopeptides in the library by rationally designed peptide tags directly showing the number and structure of individual glycosylation sites of the MUC1 tandem repeat. In this preliminary study, we selected the anti-MUC1 mAb clone VU-3C6, which was produced by immunization with breast cancer cell line ZR75-1. This anti-MUC1 mAb was chosen because it has been extensively used in previous MUC1 studies with different biological assays, and shows a high degree of selectivity among similar epitopes.[8–10,14,39–41]

Figure 2. High-throughput screening of "positive-hit glycopeptides" binding to the anti-MUC1 mAb (clone VU-3C6) as clusters on the basis of mass-based detection of the tripeptide tags. Each encoded epitope may have a different intensity in the MALDI-TOF MS, therefore, the blank control intensity of the peaks was used as a reference for the three other batches of different anti-MUC1 mAb concentrations.

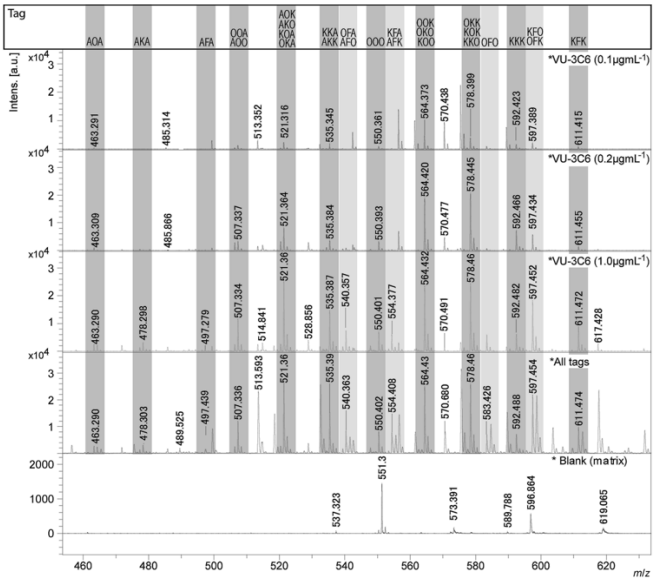


Table 2. The masses (m/z) and signal intensities of the tag peptides released from the inner layer of the corresponding core–shell bifunctional beads carrying the positive-hit glycopeptides on the outer layer.

Glycan profile[a]	TAG	M + Na (theor.)	All tags		VU-3C6 (1 μg mL^{-1})		VU-3C6 (0.2 μg mL^{-1})		VU-3C6 (0.1 μg mL^{-1})	
			M + Na	Intensity	M + Na	Intensity	M + Na	Intensity	M + Na	Intensity
S^4-T^8-S^{14}	AFA	497.5	497.4	1452.58	497.3	472.94	497.3	87.81	497.5	1.26
S^4(Tn)-T^8-S^{14} S^4-T^8-S^{14}(Tn)	KFA AFK	554.6	554.4	7011.42	554.4	5643.32	553.4	147.32	–	0.00
S^4-T^8(Tn)-S^{14}	AKA	478.5	478.3	2781.58	478.3	2509.44	478.3	98.16	478.3	52.00
S^4(T)-T^8-S^{14} S^4-T^8-S^{14}(T)	OFA AFO	540.6	540.4	7214.03	540.4	6762.61	540.4	678.06	540.3	32.55
S^4-T^8(T)-S^{14}	AOA	464.5	463.3	2250.58	463.3	2273.90	463.3	702.98	463.3	144.19
S^4(Tn)-T^8(Tn)-S^{14} S^4-T^8(Tn)-S^{14}(Tn)	KKA AKK	535.6	535.4	15329.31	535.4	13967.45	535.4	3148.81	535.3	777.02
S^4(Tn)-T^8-S^{14}(Tn)	KFK	611.7	611.5	8454.16	611.5	8309.69	611.4	1128.90	611.4	636.76
S^4(Tn)-T^8(T)-S^{14} S^4(T)-T^8(Tn)-S^{14} S^4-T^8(Tn)-S^{14}(T) S^4-T^8(T)-S^{14}(Tn)	KOA OKA AKO AOK	521.3	521.4	26161.33	521.4	24289.04	520.4	3238.81	521.3	2267.54
S^4(Tn)-T^8-S^{14}(T) S^4(T)-T^8-S^{14}(Tn)	KFO OFK	597.7	597.4	18207.88	597.4	17668.89	597.4	5728.35	597.4	1984.20
S^4(T)-T^8(T)-S^{14} S^4-T^8(T)-S^{14}(T)	OOA AOO	507.6	507.3	10735.46	507.3	10316.77	507.3	2399.04	507.3	1125.98
S^4(T)-T^8-S^{14}(T)	OFO	583.3	583.4	5255.74	583.4	5656.63	583.4	607.96	583.4	887.61
S^4(Tn)-T^8(Tn)-S^{14}(Tn)	KKK	592.7	592.5	6808.62	592.5	6032.53	592.5	7127.15	592.4	1531.20
S^4(T)-T^8(Tn)-S^{14}(Tn) S^4(Tn)-T^8(T)-S^{14}(Tn)	OKK KOK	578.7	578.5	25562.28	578.5	23676.05	578.4	20876.64	578.4	4029.84
S^4(Tn)-T^8(Tn)-S^{14}(T) S^4(T)-T^8(T)-S^{14}(Tn)	KKO OOK									
S^4(T)-T^8(Tn)-S^{14}(T) S^4(Tn)-T^8(T)-S^{14}(T)	OKO KOO	564.6	564.4	24018.64	564.4	22599.56	564.4	19012.84	564.4	4868.00
S^4(T)-T^8(T)-S^{14}(T)	OOO	550.6	550.4	6843.72	550.4	6700.50	550.4	2946.85	550.4	707.02

