Microarrays

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Tumor-Associated MUC1 Tandem-Repeat Glycopeptide Microarrays to Evaluate Serum– and Monoclonal–Antibody Specificity

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Dedicated to Prof. Helmut Ringsdorf on the occasion of his 80th birthday

The membrane-bound mucin glycoprotein MUC1 is extensively overexpressed on epithelial tumor cells and considered an attractive target in the development of cancer immunotherapy and immunodiagnostics. In epithelial tumors down-regulation of glycosyltransferases, in particular the core 2 β-1,6-*N*-acetylglucosaminyltransferase, and up-regulation of sialyltransferases results in the formation of short saccharides with premature sialylation of the extracellular domain of mucins,[1–4] exposure of the peptide backbone of these otherwise heavily glycosylated proteins, and formation of a tumor-associated epitope consisting of both the saccharide and the peptide structure.[5]

The MUC1 extracellular domain consists of numerous tandem repeats of the sequence HGVTSAPDTRPAPG-STAPPA which embodies five potential glycosylation sites (Scheme 1 a). The development of synthetic vaccines based on the exposed MUC1-tandem-repeat peptide backbone, the tumor associated glycans, such as T_N, T, sialyl-T_N, sialyl-T, and recent tandem-repeat glycopeptides has been the subject of intensive research efforts.[6–9] Such vaccines need to induce a specific immune response, which leads to the formation of antibodies that differentiate between normal and cancer epithelial cells. This requirement and the substantial effort required to synthesize and conjugate the carbohydrate and glycopeptide antigens call for the development of miniaturized analytical technologies which allow rapid and reliable mapping of antibody specificity. The immobilization of biomolecules such as peptides,[10–13] oligonucleotides,[14,15] carbohydrates,[16–18] and proteins[10,19–21] in microarray formats and their use in bioassays has proven to be a very valuable, time- and material-saving methodology; it is also a viable alternative to the established solution-phase bioassays, such as the ELISA-based formats. Herein we report on the development of a microarray platform for mapping the specificity of antibodies raised against tumor-associated mucin MUC1 glycopeptides. (for previous work on glycopeptide microarrays see reference [22]).

Recently, we synthesized vaccine constructs consisting of sialyl-T_N- and T_N-20-mer tandem-repeat MUC1 glycopeptides connected to a T_H-cell peptide epitope from ovalbumin ($OVA_{323-339}$). Immunization of transgenic mice whose T cells express a receptor recognizing the $OVA_{323-339}$ T-cell epitope, induced a humoral immune response,[6] thereby leading to the formation of antibodies directed against the MUC1 glycopeptide B-cell epitope. To evaluate the specificity of these antibodies we have now synthesized a number of MUC1 glycopeptides having variations in the glycan structure, the glycan position on the tandem-repeat peptide, and the number of tandem repeats or shorter peptides excluding parts of the repeat (Scheme 1 b and Scheme 2); for a description of the synthesis see the Supporting Information). These glycopeptides were equipped with an N-terminal triethyleneglycol spacer, immobilized on *N*-hydroxysuccinimide ester (NHS) coated microarray slides, and their recognition by the above antibodies was investigated. For additional validation, the specificity of serum antibodies generated through immunization with a MUC1 glycopeptide vaccine conjugated to the tetanus toxoid protein (TTox)[23] and a monoclonal antibody known to recognize cancer tissue (SM3, immunogen: deglycosylated mucin) were also evaluated.[24,25]

For establishing and initially validating the microarray the "stripped mucin" monoclonal antibody SM3, which recognizes cancer tissue and was produced through immunization with deglycosylated mucin from human milk, was studied (Figure 1).[24,25] The microarray analysis employed a readout of antibody binding to the glycopeptides by means of a secondary fluorescent-labeled antibody. It revealed that the SM3 antibody recognized the non-glycosylated mucin peptide **1**, and had an even stronger recognition of glycopeptide **4**. This MUC peptide differentiates itself from peptides **2** and **3** by means of an additional T_N glycosylation in the immunodo-

