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A straightforward protocol for the preparation of high performance microarray displaying synthetic MUC1 glycopeptides

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ABSTRACT

Background: Human serum MUC1 peptide fragments bearing aberrant *O*-glycans are secreted from columnar epithelial cell surfaces and known as clinically important serum biomarkers for the epithelial carcinoma when a specific monoclonal antibody can probe disease-relevant epitopes. Despite the growing importance of MUC1 glycopeptides as biomarkers, the precise epitopes of most anti-MUC1 monoclonal antibodies remains unclear.
Methods: A novel protocol for the fabrication of versatile microarray displaying peptide/glycopeptide library was investigated for the construction of highly sensitive and accurate epitope mapping assay of various anti-MUC1 antibodies.
Results: Selective imine-coupling between aminooxy-functionalized methacrylic copolymer with phosphorylcholine unit and synthetic MUC1 glycopeptides-capped by a ketone linker at *N*-terminus provided a facile and seamless protocol for the preparation of glycopeptides microarray platform. It was demonstrated that anti-KL-6 monoclonal antibody shows an extremely specific and strong binding affinity toward MUC1 fragments carrying sialyl T antigen (Neu5Acα2,3Galβ1,3GalNAcα1→) at Pro-Asp-Thr-Arg motif when compared with other seven anti-MUC1 monoclonal antibodies such as VU-3D1, VU-12E1, VU-11E2, Ma552, VU-3C6, SM3, and DF3. The present microarray also uncovered the occurrence of IgG autoantibodies in healthy human sera that bind specifically with sialyl T antigen attached at five potential *O*-glycosylation sites of MUC1 tandem repeats.
Conclusion: We established a straightforward strategy toward the standardized microarray platform allowing highly sensitive and accurate epitope mapping analysis by reducing the background noise due to nonspecific protein adsorption.
General significance: The present approach would greatly accelerate the discovery research of new class autoantibodies as well as the development of therapeutic mAbs reacting specifically with disease-relevant epitopes.

1. Introduction

MUC1 glycoprotein [1] is highly overexpressed in over 90% of breast cancer [2,3] and many other cancers such as prostate cancer, hepatocellular carcinoma, pancreatic cancer, colon cancer, or ovarian carcinoma [4,5]. In addition, the expression level of the abnormal and immunogenic truncated glycoforms such as GalNAcα1 → (Tn antigen), Galα1,3GalNAcα1 → (T or TF antigen, core 1 type disaccharide), Neu5Acα2,6GalNAcα1 → (STn antigen) are considered to increase according to up- or down-regulation of some glycosyltransferases. Owing mainly to the differences in such glycan structures at five potential glycosylation sites, antigenic structures (epitopes) of MUC1 peptide fragments may alter during tumor progression (cancer cell differentiation) [6].

It is clear that antibodies against such cancer-relevant MUC1 structures are promising candidates for the development of novel antitumor drugs. One of the representative examples of the important anti-MUC1 antibodies are monoclonal antibodies recognizing a peptide epitope Pro-Asp-Thr-Arg (PDTR) motif on tandem repeat region [7–13]. The humanized mAb with fully human glycoforms has recently completed clinical phase I study and moved to phase II clinical development in ovarian cancer [11–13]. It is noteworthy that this mAb more strongly recognizes a glycosylated peptide epitope PDTR having Tn or T antigen than naked peptide fragments while its binding profile against this peptide epitope with other glycoforms remains unclear. In 1998, a panel of 56 anti-MUC1 mAbs was investigated at the workshop of MUC1 [14]. The results of this assessment and the following studies [15] also indicated that all antibodies had different binding specificities against

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MUC1 peptide epitopes, although many of them showed high affinity with peptides involving DTR moiety. In addition, it was reported that *O*-glycosylation at the threonine residue in PDTR motif led to enhance binding affinity with most antibodies [16–18]. However, it seems also that glycan specificity of most anti-MUC1 mAbs recognizing this peptide region is not fully elucidated.

Extensive efforts have also been paid toward the development of the MUC1-targeted cancer vaccines based on carrier-conjugated MUC1 fragments [19–29]. However, it is important to note that clinical outcome of this approach might be strongly dependent on the binding specificity of the antibody to combined glycopeptide epitopes raised by immunization of MUC1 vaccines because cancer-associated antigens are generated essentially by altered posttranslational glycosylation at the individual *O*-glycosylation sites involved in the antigenic peptide sequences. It is well documented that various cancer cells often express MUC1 bearing immature aberrant *O*-glycans such as Tn, T, and their sialylated structures [30]. Therefore, immunization with MUC1-based vaccines carrying such glycoforms appears to be a promising approach to raise specific immune response against the pre-mature cancer cells, while the epitopes of the antibodies raised by vaccinations, which include both the peptide backbone as well as cancer-associated posttranslational glycosylation, have been little discussed. In addition, it is of emerging importance to profile the individual's serum levels of autoantibodies to cancer-related MUC1 fragments [31–33] because the autoantibody signature may be compromised of an accumulation of antibodies elaborated in the presence of pre-malignant cells at very early stages of tumor development [34,35].