[a] Note of abbreviations: S, Ser; T, Thr; Tn (Tn antigen), GalNAca1; T (T antigen), Galb1-3GalNAca1.

The screening was conducted on the basis of the ability of the MUC1 tandem repeat region containing truncated glycans to bind to the anti-MUC1 mAb. For Ab binding, three batches of 10 mg of the library bound to the resin (approximately 3 x 10^4 beads each) were respectively incubated with anti-MUC1 mAb (clone VU-3C6) at three different concentrations.

Following the protocol, after several washings, the hits and non-hits of the batches were separated by magnetic capture.[42,43] Thus, anti-mouse IgG Fc magnetic particles were able to attach anti-MUC1 mAbs linked to the epitope-bounded resin. This process was performed directly in a vial, which, when placed on a magnet, allowed the positive hits-bonded resin to move to the wall of the vial, whereas the non-hits remained at the bottom of the vial, thereby permitting easy separation of the two. The novelty of this procedure is the obtaining of positive hits as a pool, without the need for time-consuming and costly instrumentation. After division, the antibodies were removed from the positive epitope-bonded resin and the procedure was repeated to avoid false positives and to confirm the activity of the positive glycopeptidyl-resin. After removal of the mAb and magnetic beads, the three batches and a negative control (10 mg of the library bonded resin) underwent chemical cleavage of the tag peptides from the resin at the Met cleavage point by CNBr treatment.

This approach permitted the separation of all positive epitopes and the elucidation of the essential elements for binding to the anti-MUC1 mAb as clusters.

As shown in Figure 2, MALDI-TOF mass spectra clearly revealed “positive-hit glycopeptides” as clusters of the mass signals due to the designated tripeptides tag of the same m/z value listed in Table 2. It was possible to discriminate between low- to high-affinity epitopes by adding different concentrations of anti-MUC1 mAb (0.1, 0.2, and 1.0 mgml^1).

The results of the mass-based epitope screening on the basis of the signal intensities at the m/z due to the positive-hit tripeptide tags are summarized in Figure 3. As shown in Figure 3 A, by adding 1 mgml^1 mAb, all positive-hit glycopeptides reached saturation of binding, whereas the naked eicosapeptide showed minor levels of affinity. For higher-affinity epitopes, data produced by lower concentrations (0.2 and 0.1 mgml^1) of the anti-MUC1 mAb shed light on the multiple glycosylation effect. It was demonstrated that for monoglycosylated peptides, the affinity of the anti-MUC1 mAb VU-3C6 with MUC1 peptides depends significantly on glycosylation at the Thr8 involved in the PDTR motif when compared with the other two glycosylation sites tested (Figure 3 B). This result is consistent with previous studies that highlighted the importance of glycosylation on this residue for VU-3C6 mAb. Notably, in our library, peptides carrying two glycans do not show much difference, irrespective of glycosylation of Thr8 (Figure S3 in the Supporting Information).

Interestingly, it seems likely that the strength ofthe interaction was influenced strongly by the glycan valency rather than the glycoform, because clusters encoded by K3 (KKK), K2O1 (OKK, KOK, KKO), K1O2 (KOO, OKO, OOK), and O3 (OOO) exhibited similar affinities with the antibody that were much higher than other clusters such as monoand di-glycosylat-ed glycopeptides (Figure 3 C). These results may indi-cate that cluster screening based on the mass tagOBOGP format is an innovative and high-throughput approach with the potential to both identify the pos-itive hits from the low-binding strength to high-affini-ty epitopes and distinguish between the affinities ofthese epitopes. However, it should be noted that the mass intensities in MALDI-TOFMS are not suited for quantitative analysis to compare the strength of the affinity between positive-hit epitopes, whereas this protocol allows for the rapid and facile screening of large-scale compound libraries to identify the clusters of positive-hits.