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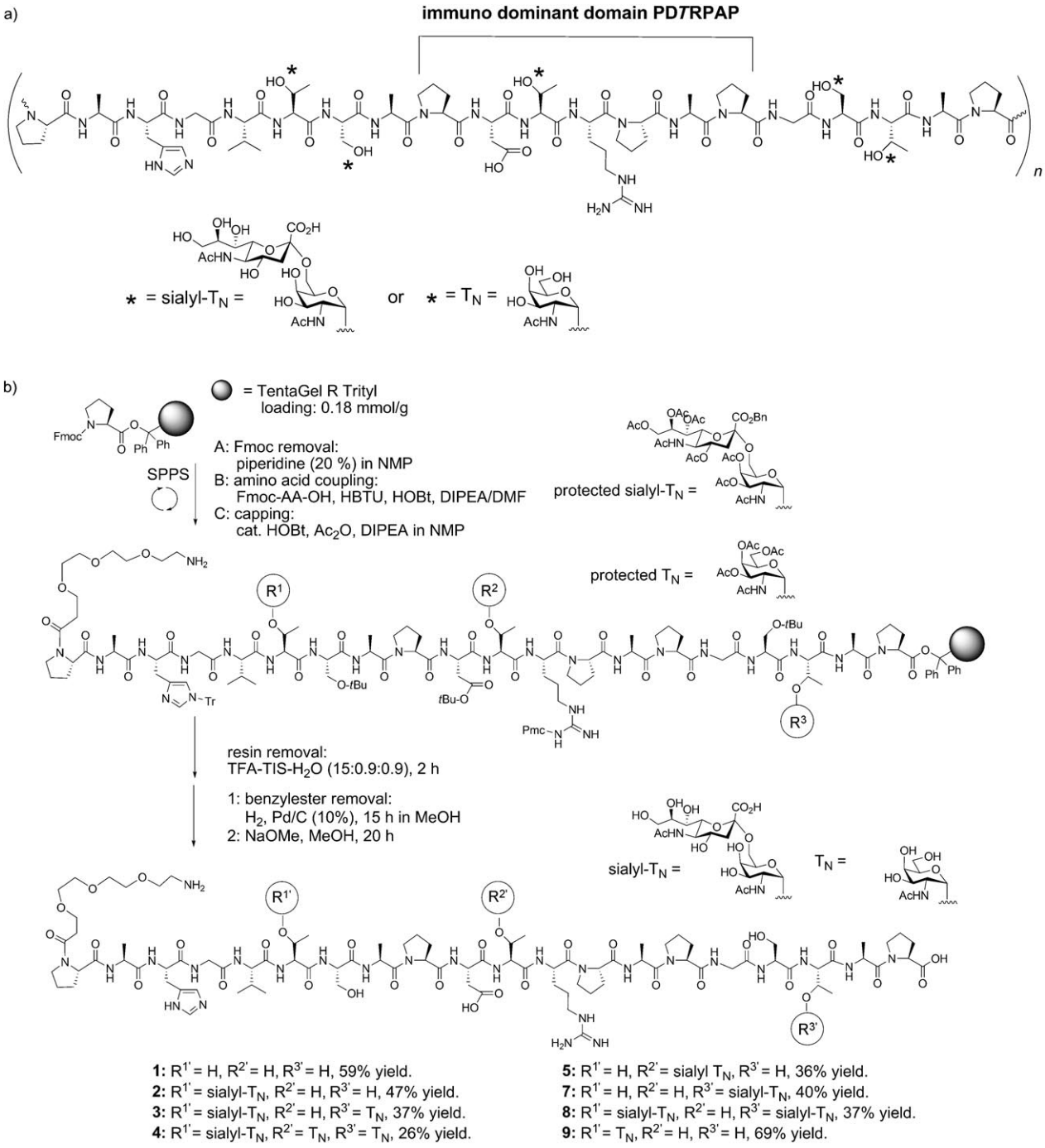
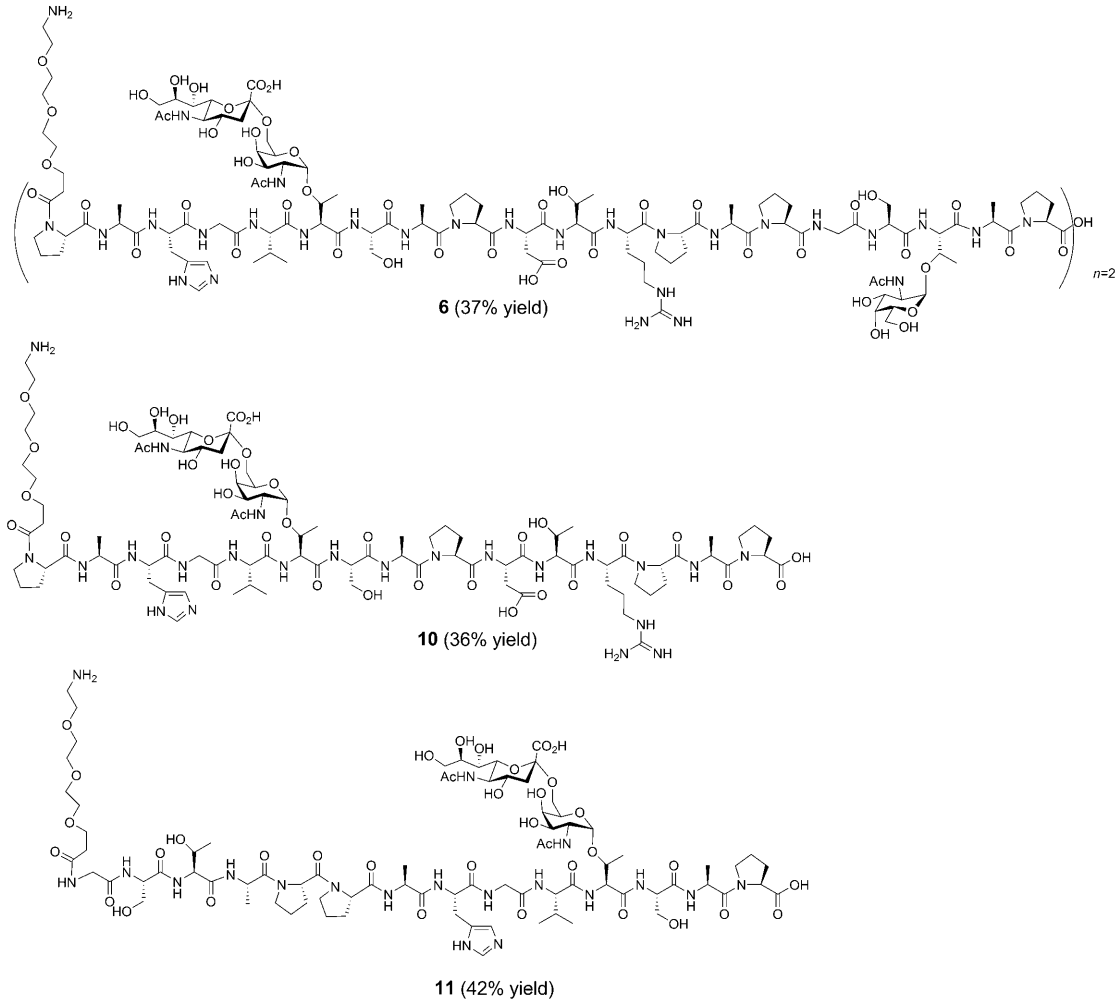