On the other hand, shedding of the MUC1 fragments from cancer cells into the circulation in the bloodstream is also observed in many cancer patients [36,37]. MUC1 antigens known as CA15-3, CA27.29, CD227, EMA, PEM, or PUM with different properties between normal and cancer cells have therefore been considered as promising biomarkers [38]. CA15-3 is one of the potent diagnostic MUC1-based serum markers known for an early recurrence of breast cancer [39] and for evaluating the efficacy of treatment for metastatic breast cancer [40]. However, it is well known that CA15-3 is detected from patients' serum not only in breast cancer but in many other adenocarcinoma such as lung, pancreas, kidney, ovary, uterus, colon, and even in cases for endometriosis, pelvic inflammatory diseases and hepatitis [41–43]. At present, circulating CA15-3 is detected by the sandwich assay using two key mAbs, namely DF3 and 115D8. DF3 was generated against a human breast carcinoma metastatic to liver [44], while 115D8 was raised against human milk-fat globule membrane that should exhibit a broad binding affinity to most MUC1 fragments [14,45]. Though the epitopes of DF3 appeared to locate at a TRPAPGS region in the tandem repeat [46], its binding specificity and affinity strength toward the peptides having different glycoforms at the threonine residue is unknown. This may indicate that glycans attached at this epitope influence greatly to the specificity in the interaction between DF3 and circulating CA15-3 in sera of patients suffering from abovementioned various diseases, leading to inaccurate conclusions. Krebs von den Lungen-6 (KL-6) is also one of the clinically important MUC1-related antigens currently used as a practical serum biomarker for diagnosing various lung diseases associated with pneumonitis [47,48]. Anti-KL-6 mAb is a promising probe for detecting KL-6 in bloodstream and the minimal epitope structure recognized by this mAb has recently been discovered by our group to be heptapeptide PDTRPAP having sialyl T antigen (Neu5Acα2,3Galβ1,3GalNAcα1→) at threonine residue [49]. Given that anti-KL-6 mAb reacts with serum MUC1 in patients suffering from lung adenocarcinoma, breast cancer, colorectal adenocarcinoma, and hepatocellular carcinoma in addition to interstitial pneumonia [50–54], it is obvious that elucidation of accurate glycopeptide epitope for such mAbs is of emerging importance to reveal the false positive cross-reactions due to "tolerance in the glycan recognition" [49].

Microarray displaying antigenic glycopeptides is an attractive and powerful tool for high throughput screening of antibodies and its epitope mapping analysis including glycan-binding profiles. Recently, some pioneering studies [55,56] demonstrated the occurrence and clinical potentials of serum autoantibodies against glycans [57–60], while their exact specificities and functions seem still very elusive. On the other hand, serum antibodies to combined glycan-peptide epitopes are easier to define and more promising probes to react with disease-relevant mucin glycopeptides [31–33]. However, it seems likely that only a few are in current use for large-scale glycan/glycopeptide microarrays intended for diverse applications [55,59,61]. We considered that a much more efficient and versatile method for the preparation of MUC1-glycopeptides microarray would accelerate greatly a creation of new class diagnostic and therapeutic anti-MUC1 mAbs with a promising clinical outcome. In the present study, we report a facile and standardized protocol for the fabrication of high performance microarrays displaying robust peptides/glycopeptides library on the basis of "glycoblotting method", a general protocol for the site-specific immobilization of compounds with an aldehyde or an equivalent ketone functional group under a mild aqueous condition without any coupling reagent [62]. Advantage of our array format is demonstrated by highly sensitive and quantitative profiling of anti-MUC1 mAbs and IgG autoantibody signature to MUC1 fragments in healthy human serum.

2. Experimental section

2.1. Materials

All commercially available solvents and reagents were used without purification. TentaGel S RAM resin was purchased from Rapp Polymere GmbH (Tübingen, Germany), and N^{α}-Fmoc-amino acid derivatives except for glycosylated compounds and recombinant rat α2,3-(*O*)-sialyltransferase (α2,3-SiaT) were purchased from Merck Millipore (Darmstadt, Germany). N^{α}-Fmoc-glycosylated amino acids such as Fmoc-(Ac_3GalNAcα)serine/threonine and Fmoc-(Ac_4Galβ1, $3Ac_2$GalNAcα)serine/threonine were synthesized according to the method reported previously [63,64]. 1-[Bis(dimethylamino)methyliumyl]-1H-benzotriazole-3-oxide hexafluorophosphate (HBTU), 1-hydroxybenzotriazole monohydrate (HOBt) and *N*,*N*-diisopropylethylamine (DIEA) were purchased from Kokusan Chemical Co., Ltd (Tokyo, Japan). *N*,*N*-Dimethylformamide (DMF) and 20% piperidine in DMF was purchased from Watanabe Chemical Industries, Ltd. (Hiroshima, Japan). Acetic anhydride and 2,2,2-trifluoroacetic acid (TFA) were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 5-Oxohexanoic acid was from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Cytidine-5′-monophospho-β-D-*N*-acetylneuraminic acid, disodium salt (CMP-Neu5Ac) was from Yamasa Corporation (Chiba, Japan). Preparative HPLC separations were performed using a Prominence Shimadzu High Performance Liquid Chromatographs (HPLC) system equipped with two LC-6AD pumps, an SPD-20A UV/VIS detector and Inertsil ODS-3 reversed-phase C18 column (250 × 20 mmI.D.). Purified compounds were analyzed using a Waters Acquity Ultra Performance LC system (see also, Supporting Information). High-resolution electrospray ionization mass spectra (ESI-HRMS) using JEOL JMS-700TZ and amino acid analyses using JEOL JLC/500 equipped with ninhydrin detection system were performed in Center of Instrumental Analysis at Hokkaido University. Monoclonal antibodies, VU-3D1, Ma552, VU-12E1, and VU-11E2, were obtained from Sanbio B. V. (Uden, the Netherlands), SM3 mAb was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), VU-3C6 mAb was from Exalpha Biologicals, Inc. (Shirley, MA, USA), and DF3 mAb was from Covance Research Products, Inc. (Emeryville, CA, USA). Anti-KL-6 mAb labeled with alkaline phosphatase from Lumipulse® KL-6 Eisai (a reagent kit to determine KL-6 antigen) was purchased from Sanko Junyaku Co., Ltd. (Tokyo, Japan). FluoroLink™ Cy™3-labeled goat anti-mouse IgG (H + L) was from Amersham Biosciences (Buckinghamshire, UK). Biotin–goat anti-human IgG (H + L) conjugates (ZyMax™ Grade) were from Invitrogen Corporation (Carlsbad, CA, USA). Amersham Cy™3-streptavidin and Amersham Cy™3 mono-reactive dye pack