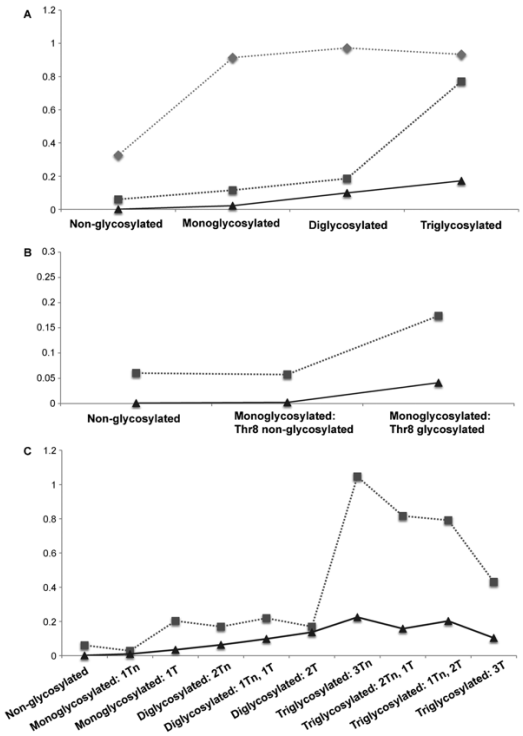
The adaptive immune system is a complex and perfectly organized system that can recognize specific molecular elements. MUC1 multiglycosylated peptide profiling for VU-3C6 mAb has been under investigation but it should be noted that other anti-MUC1 mAb may specifically recognize different positions and glycosylation patterns.[14,44]

MUC1 recognition is not limited to mAbs because innate immune system cells equipped with C-type lectins are also able to recognize changes in glycosylation, as human macrophage galactose-type lectin (hMGL) that binds to truncated O-glycans on MUC1.[45–47] Furthermore, in a recent work with collaborators, we have already found the structural elements of

the molecular recognition of monoglycosylated MUC1 peptide by STD NMR studies with hMGL protein.[48] Thus, the approach described in the present work has further applications in the study of lectin binding to glycopeptide libraries.

Here, we have demonstrated that this approach is a powerful tool for combinatorial chemistry and high-throughput screening of a glycopeptide library with different glycosylation sites and moieties. In comparison with conventional single bead characterization of positive hits, we could cleave and analyze the whole set of positive epitopes together without time-consuming separation and expensive instrumentation. Positive hits provide preliminary information of the essential elements for recognition in cluster, allowing smaller libraries to be designed and synthesized based on those results for further biological assays.

Figure 3. The affinity of the anti-MUC1 mAb (VU-3C6) for MUC1 glycopeptides as clusters revealed using the OBOGP method. Relative binding affinity was judged by employing different mAb concentrations: 1.0 mgml^{1} (diamond), 0.2 mgml^{1} (square) and 0.1 mg ml^{1} (triangle). a) Clusters focusing on the glycan valency. b) Evaluation of monoglycosylated peptides, focusing on Thr8. c) Clusters focusing on the glycan valency and glycoform combination. The graphic refers to the different epitope clusters in relation to the MS intensity relative ratio.



Epitope mapping of the oriented diversity MUC1 peptides by using microarrays

The microarray technique is a powerful tool for performing ligand–protein binding assays; therefore, we decided to use this approach to confirm the identified positive hits. To immobilize the glycans and glycopeptides on the microarray, several options based on the reactive functional group on the microarray slide are available.[49] In the present study, we chose to use a method of immobilization based on the "glycoblotting" approach, because it permits the chemoselective reaction with al- dehyde or ketones under mild conditions.[50] The advantage of our array format is demonstrated by the highly sensitive and quantitative profiling of the anti-MUC1 mAbs and IgG autoantibody signatures in relation to MUC1 fragments in human serum.[14]

For the purpose of this study, we designed and synthesized a small focused compound library selected from the diverse MUC1 species and performed a more precise screening assay by using the microarray technique. Among the 27 glycopeptides tested by the OBOGP method, we selected the oriented diversity MUC1 glycopeptides as "positive-hits" for further assessment by means of the microarray platform, because the results obtained by the rapid mass-based screening of OBOGP indicated that the valency of glycans significantly influences the antibody affinity of the mAb (VU-3C6). Thus, only six MUC1 peptides from all over the map were synthesized as focused positive-hit glycopeptides having ketone-capped N-termini to become immobilized on an aminooxy-functionalized microarray platform (Figure 4 A).[14] In addition to the naked MUC1 due was glycosylated, as is the case for compounds 3–6, whereas glycopeptide 2, bearing a Tn antigen at Ser4, showed a similar binding profile to naked peptide 1. Interestingly, further O-glycosylation at Ser4 and Ser14 drastically enhanced the affinity of mono-glycosylated 3 for VU-3C6 mAb. Moreover, it is clear that the valency of the attached glycans strongly influences the interaction with this antibody, because glycopeptide 5, carrying three Tn antigens at Ser4, Thr8, and Ser14, showed much higher affinity for the antibody than MUC1 peptide 4 with two Tn antigens at Ser4 and Thr8. It was also revealed that VU-3C6 mAb appeared to have a high binding affinity with multiglycosylated peptides 5 and 6.