1: $R^{1'}$ = H, $R^{2'}$ = H, $R^{3'}$ = H, 59% yield.
2: $R^{1'}$ = sialyl-T_N, $R^{2'}$ = H, $R^{3'}$ = H, 47% yield.
3: $R^{1'}$ = sialyl-T_N, $R^{2'}$ = H, $R^{3'}$ = T_N, 37% yield.
4: $R^{1'}$ = sialyl-T_N, $R^{2'}$ = T_N, $R^{3'}$ = T_N, 26% yield.
5: $R^{1'}$ = H, $R^{2'}$ = sialyl T_N, $R^{3'}$ = H, 36% yield.
7: $R^{1'}$ = H, $R^{2'}$ = H, $R^{3'}$ = sialyl-T_N, 40% yield.
8: $R^{1'}$ = sialyl-T_N, $R^{2'}$ = H, $R^{3'}$ = sialyl-T_N, 37% yield.
9: $R^{1'}$ = T_N, $R^{2'}$ = H, $R^{3'}$ = H, 69% yield.

Scheme 1. Structure of the MUC1 tandem repeat and synthesis of mucin peptides **1**–**11**. a) Structure of the 20-mer tandem-repeat tumor-associated MUC1 glycopeptide which defines the immunodomain of MUC1. Potential glycosylation sites are marked with an asterisk (*). The glycosylation pattern may include the T_N or the sialyl-T_N saccharide. b) Synthesis of glycopeptides **1**–**9**; for a description of the synthesis see the Supporting Information. R^1, R^2, R^3 = H, protected sialyl-T_N, or protected T_N. Fmoc = 9-fluorenylmethoxycarbonyl, NMP = *N*-methylpyrrolidone, HBTU = *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, HOBt = 1-hydroxybenzotriazole, DIPEA = diisopropylethylamine, DMF = *N*,*N*-dimethylformamide, TFA = trifluoroacetic acid, TIS = triisopropylsilane, AA = amino acid.

minant PD*T*RPAP domain. Peptide **5**, which is monoglycosylated with a sialyl-T_N structure in the immunodominant domain, and peptide **7** having a sialyl-T_N glycosylation in the GS*T*A region, are also very strongly recognized by the SM3 antibody. The other glycopeptides were only weakly recognized and the shorter peptide **11**, lacking the DTRPAP region was not bound at all. On the basis of these results, and as expected, the antibody does not only recognize the peptide backbone, but also shows specificity for glycosylation in individual positions. This result is in agreement with previous findings since the immuno dominant PD*T*RPAP region of the tandem repeat is known to be the binding epitope of this antibody.[26] It is not surprising that this antibody also accepts short glycans in some positions, since the immunogen used in



Scheme 2. Structure of peptide **6** with two tandem repeats; peptide **10** without the GSTA domain; and peptide **11** without the immunodominant DTRPAP region. Peptides **6, 10,** and **11** were synthesized according to the procedure described in Scheme 1 b for the tandem-repeat peptides **1**–**9**. Given yields are overall yields determined after purification by HPLC methods and calculated from individual resin loadings.

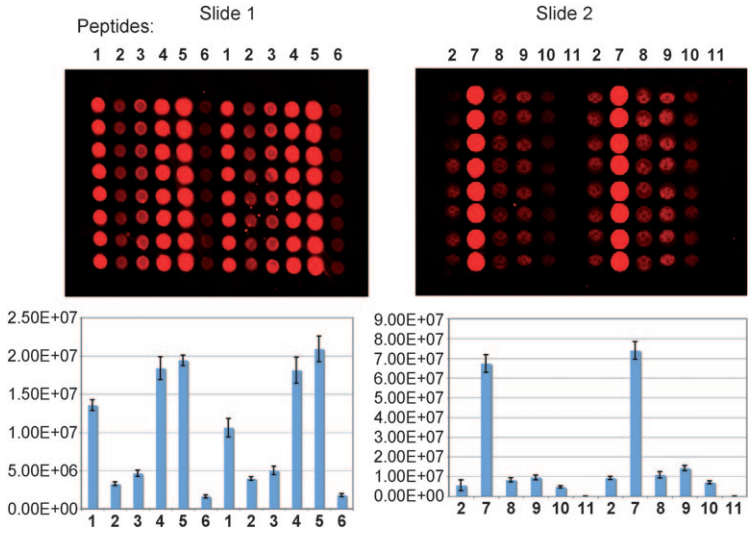


Figure 1. Analysis of the specificity of antibody SM3 for recognition of MUC1 glycopeptides. Peptides **1**–**6** were printed on slide 1 and peptides **2, 7**–**11** were printed on slide 2 (repeated twice, 8 spots), and the slides were incubated with SM3 monoclonal antibody (1/100 dilution, deglycosylated mucin as immunogen). Cy5-labeled anti-mouse antibody (1/500) was used as secondary antibody. Each column in the diagram shows the average detected fluorescence area of the 8 spots, including standard deviation.

its production consisted of both partially deglycosylated (1 h HF treatment) and completely deglycosylated mucin (3 h HF treatment). Previous studies have also shown that glycosylation with a GalNAc residue in the immunodominant domain increases antibody binding.[27,28] In addition, our results show that glycosylation with sialyl-T_N in both the PD*T*RP and the GSTA regions increases binding to the antibody whereas sialyl-T_N or T_N glycosylation in the HGV*T* domain decrease binding. In a previous study with the SM3 antibody the binding epitope and conformation of a GalNAc-glycosylated PD*T*RP 5-mer peptide and a non-glycosylated peptide were compared using saturation transfer difference(STD) NMR and trNOESY methodologies for readout.[29] It was observed that glycosylation with GalNAc stabilized the otherwise flexible peptide backbone conformation. Direct antibody binding to the glycan was also confirmed by a strong STD signal to the *N*-acetyl group.