were from GE Healthcare Bio-Sciences KK (Tokyo, Japan). Microarray slides were basically manufactured by Sumitomo Bakelite Co., Ltd. (Tokyo, Japan) as follows: Glass-like raw materials made of cyclic polyolefin were molded into slides. The surface of naked slides were coated by methacrylic polymer, (2-methacryloyloxyethylphosphorylcholine-cyclohexylmethacrylate-*N*-[2-[2-[2-(t-butoxycarbonylaminooxyacetyl-amino)ethoxy]ethoxy]ethyl]-methacrylamido copolymer (*N*-protected AO/PC-copolymer) [65,66]. The size of a slide is 75-mm long, 25-mm wide and 1-mm thick. Hybridization covers (60 × 25 × 0.7 mm) were also obtained from Sumitomo Bakelite Co., Ltd. SIMplex 16 multi-array system was from GenTel BioSciences, Inc. (Madison, WI, USA). "*Reaction buffer*" that specified in the present study is a solution of 50 mM Tris–HCl, 100 mM NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 0.05% Tween 20, pH 7.4. "*Washing buffer*" that specified in this paper is a solution of 50 mM Tris–HCl, 100 mM NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 1 mM $MgCl_2$, pH 7.4. Hybri Container was purchased from Greiner-Bio-One Co., Ltd. (GmbH, Germany) for washing slides. Slides adapter CombiSlide® was from Eppendorf Co., Ltd. (Hamburg, Germany) as a centrifugal support of slides for drying. AFM images were measured in the range of 500 × 500 nm with NanoScope (Digital Instruments, Santa Barbara, CA, USA). Fluorescence images of microarray slides after assays were measured at 10 μm resolution on a Typhoon Trio plus variable mode imager (GE Healthcare) with a green (532 nm) laser and a 580 BP 30 filter at a PMT voltage of 600 V and normal sensitivity. The digital images of fluorescence responses were analyzed using ArrayVision™ software version 8.0 (GE Healthcare) and were graphed using GraphPad Prism 5 software (San Diego, CA, USA).

2.2. General methods for the solid-phase synthesis of peptides and glycopeptides

Peptides (**1**–**41** and **51**) and glycopeptides (**42**–**47** and **52**–**57**) were synthesized by using an Apex 396 automated multiple peptide synthesizer (AAPPTec, Louisville, KY, USA) in a general manner of solid-phase peptide synthesis. TentaGel S RAM resin (100 mg each, loading 0.24 mmol g^{-1}) was swollen with DMF for 1 h at ambient temperature prior to use. Protective groups for masking side chain of Fmoc-amino acids are selected as indicated below: *tert*-butyl for Ser, Thr and Asp; trityl for His; and 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl (Pbf) for Arg residue, respectively. As for the coupling reactions, Fmoc-amino acids or Fmoc-glycosylated amino acids (5 equiv) were incorporated by use of HBTU (5 equiv), HOBt (5 equiv) and DIEA (10 equiv) in DMF for 2 h at ambient temperature. For the capping at *N*-terminus, a cocktail of acetic anhydride (4.75%, v/v), DIEA (2.25%, v/v), and HOBt (1.99% w/v) in DMF was treated for 10 min at ambient temperature. Fmoc group was removed by treating with 20% piperidine in DMF for 2 × 10 min at ambient temperature. The resin was washed at every step with DMF for 2 min five times at ambient temperature. 5-Oxohexanoic acid (5 equiv) was introduced to *N*-terminus of target (glyco)peptidyl-resins according to the same coupling procedure at a final step. Cleavage of the product from resin and removal of acid-labile protective groups were carried out concurrently by treating the resin with 90% aqueous TFA for 2 h at ambient temperature. The solution was filtered and concentrated by a flow of nitrogen gas, and the crude peptides or glycopeptides having *O*-acetylated glycan were precipitated using cold diethylether. Glycopeptides **42**–**47** and **52**–**57** were obtained by treating with alkaline condition (pH 12.4) in MeOH for 1.5 h according to the previously reportedly [45,67–69]. Finally, crude peptides and glycopeptides were purified by preparative HPLC. High-resolution ESI-MS, UPLC chromatograms and amino acid analyses of all compounds were described in Supporting Information.

2.3. Enzymatic sialylation of glycopeptides having T antigen

To the stock solutions of 8 mM glycopeptides (**45**–**47** and **55**–**57**) in water were added 1 M HEPES-NaOH buffer (pH7.0), 1 M $MnCl_2$, 100 mM CMP-Neu5Ac, 1.4 U ml^{-1} α2,3-SiaT and Milli-Q water to adjust the final concentration as follows: [49] 1 mM glycopeptide, 50 mM HEPES-NaOH buffer, 10 mM $MnCl_2$, 5 mM CMP-Neu5Ac and 20 mU ml^{-1} α2,3-SiaT. After gently shaking for 24 h at ambient temperature, the reaction mixture was subjected to the purification by preparative RP-HPLC to afford compounds **48**–**50** and **58**–**60**. High-resolution ESI-MS, UPLC chromatograms and amino acid analytical data of all compounds were summarized in Supporting Information.