As suspected, the naked MUC1 peptide had no affinity for the anti-MUC1 monoclonal antibody, which correlated with the fact that tumoral cells exhibit aberrant O-glycosylations.[5–7] It was demonstrated that the binding affinity of VU-3C6 mAb for the PDTR motif improved significantly only when the Thr8 residue was glycosylated, as is the case for compounds 3–6, whereas glycopeptide 2, bearing a Tn antigen at Ser4, showed a similar binding profile to naked peptide 1. Interestingly, further O-glycosylation at Ser4 and Ser14 drastically enhanced the affinity of mono-glycosylated 3 for VU-3C6 mAb. Moreover, it is clear that the valency of the attached glycans strongly influences the interaction with this antibody, because glycopeptide 5, carrying three Tn antigens at Ser4, Thr8, and Ser14, showed much higher affinity for the antibody than MUC1 peptide 4 with two Tn antigens at Ser4 and Thr8. It was also revealed that VU-3C6 mAb appeared to have a high binding affinity with multiglycosylated peptides 5 and 6.

Figure 5 shows the comparison between the OBOGP and microarray methods when compound 5 (MUC1 glycopeptide containing three Tn antigens) was used as a positive standard. Both curves showed the same tendency, especially with high affinity epitopes such as glycopeptides 5 and 6. Given that the microarray is a more sensitive technique, low-affinity epitopes (compounds 1–4) appeared to exhibit higher and more reliable Ab binding using the microarray technique than they did using the OBOGP method. In both cases, the results are in concordance, and we could observe that for monoglycosylated MUC1 peptides, glycosylation at Thr8 significantly enhances the binding in comparison with glycosylation at Ser4. Furthermore, it was also indicated that higher glycan valency in the tandem repeating unit is proportional to higher affinity with VU-3C6 mAb, although this effect might be influenced strongly by the conformational impact of the glycosylation at the O-glycosylation site adjacent to the epitope region.[13,51] These results demonstrate that the microarray technique validates the combinatorial chemistry approach, and the synergy of both techniques represents a method for binding assays with multiply glycosylated peptides.

Figure 4. Epitope mapping analysis of the oriented diversity MUC1 glycopeptides using a microarray platform. a) Six MUC1 peptides/glycopeptides selected from the results of the rapid mass-based screening of the 27 OBOGP library. b) Interaction of the anti-MUC1 mAb (VU-3C6, 0.1 mg ml^{1}) with six selected MUC1 peptides/glycopeptides. Compounds 1–6 were printed at eight concentrations (3.90, 7.81, 15.6, 31.2, 62.5, 125, 250, and 500 mm) onto an aminooxy-functionalized microarray in quadruplicate. Relative fluorescence units (RFU) due to the binding of the Cy3-labeled secondary antibody were measured and represented as mean values in a bar chart.

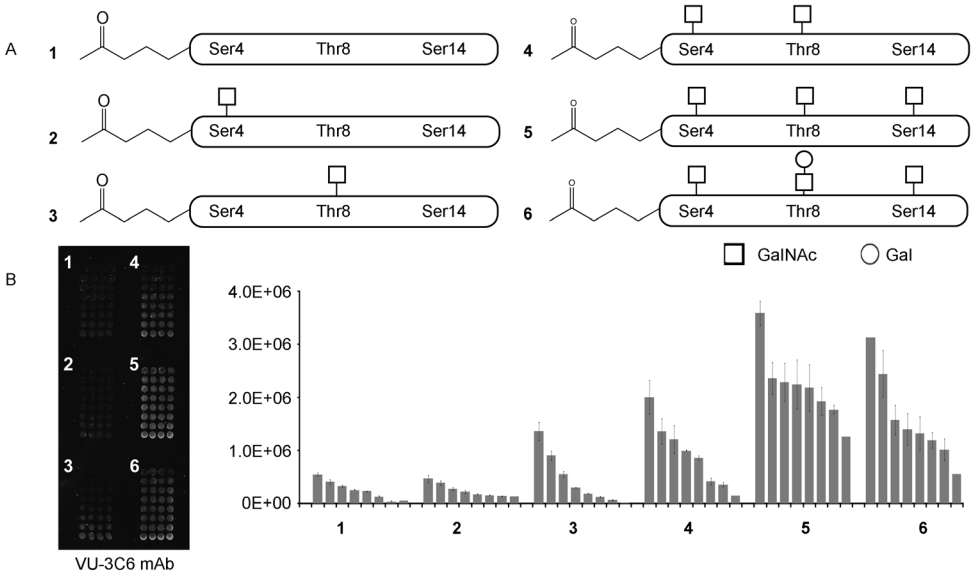
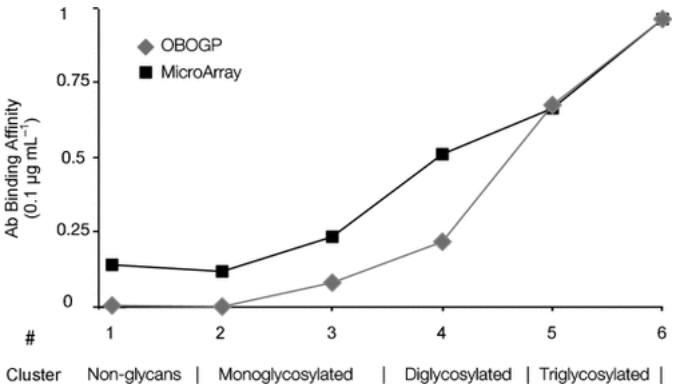


Figure 5. Comparison of VU-3C6 mAb assays between OBOGP and microarray platforms. Relative affinity is calculated by dividing the intensity of fluorescence or mass tags of each cluster composed of nonglycans (1), mono- (2 and 3), di- (4), or tri-glycosylated peptides (6) by that of compound 5 as a positive reference. (The results of the microarray assay in which 125 mm of the compounds were printed are used here because this was the highest concentration of every compound prior to saturation of mAb binding.)