Encouraged by these findings we analyzed serum from mice immunized with the monoglyco-sialyl-T_N-MUC1 $OVA_{323\text{-}339}$ and the diglyco-sialyl-T_N-T_N-MUC1 $OVA_{323\text{-}339}$ vaccines.[6] As shown in Figure 2a the results revealed that immunization with the synthetic MUC1 antigens induced the formation of highly specific antibodies. The antibodies were generated after immunization with the mono- and diglyco vaccines, respectively, and displayed a similar pattern of ligand recognition. A high specificity for the sialyl-T_N glycan connected to the threonine in the HGV*T* domain of the tandem-repeat was found. If the sialyl-T_N glycan was positioned on the threonine in the immunodominant domain PD*T*RPAP (**5**) or in the GS*T*A domain (**7**) instead of being located in the HGV*T* domain (**2**), the recognition by the antibody was lost. When the sialyl-T_N glycopeptide was completely removed from the peptide backbone (**1**) the recognition was lost as well. The tandem-repeat peptide **9**, which embodies T_N instead of sialyl-T_N (**2**), only very weakly bound to the serum antibodies. The shorter 15-mer sialyl-T_N peptide **10** without the GSTA region of the tandem repeat was recognized, similar to that of the whole tandem-repeat peptide **2**. This GSTA region is not essential for binding. However, when the immunodominant domain was removed as in the case of peptide **11** no binding was detected. Therefore the antibodies are highly specific not only for sialyl-T_N glycosylation in a certain position (HGV*T*), but also to the peptide backbone in particular for the immunodominant domain. Even if the serum antibodies from the OVA vaccines are highly specific they still accept additional glycosylation in other positions. Peptide **3** having additional T_N glycosylation in the GS*T*A region, peptide **4** having additional T_N glycosylation in the immunodominant domains PD*T*RPAP and GS*T*A, and peptide **8** having an extra sialyl-T_N glycan in the GS*T*A part are all well recognized by the serum antibodies. Longer peptides such as the glycopeptide **6** consisting of two tandem repeats are also accepted by the antibodies, although with slightly lower binding affinity compared with the one tandem-repeat peptide. The significance of this finding will require further studies with longer and more protein-like tandem-repeat glycopeptides. Recognition of peptides/proteins with more than one tandem repeat and some flexibility with respect to additional glycosylation on the tandem repeats are preferable since the MUC1 protein on the tumor epithelial