2.4. Preparation of glycopeptide microarray

Slides coated by *N*-protected AO/PC-copolymer were immersed in 2 M HCl for 4 h at 37 °C, and then washed twice with water and dried by centrifugation (2000 rpm, 2 min, ambient temperature). Synthetic peptides and glycopeptides were spotted on the slides prepared above as follows: Quadruplicates of 5 mM **1**–**20** in 25 mM sodium acetate (pH 5.0) with 0.00025% (w/v) Triton X-100 were spotted on BioChip Arrayer (Filgen Inc., Aichi, Japan) with a 0.8-mm pitch using a Filgen solid pin (400 μm pin diameter). Quadruplicates of 0.25 mM **21**–**40** or 0.25 mM **41**–**60** in 25 mM acetate buffer (pH 5.0) with 0.00025% (w/v) Triton X-100 were spotted on OmniGrid® Micro (Digilab Inc., Marlborough, MA, USA) with a 0.8 mm pitch using four ChipMaker™ CMP6 micro spotting pins (200 μm spot diameter, ArrayIt Corporation, Sunnyvale, CA, USA). The slides after spotting were placed in a dry oven for 1 h at 80 °C to complete oxime bond formation according to the general condition used in glycoblotting of various hemiacetals. Recently, it was demonstrated that ketone-capped glycopeptides can be immobilized quantitatively at room temperature as reported previously [67]. After washing once with Milli-Q water, the slides were immersed in an aqueous solution of 10 mg ml^{-1} succinic anhydride for 1 h at ambient temperature for capping free aminooxy groups remained on the slide surface. The slides after washing twice with Milli-Q water were dried by centrifugation (2000 rpm, 2 min, at ambient temperature) and then used for further binding assay of anti-MUC1 mAbs. The dynamic range and the detection limit were investigated preliminarily by means of anti-MUC1 mAb (VU3C6) as an example (Figures S1 and S2 in the Supporting Information). Briefly, it was indicated that the binding of VU3C6 with an array prepared by spotting the solution of a positive MUC1 glycopeptide (1.0 mg ml^{-1}) can be monitored quantitatively by incubation with a fluorophore-tagged secondary antibody against this primary antibody at a range from 100 − pg − ml^{-1} to 50 ng ml^{-1}. Calculated from the concentration of stock solutions and the performance of the spotting pins (~1.8 nl per spot), approximately 10^{-12}–10^{-11} mol of peptides/glycopeptides could be immobilized.

2.5. Epitope mapping of anti-MUC1 mAbs using peptide/glycopeptide microarray

Anti-MUC1 mAbs were diluted with reaction buffer to make 1 μg ml^{-1} mAb solutions. A hybridization covers were mounted on the slides, and the diluted mAb solutions (70 μl each) were carefully infused through narrow gaps between slides and covers. After filling the void with the solution, the slides allowed to remain for 2 h at ambient temperature. The hybridization covers were removed and the slides were washed with washing buffer for 2 × 2 min, and then dried by centrifugation (2000 rpm, 2 min, ambient temperature). Unused hybridization covers were mounted to the slides, and 1 μg ml^{-1} Cy™3-labeled goat anti-mouse IgG antibody in reaction buffer (70 μl each) was infused to fill the void. After standing for 1 h at ambient temperature, the uncovered slides were washed with washing buffer for 2 × 2 min, followed by water for 1 × 1 min. The slides dried by centrifugation (2000 rpm, 2 min, ambient temperature) were subjected to fluorescent image scanning on a Typhoon Trio plus and the fluorescent responses were measured and analyzed using an ArrayVision software as described in the above (see also, Figure S3 in the Supporting information).

2.6. Analysis of human serum autoantibodies binding with synthetic MUC1 fragments

This study was performed in accordance with the ethical standards the World Medical Association Declaration of Helsinki and approved by the institutional ethical committee of Faculty of Advanced Life Science, Hokkaido University. Informed consent was obtained from all serum donors. Human sera were collected from six healthy volunteers (male n = 3, female n = 3, mean age 57.3 ± 7.3 years) after written informed consent was acquired. Human sera (3 μl) were diluted with reaction buffer (297 μl) and the solutions were subjected to centrifugal filtration (12,000 ×*g*, 3 min, 4 °C) with Amicon Ultrafree®-MC centrifugal filter units (pore size 0.45 μm, Durapore PVDF membrane) that purchased from Merck Millipore (Billerica, MA, USA). Duplicates of 5 mM **41**–**60** in 25 mM sodium acetate (pH 5.0) with 0.00025% (w/v) Triton X-100 were spotted to the pretreated slides on BioChip Arrayer with a 0.8-mm pitch using a Filgen solid pin (400 μm pin diameter). Cy3 mono-reactive dye (1 μg ml^{-1}) that dissolved in the same spotting buffer was also spotted on the same slides as grid markers. Washing, capping and drying of the slides were performed as the same procedure described previously. After installing the slides to SIMplex™ 16 gaskets, 100 μl of human serum solution was applied to each well and the slides were allowed to remain for 2 h at ambient temperature. After removal of the solutions by aspiration, each well was washed with washing buffer (100 μl) for 2 min three times and 0.1 μg ml^{-1} biotin-labeled goat anti-human IgG conjugate in reaction buffer (100 μl) was next added. After standing for 1 h at ambient temperature, each well was washed as the same and Cy™3-labeled streptavidin in reaction buffer (1.0 μg ml^{-1}) was finally added. After standing for 1 h at ambient temperature, the slides were washed as the same manner, and the slides uninstalled gaskets were rinsed with Milli-Q water for 1 × 1 min. The slides dried by centrifugation (2000 rpm, 2 min, ambient temperature) were subjected to scan of fluorescent images on a Typhoon Trio plus and the fluorescent responses were analyzed using an ArrayVision software.