Conclusion

Rapid detection and evaluation of disease-relevant epitopes for anti-MUC1 mAbs is of growing importance for cancer detection and immunotherapy applications. The aim of this work is to provide tools for the fast screening of a MUC1 glycopeptide library, including glycosylation at different positions, multiglycosylations, and different types of glycans. The significance of the method lies in the use of topographically segregated resins that are employed to synthesize the glycopeptides on the outer surface and the encoded peptide in the inner layer of the bead. Glycopeptides were synthesized by the Fmoc method and the encoded tag was generated by following an orthogonal method based on the reduction of the deprotected Aloc group. After elongation and removal of the lateral-chain- protecting groups, a biological assay of the anti-MUC1 mAb was performed directly on the bead. As part of the novelty of the method, screening was performed with magnetic beads carrying a secondary Ab, which permitted the discrimination using a pool instead of individual resin beads. Furthermore, different dilutions of the anti-MUC1 mAb (Clone VU-3C6) allowed the separation and classification of the epitopes from low to high affinity. After the cleavage and characterization of encoded tags, the essential elements for Ab binding were elucidated. Thus, this fast and easy procedure permits the main aspects of the high affinity epitopes to be delineated in clusters. This information reveals the best possible epitopes, and allows a prediction of the next generation of MUC1 glycopeptides library. The results illustrated that the clear element in high-affinity binding is the multiple glycosylated peptides and glycosylation at Thr8 in the PDTR

motif is a key glycosylation site. A diversity-oriented library based on the results of the OBOGP assay was subjected to a validation assay using the microarray platform. It was demonstrated that the combination

of the OBOGP method and the microarray platform allows for rapid and highly reliable epitope mapping analysis of antibodies that bind to glycopeptides known to be clinically important biomarkers for the development of new diagnostic and therapeutic reagents.

Experimental Section

Materials and general methods

Commercially available reagents and compounds were used without further purification. The Tentagel resins were obtained from Rapp Polymere GmbH (Tuebingen, Germany). Anti-MUC1 monoclonal antibody clone VU-3C6 was purchased from Exalpha biologicals Inc. (MA, USA) provided at 0.2 $mgml^{1}$ concentration. Anti-mouse Fc IgG biomagnetic beads were obtained from Polysciences Inc. (PA, USA) provided at 5 mg ml^{1} and binding capacity > 0.20 $mgml^{1}$. Fmoc-N-protected l-amino acids were supplied from Novabiochem Merck KGaA (Darmstadt, Germany). Fmoc-Ser(Ac3-a- GalNAc)-OH (8), Fmoc-Thr(Ac3-a-GalNAc)-OH (9), Fmoc-Ser(Ac4-b- Gal(1–3)-Ac3-a-GalNAc)-OH (10), and Fmoc-Thr(Ac5-b-Gal(1–3)-Ac3- a-GalNAc)-OH (11) were synthesized according to the methods reported previously[26, 52–56] and characterized by ^{1}H NMR spectroscopic analysis (see the Supporting information).

Aloc-N-protected l-amino acids were obtained from Bachem AG (Bubendorf, Switzerland). Aloc-Met-OH can be prepared on solid- phase by attaching commercially available Fmoc-Met-OH.[57] Experimental details are described in the Supporting Information.

Ninhydrin test kit was supplied by Funakoshi Co. Ltd. (Tokyo, Japan). Other solvents and chemicals used in this study were supplied by Wako Pure Chemical Industries (Osaka, Japan), Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan) and Sigma–Aldrich Co. LLC (Milwaukee, USA) and were of the highest purity available. Thin-layer chromatography was performed on silica gel. Detection was accomplished by irradiation using a UV lamp or by staining with ninhydrin or anisaldehyde. The chromatographic separations were achieved on silica gel columns. Reactions assisted with microwave energy were performed in a closed reaction vessel (IDX Corporation microwave system); the system was fixed to a maximum of 50 8C, and wattage rating was 0–50 W. The ^{1}H NMR spectra were recorded at an operating frequency of 500 MHz and a temperature of 300 K with a Varian 500 MHz spectrometer. Chemical shifts are reported in parts per million (d) relative to TMS or to solvent as the internal standard. All measurements of MALDI-TOFMS were performed by using an Ultraflex TOF/TOF mass spectrometer equipped with a reflector, and controlled by the Flexcontrol 1.2 software package according to the general protocols. In MALDI- TOFMS positive reflector mode, ions generated by a pulsed UV laser beam (nitrogen laser, l=337nm, 5Hz) were accelerated to a kinetic energy of 23.5kV. Reverse-phase analytical high-performance liquid chromatography (RP-HPLC) was carried out with a Hitachi instrument [detector l-2400 (220 nm) and pump l-2130]. The conditions are described for each compound. Glycan Array was a kindly gift from Sumitomo Bakelite (Tokyo, Japan). For the microarray assay, peptides were purified to a more than 90% purity by RP-HPLC using a semi-preparative column [Inertsil ODS-3 (f 20x250 mm) (GL Science Inc.)], flow rate 5 $mLmin^{1}$. Gradient conditions depending on each peptide. High-resolution electro- spray ionization mass spectra (ESI-HRMS) with a JEOL JMS-700TZ and amino acid analyses with a JEOL JLC/500 equipped with ninhydrine detection system were performed at the Center of Instrumental Analysis at Hokkaido University.