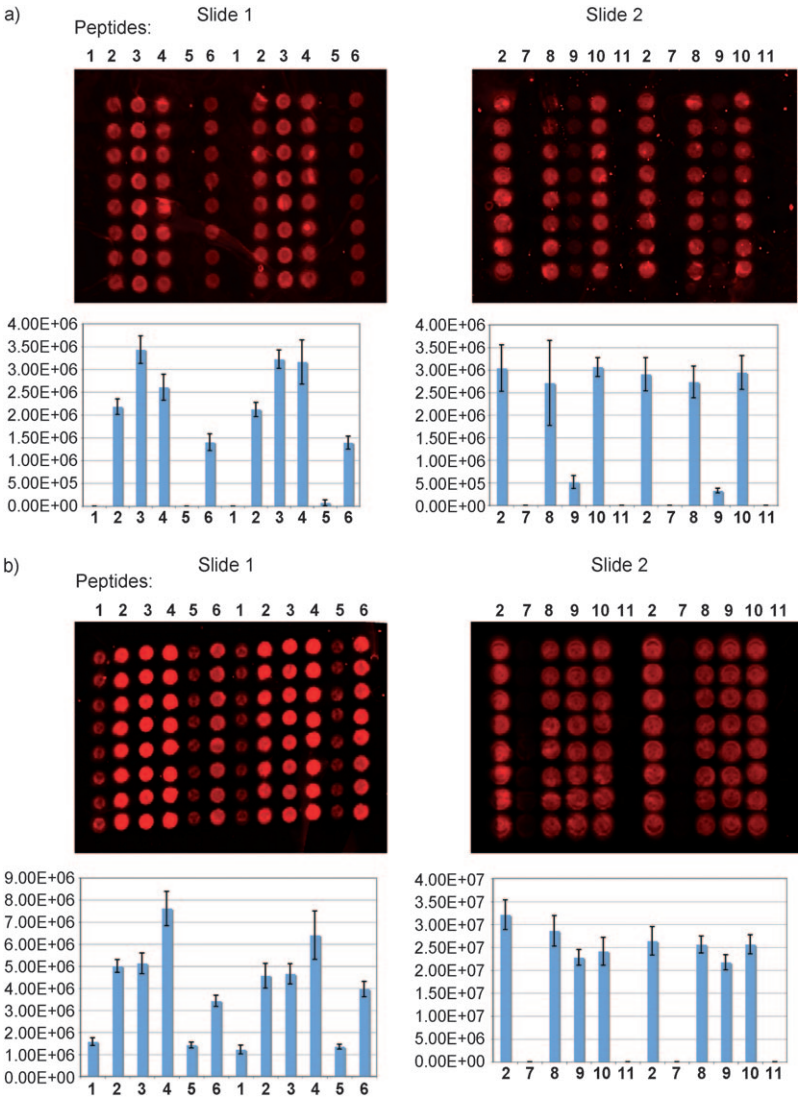


Figure 2. Analysis of the specificity of mouse serum immunized with monoglyco-sialyl-T_N-MUC1 $OVA_{323-339}$ or immunized with monoglyco-sialyl-T_N-MUC1 tetanus toxoid protein (TTox) for recognition of MUC1 glycopeptides. Peptides **1**–**6** were printed on slide 1 and peptides **2, 7**–**11** were printed on slide 2 (repeated twice, 8 spots), and the slides were incubated with a) mouse 4 serum[6] (1/100 dilution, after 6th immunization) immunized with monoglyco-sialyl-T_N-MUC1 $OVA_{323\text{-}339}$, b) mouse 8 serum[23] (1/1000 dilution, after second immunization) immunized with monoglyco-sialyl-T_N-MUC1 TTox. Cy5-labeled anti-mouse antibody (1/500) was used as secondary antibody. Each column in the diagrams shows the average detected fluorescence area of the 8 spots including standard deviation.

cells consists of numerous tandem repeats and other threonine and serine residues within the repeat can be potential glycosylation sites.

To additionally strengthen the validity of the described method we analyzed antibodies obtained from the immunization of mice with the same sialyl-T_N monoglyco MUC1 B-cell epitope but conjugated to the tetanus toxoid protein (TTox)[23] instead of the T-cell peptide epitope $OVA_{323-339}$. Microarray analysis of the generated serum antibodies revealed a similar recognition pattern, but with lower specificity when compared with antibodies generated with the OVA vaccines[6] (Figure 2b). A lower specificity of the TTox serum antibodies was expected because of the much stronger immune response with an up-regulation of the whole immune system compared to the OVA vaccine,[23] although individual variation in mice can not be completely excluded. Thus, weak binding to the non-glycosylated MUC1 peptide **1** and to peptide **5** glycosylated with sialyl-T_N in the immunodominant domain was detected. In contrast, the T_N-peptide **9**, which was only weakly detected in the MUC1 OVA serum, was strongly recognized. Thus, in this case the sialyl part is less important. However if the sialyl-T_N is moved from the HGV*T* position (**2**) to the GS*T*A domain (**7**) no binding could be detected. Also in this case the immunodominant domain appears to be important for recognition and no binding to peptide **11** without the DTRPAP region could be found. Substantial binding to peptides **2, 3, 4, 6, 8**, and **10** was apparent, a finding similar to the results recorded for the OVA serum as described above.

In conclusion, we developed a novel glycopeptide microarray-based approach to study the specificity in recognition by antibodies raised against different MUC1 epitopes. The technique allowed identification of new binding epitopes and clearly revealed differences in the selectivity patterns for antibodies raised against different, yet closely related, glycopeptide antigens. The glycopeptide microarray platform promises to be a valuable tool to efficiently study MUC1 glycosylation and evaluate antibodies for immunotherapy and immunodiagnostics employing minimum amounts of precious glycopeptides and serum/monoclonal antibodies. By analogy the method should be applicable to other glycopeptide antigens as well.

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