3. Result and discussion

3.1. A novel microarray platform to display robust synthetic peptides/glycopeptides

The primary aim of the present study was to establish a versatile and highly sensitive microarray platform for the discovery and characterization of antibodies reacting specifically with glycopeptides as disease relevant epitopes. We set the criteria required for new class peptide/glycopeptide microarray as follows: (i) *A microarray slide having non-fouling surface* is made of a base material with more advantages than a glass in disposability, safety, shape forming, and most importantly low background noise, (ii) *Covalent immobilization of multifunctional peptides/glycopeptides* can be achieved by a straightforward scheme from the synthesis to the site-specific immobilization in a chemoselective, spontaneous, and uniform fashion, and (iii) *A standardized protocol* can be applied widely for the fabrication of microarrays carrying robust compound libraries of not only peptides/glycopeptides but a variety of glycoconjugates including free *N*/*O*-glycans, glycosphingolipids, and glycosaminoglycans as potential candidates for disease specific antigens.

The surface of a glass slide scaffold used for printing peptides/glycopeptide microarray is usually functionalized with *N*-hydroxysuccinimide (NHS) esters in direct or polymer coating manner for immobilizing compounds with amino group *via* amide bond formation [55,56]. However, this conventional approach has disadvantage to react with peptides/glycopeptides on the surface in an undesired fashion due to the difficulty in the site-specific coupling reaction of NHS ester with compounds bearing multiple amino groups and other potentially reactive sites rather than the targeted *N*-terminus [70]. Unfavorable tethering peptides/glycopeptides leads to possible misinterpretation in the specific interaction of the antibodies with a target antigen more severely rather than the cases for the immobilization of macromolecular proteins. In addition, glass-based surfaces involve the risk of cracks and breaks during transportation, use, and disposal cause of accidental injuries and subsequent infections, which are not favorable features for the clinical applications. Although NHS ester-activated slides have also been used extensively for the preparation of glycans [71] and glycosphingolipids microarray [72], prerequisite modification of every carbohydrates to attach an amino-handle is needed to print on this surface. On the solid-phased assay platforms, non-specific adsorption of various abundant biomolecules in human sera is one of the most serious and problematic phenomena that leads to enhance background signals. Non-specific binding may usually be minimized by saturating the unoccupied sites other than immobilized compounds with blocking reagents such as bovine serum albumin, skin milk, gelatin, and so on. These reagents, however, sometimes mask even compounds displayed and cause a significant decrease of the signals of interest. Consequently, it is likely that the establishment of assay protocols becomes more cumbersome and complicated due to optimization of blocking condition as well as procedures for the immobilization of individual compounds of interest. It was also reported that glass slides coated with functionalized hydrogel [73] have been shown to give very little background in serum binding studies [71].

As a potential and alternative material, we selected a cyclic polyolefin plastic with adequate properties of physical strength, optical transparency, and low autofluorescence as a base material of a microarray slide. This plastic slide satisfies our requirement for disposal use and safety manipulation, and it is easily moldable not only into slide format but into other expansive formats such as film, tube, and plate. Merit of the use of such sophisticated plastic material is evident because the microarray technique could expand to the fabrication of microtiter plates, microfluidic chips, and various types of diagnostic sensor devices. It was hypothesized that integration of chemoselective ligation, notably "glycoblotting method [62]" for capturing compounds involving a ketone/aldehyde functional group, into the surface of such plastic material would facilitate greatly to tether peptides/glycopeptides. We have previously reported the feasibility of aminooxy (AO)-functionalized polyacrylamide copolymer in a seamless chemical and enzymatic synthesis of robust compound libraries of mucin glycopeptides [67,74,75]. Our strategy allowed for rapid and high throughput synthesis of highly complicated glycopeptides by connecting two different polymer platforms, solid phase and water soluble supporting materials. It was demonstrated that peptides/glycopeptides can be captured quantitatively by AO-functionalized polymers through an oxime-bond formation when the *N*-terminus is capped by a reagent involving ketone/aldehyde auxiliary. Advantage of glycoblotting method is clear because this reaction proceeds smoothly under a mild aqueous condition without any side reactions. This method allowed for the conjugation with glycans/oligosaccharides, glycopeptides and a variety of compounds library in a site-specific manner, although the optimization is often needed for fixing the suited reaction time due to the reversible oxime bond formation [62,76–78].