Synthesis

General methods of solid-phase synthesis: Manual solid-phase peptide elongation and other solid-phase manipulations were carried out in polypropylene syringes, each fitted with a porous disk. Standard side-chain protecting groups were used. Solvents and soluble reagents were removed by suction. Previous washings of the Tentagel S NH_2 resin were performed with CH_2Cl_2 (5x1min) and DMF (5x1min). Washings between deprotection, coupling and subsequent deprotection steps were carried out at RT with DMF (3x1 min), CH_2Cl_2 (3x1 min) and DMF (3x1 min). Fmoc removal was carried out with pip-DMF (1:4) (1 x 3 min, MW assisted). The coupling of non-glycosylated amino acids (4 equiv) was carried out with HBTU–HOBt–DIEA (4:4:6) in DMF for 10 min assisted by MW energy. Glycosyl amino acids were achieved following double activation protocol.[38] This method incorporates amino acids bearing carbohydrates by employing 1.2 equiv of glycosylated amino acids and the reagents PyBOP–HOAt–DIEA (1.2:1.2:1.8) in DMF. After 10 min reaction assisted by MW energy, without filtering, more PyBOP–HOAt (1.2 equiv) was added and reacted for an further 10 min. In case of Aloc protected amino acids, the Aloc group was removed by $Pd(PPh_3)_4$ (0.1 equiv) in the presence of the scavenger $PhSiH_3$ (10 equiv) in CH_2Cl_2 (3 x 15 min) under an Ar atmosphere; washings with CH_2Cl_2 followed each treatment.[58] Every coupling and deprotection step was checked by a ninhydrin test to ensure completion.

Generation of bifunctional topographically segregated resin for OBOGP synthesis: This protocol is based on an adapted protocol from Liu et al.[14, 26] as follows : 100 mg of Tentagel S NH_2 (90 mm, 0.27 mmolgr^{1}) beads were swollen in H_2O and vigorously shaken overnight. Water was removed by filtration, keeping water inside the resins to avoid incorporation of the AA in the interior. Next, Fmoc-Thr(tBu)-OH was incorporated on the surface with DIC in CH_2Cl_2/Et_2O (55:45, 0.5 mL) at RT for 2 h. Washing with CH_2Cl_2/Et_2O (55:45, 3x1min) and DMF (6x1min) then removed water from inside the resin. A few beads were separated for observation of the bifunctional topographically segregated resin (see the Supporting Information). Aloc-Met-OH was incorporated in the interior of the bead by conventional peptide synthesis protocol. By measuring the UV absorbance at 290 nm of the Fmoc-adduct after Fmoc group removal of first Fmoc-Thr(tBu)-OH, providing a 62.7% of the positions occupied by Fmoc-AA-OH.

Glycopeptide library (Scheme1): Glycopeptide elongation was performed as described in the general methods. At diversity point, the resin was split in three and, after amino acid incorporation, the Aloc group in the interior of the resin was removed and a new tag amino acid was incorporated. The resin was then combined again to elongate the peptide. After the last amino acid incorporation, the N-terminal was acetylated. However, the tag peptide inside of the resin remained protected with the Aloc group to improve detection by mass spectrometry. In general, removal of the amino acid protecting groups was performed simultaneously with the cleavage of the peptide from the resin due to the acid-labile anchor. In this case, the testing compounds should be evaluated directly on-resin, therefore, the glycopeptide was directly incorporated into the resin without a linker. Previous tests demonstrated that the glycopeptide was completely deprotected after both consecutive acid and basic treatments, without cleavage of the compound from the resin (data not shown). Lateral protection groups were removed by the addition of cleavage cocktail (TFA–thioanisole–H_2O–phenol–TIS (82.5 :5 :5 :5 :2.5), 1 mL) for 90 min shaking at RT. The resin was then washed with AcOH (4x1min), DMF (2x 1 min), CH_2Cl_2 (2 x 1 min), DIEA–CH_2Cl_2 (1:9, 3 x 1 min), CH_2Cl_2 (2 x 1 min), DMF (2 x1 min), and MeOH (2 x 1 min). Acetylated groups at hydroxyl groups of the glycans were removed on resin by treatment with NH_3 (2m in MeOH, 1mL) for 2h at RT. The resin was then thoroughly washed before biological assays on resin. Washings were done as follows: MeOH (4x1min), DMF (4x1min), CH_2Cl_2 (2 x 1 min), TFA–CH_2Cl_2 (0.1 %, 2 x 1 min), CH_2Cl_2 (2 x 1 min), DMF (2x1 min), MeOH (2x1 min), and H_2O (4x1 min). From here, the glycopeptide library was set for biological screening (see the biological assay section).

Synthesis and characterization of glycopeptides 1–6: Compounds 1–6 were synthesized in parallel by using Tentagel S Rink Amide resin (20 mg, 0.24 mmol g^{1}). Elongation was performed as described in the general methods. Once peptides were achieved, PEG spacer (Fmoc-8-amino-3,6-dioxaoctanoic acid) was added and, after Fmoc deprotection, 5-oxoketohexanoic acid was incorporated as N-terminus linker for microarray analysis. The glycopeptides were cleaved from the resin with simultaneous removal of sidechain protecting groups by treatment with TFA–thioanisole–H_2O– phenol–TIS (82.5:5:5:5:2.5) for 90 min at RT. A ratio of 10 mL of cleavage cocktail per 1 mg of resin was used. After the cleavage reaction, peptides were precipitated by adding cold tert-butylmethyl ether. Following centrifugation, the solution was decanted and this process was repeated twice. Finally, peptides were dissolved in H_2O–CH_3CN (1:1) and lyophilized. For the removal of the acetyl protecting groups of the glycosyl moieties in compounds 2–6, glycopeptides were dissolved in MeOH (5 mL). To the solution was added 1n NaOH, keeping the solution at pH 12.5. Deacetylation reaction was followed by MS spectroscopy. After completion, the reaction mixture was neutralized by addition of AcOH. The solvents were evaporated in vacuo. The deprotected glycopeptides were dissolved in H_2O and characterized by RP-HPLC. Reverse-phase analytical high-performance liquid chromatography (RP-HPLC) was carried out with a Hitachi instrument [detector l-2400 (220 nm) and pump l-2130]. The conditions are described for each compound. For the microarray analysis, peptides were purified to more than 90% purity by RP-HPLC, gradient conditions depending on each peptide. Composition and isolated yield were determined by amino acid analysis. Mass spectra were obtained with an Ultraflex MALDI-TOF instrument with a solution of DHB (10 mgml^{1}) in H_2O–CH_3CN (1:1), 0.1% TFA. Characterization of 1–6 is described in the Supporting Information.

Biological assay on resin: The library of glycopeptides linked to the resin was directly employed for subsequent biological screening.[26,27] The following buffers and solutions were used in this section: Blocking buffer: 50 mm Tris-HCl, 100 mm NaCl, 1 mm $CaCl_2$, $MnCl_2$, $MgCl_2$, 0.05% Tween-20, 5% bovine serum albumin (BSA), pH 7.4. Reaction buffer: 50 mm Tris-HCl, 100 mm NaCl, 1 mm $CaCl_2$, $MnCl_2$, $MgCl_2$, 0.05% Tween-20, 0.1% BSA, pH 7.4. Washing buffer: 50mm Tris-HCl, 100mm NaCl, 1mm $CaCl_2$, $MnCl_2$, $MgCl_2$, 0.05% Triton X-100, pH 7.4.

Protocol to evaluate the OBOGP library : a) Blocking : The resin was washed with H_2O (4 x 1 min) and washing buffer (1 x1 min). Blocking buffer (10–50 mL per 1 mg resin) was added to the resin and the mixture was vigorously shaken overnight at RT. The solution was then discarded and the resin was washed with washing buffer (10 x 1 min). b) Incubation with the anti-MUC1 mAb : The antiMUC1 mAb solution (1 , 0.2, and 0.1mgml^{1}) was added to the resin (10 mL per 1 mg resin) and the mixture was shaken for 2 h at 37C. The resin was then thoroughly washed with washing buffer (10 x 1 min). c) Secondary Ab, magnetic beads (Anti Fc mouse Magnetic beads): The resin was washed with H_2O (5 x 1 min). The secondary antibody was added to the reaction vessel (10 mL per 1 mg resin) in high excess and the mixture was shaken overnight at RT. The resin was then washed with H_2O (10 x 1 min) to remove nonreacting