On the other hand, it has also been documented that phosphorylcholine moiety of phosphatidylcholine, one of major components of biomembrane lipids, gives an excellent non-fouling surface when incorporated into the coating materials such as synthetic polymers and alkanethiol derivatives [79,80]. Recently, we demonstrated that combined use of an AO-terminated alkanethiol derivative, 11,11′-dithio bis[undec-11-yl 12-(aminooxyacetyl)amino hexa (ethyleneglycol)], and a phosphorylcholine (PC) derivative, 11-mercaptoundecylphosphorylcholine, provides quantum dots (QDs) with novel functions for the chemical ligation of ketone-functionalized compounds and the prevention of non-specific protein adsorption concurrently [81,82]. It should be emphasized that non-fouling characteristic of highly stable PC self-assembled monolayers-coated AO-QDs (AO/PC-QDs) makes *in vivo* direct monitoring of glycan-specific interaction

and distribution of PC-QDs displaying various glycans after injection into the vein of mouse tail possible [81]. Taking all these observations into account, we decided to synthesize a novel multifunctional methacrylic polymer (*N*-protected AO/PC-copolymer) as a coating material of cyclic polyolefin molded into array slides. A general procedure for the chemical and enzymatic synthesis (Fig. 1A) and site-specific immobilization of peptides/glycopeptides (Fig. 1B) were outlined. The *tert*-butoxycarbonyl (Boc) protecting groups of AO-functions of the surface copolymer were easily removed by treatment with aqueous hydrochloric acid prior to use. Next, AO-functional groups of microarray were subjected directly to the selective reactions with peptides/glycopeptides capped with 5-oxohexanoic acid without any side reactions even in the presence of undesired peptides/glycopeptides or contaminated impurities lacking a reactive ketone auxiliary. After blocking the redundant AO-functions by treating with succinic anhydride, microarray displaying peptides/glycopeptides can be used for further analysis without any other specific treatments. Although we employed 4 fixed concentrations (1.0, 2.5, and 5.0 mg mL^{-1}) of peptides/glycopeptides at 80 °C for complete imine-coupling reaction with aminooxy-groups of the microarray (Supporting Information, Figure S1), the optimization might be needed for improving the sensitivity or detection limit due to the difference of the affinity with monoclonal antibodies tested.

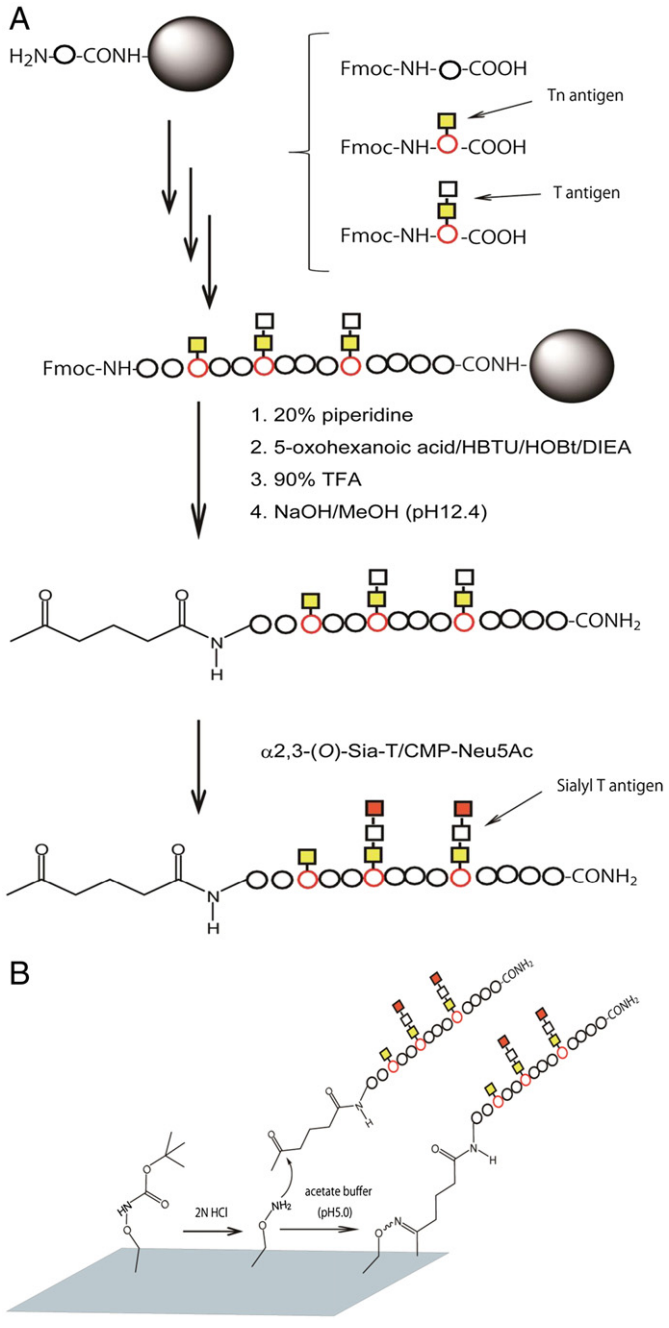

Fig. 1. A seamless scheme for the preparation of glycopeptide microarray platform. (A) Schematic image for the synthesis of *N*-(5-oxohexanoyl)-MUC1 peptides and glycopeptides based on chemical and enzymatic approach. (B) A general protocol for chemoselective immobilization of MUC1 peptides and glycopeptides on the basis of a concept of "Glycoblotting method". *N*-Protected AO/PC-copolymer coated on the base material is treated with 2 M HCl at 37 °C for 4 h and allowed subsequently to couple with *N*-(5-oxohexanoyl)-peptides/glycopeptides in 25 mM acetate buffer (pH 5.0) at 80 °C for 1 h.

Needless to say that this protocol is feasible for the direct immobilization of free glycans as demonstrated in the large-scale glycan analysis [77,83–85]. Atomic force microscopy (AFM) demonstrated that coating by methacrylic copolymer having PC moiety prevents perfectly the surface of a polypropylene-based slides from non-specific protein adsorptions (Fig. 2). No significant adsorption of hydrophobic TGF-β, basic histone H1, and glycoprotein hormone erythropoietin was detected on the surface coated by the PC-copolymer while considerable accumulation of all these proteins was observed on the naked plastic surface when exposed to the protein solutions. However, it is still not clear that the present plastic format is superior to other glass-based microarrays because no quantitative study using other available format has been carried out.

3.2. Microarray displaying synthetic MUC1 peptides/glycopeptides

To validate the potentials of the present microarray format in the precise epitope mapping analysis of anti-MUC1 mAbs, we designed a compound library composed of 60 MUC1 peptides/glycopeptides, which are generated by cutting MUC1 tandem repeat sequence into overlapping fragment with equal length (Table 1).

The library can be divided into three categories of 10-mer (**1**–**20**), 15-mer (**21**–**40**), and 23-mer (**41**–**60**) in size. MUC1 peptides (**1**–**40**) are non-glycosylated peptides with one amino acid residue shifted between two proximate fragments. On the other hand, 23-mer MUC1 fragments (**41**–**60**) were designed by focusing on the glycoforms at five potential *O*-glycosylation sites and its distribution in the two different tandem repeating units involving three extra amino acid residues that can be regarded as a part of neighboring repeating unit. To assess the effect on the antibody bindings caused by altering cleavage sites in the MUC1 tandem repeating sequence, we assigned two sequentially-identical peptide fragments with two different *N*-terminus starting from GVT (**41**–**50**; fragment I) and STA (**51**–**60**; fragment II) motifs. MUC1-peptide/glycopeptide library was constructed by a general procedure indicated in Fig. 1A on TentaGel resin with RinkAmide linker using Fmoc solid-phase peptide synthesis protocol on a standard peptide synthesizer. With respect to *O*-glycan modification, three tumor-associated *O*-glycans of Tn (GalNAcα1→), T (or TF; Galβ1, 3GalNAcα1→), and sialyl-T (Neu5Acα2,3Galβ1,3GalNAcα1→) were singly linked to one of the five serine/threonine resides that are potential sites to be *O*-glycosylated in a tandem repeat unit. All peptides/glycopeptides were terminated by capping with a 5-oxohexanoyl group at *N*-terminal end and a carboxamide group at *C*-terminal end of the sequence. All compounds (**1**–**60**) were employed for the site-specific immobilization by a standardized protocol indicated in Fig. 1B.

3.3. Accurate epitope mapping of anti-MUC1 mAbs

To test the performance of our newly established microarray format displaying peptides (**1**–**41**, and **51**) and glycopeptides (**42**–**50**, and **52**–**60**), the epitope mapping analysis of a panel of eight commercially available anti-MUC1 mAbs listed in Table 2 was carried out. Judging from clear fluorescence images illuminated by secondary antibody (Cy3-labeled goat anti-mouse IgG antibody) shown in Fig. 3 (see also,

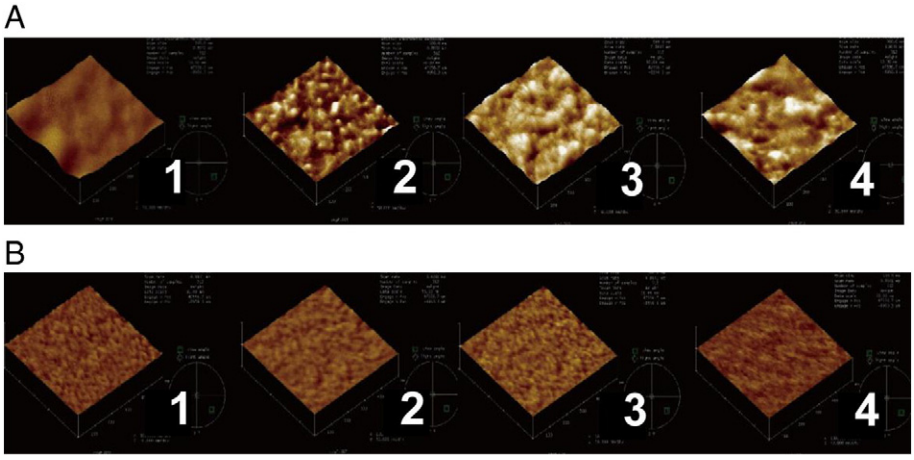

Fig. 2. AFM views of non-specific protein adsorption to a base plastic material (naked polypropylene slides) (A) and the surface after coating with PC-methacrylic copolymer (B). Silds were exposed to pure water (1; negative control), or 10 nM aqueous solutions of TGF-β (2; a hydrophobic protein), histone H1 (3; a basic protein), and erythropoietin (4; a glycoprotein hormone) as typical model proteins. After incubating with the solution for 1 h at 37 °C, the slides were washed three times with 0.05% (w/v) Tween 20 in deionized water and then air-dried.