mAb. d) Screening: Finally, 500 mL H_2O was added to the resin and the solution was transferred to a centrifuge tube. By using a magnet carrying the secondary Ab we could screen for positive versus negative beads. Positive beads could be easily separated because they were attracted by the magnet, and attached to the wall of the tube. Positive beads were washed with H_2O (10x 1 min) and 8 m guanidine-HCl (3 x 15 min) to remove the antibody. Positive beads were evaluated again to confirm activity of glycopeptidyl-resin. To cleave tag peptides from the resin through Met cleavage point, cyanogen bromide (CNBr) treatment was performed.[27] The chemical cleavage was done as follows: A solution of 0.25m CNBr (Caution: very toxic!!!) in 70% formic acid was added to a sample of resins (10 mL per 1 mg of resin). The reaction mixture was allowed to stand at RT for 12 h. Solvents were then evaporated, and the dry solid was dissolved in H_2O and evaporated (this step was repeated twice). Finally, the peptide was dissolved in H_2O–CH_3CN (2 :8, 20 mL) for further characterization by MALDITOFMS and/or lyophilized. e) Identification of positive epitopes by MALDI-TOFMS: Mass spectra were obtained with an Ultraflex MALDI-TOF instrument. The matrix used was a solution of DHB (10 mg ml^1)–DHB-Na (10 mg ml^1) in a 9 :1 mixture in H_2O–CH_3CN (7:3), 0.1% TFA. Under this matrix, single charged species forming sodium adduct were followed. In all cases, maximal intensity range in the vertical coordinate was adjusted to 5000. Mass spectra of the full pool of tags and the positive hits after screening with anti- MUC1 mAb VU-3C6 were analyzed and measured (Figure 2, Table 2 and Figure S2 in the Supporting Information).

Microarray assay: “Glycan Array” with Boc-oxime linker (Glycan Array was a kindly gift from Sumitomo Bakelite) were used as microarray slides. Glycan Array is a cyclic polyolefin plastic coated by aminooxy-functionalized methacrylic copolymer with phosphorylcholine unit. Boc-N-protected slides were immersed in 2n HCl overnight at RT, and then washed with H_2O (2x1min) and dried by centrifugation. Synthetic peptides and glycopeptides solved in 25 mm AcOH-Pyr, 0.0025 wt %, Triton X-100 were printed in quadruplicate at different concentrations to Glycan Array slides (see FigureS4 in the Supporting Information). For the printing, compounds 1–6 were spotted by OmniGrid Micro (Digilab Inc., Marlborough, MA, USA) with a 0.8 mm pitch using four ChipMakerTM CMP6 microspotting pins (200 mm spot diameter, ArrayIt Corporation, Sunnyvale, CA, USA). Each compound was printed in quadruplicate with 0.8 mm distance between spots of the same compound and 3.2 mm gap between different compounds. After printing the peptides, slides were incubated at 80 8C for 90 min to complete oxime bond formation and then washed with H_2O (1 x1 min). After, nonreactive aminooxy groups were capped with succinic anhydride (10 $mgml^1$) for 4h at RT, followed by H_2O washing (2x1 min) and drying by centrifugation, the slides were used for further binding assay of anti-MUC1 mAb (VU-3C6).

For the binding assay, we employed the same reaction buffer and washing buffer as “biological assay on resin”. Slides were placed in a reaction vessel keeping high humidity. For the Ab incubation, hybridation covers were mounted on slides and 70 mL of anti-MUC1 mAb (VU-3C6) solution in reaction buffer was carefully infused through narrow gaps between cover and slide. After filling the void with the solution, slides were kept at RT for 2 h. Hybridation covers were then removed and slides were washed with washing buffer (3x1min), H_2O (1x1min), and dried by centrifugation. For the analysis of the binding, secondary Ab (Cy3-labeled Ab) was diluted to 1 $mgml^1$ in reaction buffer and infused between unused hybridation covers and slides as explained before. After standing at RT for 1 h in the dark, slides were washed with washing buffer (3 x 1 min). For storage, the slides were degassed under vacuum and kept at 48C. Slides were subjected to fluorescent image scanning with a Tryphoon Trio Plus instrument (GE Healthcare). Array Vision software was used to quantify the fluorescence of each spot. The median value of relative fluorescence intensity was used; spot intensities were determined by subtracting the average pixel intensity from the median pixel intensity of the local background within the spots. The fluorescence of each spot is shown as the average of four replicate spots used to construct histograms showing the antibody-binding profile. Error bars are included showing the standard deviation for each interaction peptide-mAb.

Abbreviations: Abbreviations used for amino acids and carbohydrates follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature. The following additional abbreviations are used: AAA, amino acid analysis; Ab, antibody; ACN, acetonitrile; Aloc, allyloxycarbonyl ; CNBr, cyanogen bromide ; DCM, dichloromethane ; DIC, DMF, N,N’-diisopropylcarbodiimide ; DIEA, ethyldiisopropylamine ; N,N-dimethylformamide ; Fmoc, 9-fluorenylmethoxycarbonyl ; IgG, immunoglobulin G ; mAb, monoclonal antibody ; MALDI, matrix-assisted laser desorption/ionization; MeOH, methanol; MUC, mucin; MS, mass spectrometry; OBOC, one-bead one-compound; OBOGP, one-bead one-glycopeptide ; PEG, polyethylene glycol ; PG, protecting group; RFU, relative fluorescence units; RP-HPLC, reversed-phase high-performance liquid chromatography ; SPPS, solid-phase peptide synthesis; T, Galb1–3GalNAca1; TFA, trifluoroacetic acid; Tn, GalNAca1; TOF, time-of-flight.

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