Figure S3), our microarray slides were proved to satisfy abovementioned criteria required for accurate determination of the essential epitopes of antibodies with high sensitivity and reproducibility. It was revealed that 6 antibodies (VU-3D1, VU-12E1, Ma552, VU-3C6, SM3, and DF3) have a quite similar binding profile towards the naked MUC1 peptides (**1**–**40**). As anticipated, no remarkable response with these naked MUC1 peptides was observed in case of anti-KL-6 mAb [49]. Epitopes identified as overlapping sequences of positive peptides were summarized in Table 3, in which values equal to or more than 20% relative intensity of a maximum signal arbitrarily were defined for elucidating dominant peptide epitopes of mAbs. The peptide epitopes with immunodominant PDTR motif concluded by the present microarray were largely consistent with those reported previously using different assay platforms [11,15]. Importantly, differences in the results of 10-mer from 15-mer microarray suggested that the location of the *C*-terminal proline residue included in the DTRPAP motif in the MUC1 fragments influence significantly the binding affinity with VU-3D1, VU-12E1, Ma552, VU-3C6, and DF3. More clearly, VU-11E2 showed much higher affinity with peptides carrying DTRP motif when the proline residue is placed at *C*-terminal end [2,22] than peptides **1** and **2**, in which *C*-terminal ends were modified with alanine residue. It should be emphasized that non-fouling nature of the microarray allowed for such highly sensitive detection and differentiation of the binding profiles based on relatively weak and broad affinity of these mAbs with naked MUC1 peptide fragments immobilized on the solid-plastic surface. However, it is important to note that the density of the immobilized peptides/glycopeptides significantly might influence the sensitivity in the detection, in which the density can be controlled by the printing concentration of peptide (Figure S1–3). Especially, there seemed to be a need of relatively high printing concentration as indicated in case for the analysis of short 10-mer peptides exhibiting lower affinity with most antibodies when compared with those of glycopeptides. In addition, both *N*- and *C*-terminal amino acids in the short peptides may affect the interaction with antibody even when the peptide contains the minimal epitope region, suggesting that mapping analysis using robust compound library of various fragments containing the proposed epitope would become a promising approach to identify an essential epitope structure against the target antibody.

Next, our attention was directed to the effect of *O*-glycosylation at the immunodominant PDTRP motif on the interaction of mAbs with MUC1 fragments. In the present study, modifications of two 23-mer peptides with Tn, T, and sialyl-T antigens were performed at five potential glycosylation sites, PDTRP [42,45,48,54,57,60], GSTA [43,44,46,47,49,50], and VTSA [52,53,55,56,58,59], respectively. Microarray slides displaying compounds **41**–**60** were prepared by a standardized protocol illustrated in Fig. 1B and employed for assessing the binding profile of mAbs. Naked MUC1 peptides **41** and **51** were not recognized by all mAbs tested because a key proline residue of the DTRP region is far from the *C*-terminus as indicated by the results of 10-mer and 15-mer peptides (Fig. 3). However, seven mAbs except anti-KL-6 mAb exhibited distinctly enhanced affinities with peptides glycosylated at DTRP motif [42,45,48,54,57,60] while any *O*-glycosylation at other sites (GSTA and VTSA) did not affect their binding profile at all. This result clearly indicates that the glycosylation at the threonine residue is essential for converting the PDTRP motif into an immunodominant epitope within the macromolecular MUC1 tandem repeats, even though these mAbs did not show any glycoform specificity. It was also reported by using conventional ELISA assay format that attachment of GalNAc residue at the DTR motif improved binding to anti-MUC1 mAbs involving VU-3D1, VU-12E1, VU-11E2, Ma552, VU-3C6, and SM3 [14,86]. It is well documented that α-*O*-glycosylation at Thr residues by GalNAc forces the peptide backbone into a stable extended conformation, which minimizes the steric effects by further modifications yielding bulky branched *O*-glycan chains [87–91] and serves as the preferred peptide conformations seen in cancer-relevant peptide epitopes recognized by many anti-MUC1 mAbs [19–21,92].

On the contrary, it was demonstrated that the binding specificity of anti-KL-6 mAb with peptides **48** and **60** (PDTRP motif bearing sialyl-T antigen) is remarkable among 8 mAbs tested herein. The present results were in good agreement with our previous study identifying an essential MUC1 epitope of anti-KL-6 mAb, PDT(sialyl-T)RPAP [49]. Interestingly, it seemed that the binding profiles of 7 mAbs (VU-3D1, VU-12E1, VU-11E2, Ma552, VU-3C6, SM3, and DF3) with positive glycopeptides [42,45,48,54,57,60] are obviously different between fragments I and II (Fig. 4). Although all these mAbs showed a similar level of affinity both with fragments I and II carrying Tn antigen [42,54], affinity with peptides bearing T and sialyl-T antigen [45,48,57,60] was strongly influenced by the location of PDTRP motif in these designated MUC1 fragments. Considering the occurrence of the site-specific conformational alteration during the glycosylation at this important epitope region [91], it seems that such conformational impact by glycosylation may depend on the size of the fragment and the location of the epitope region as well as the glycoform. However, it should be emphasized that anti-KL-6 mAb did not show any changes in the binding profile between the two fragments. In other words, this result clearly indicates that the distal sialylation of T antigen attached to PDTRPAP is a crucial step to provide this peptide sequence with an essential combined-glycopeptide epitope of anti-KL-6 mAb through the site-specific conformational alteration [91].

Table 1
Peptides and glycopeptides used in this study.
Each compound has a 5-oxohexanoyl group at *N*-terminus and an amide group at *C*-terminus. Shown in white letters highlighted in black are amino acids that are modified with tumor-associated *O*-linked carbohydrate antigens specified in the table.

Entry	Sequence (N→C)	Entry	Sequence (N→C)	Entry	Sequence (N→C)	Glycan
1	V T S A P D T R P A	21	P P A H G V T S A P D T R P A	41	G V T S A P D T R P A P G S T A P P A H G V T	
2	G V T S A P D T R P	22	A P P A H G V T S A P D T R P	42	G V T S A P D **T** R P A P G S T A P P A H G V T	Tn
3	H G V T S A P D T R	23	T A P P A H G V T S A P D T R	43	G V T S A P D T R P A P G **S** T A P P A H G V T	Tn
4	A H G V T S A P D T	24	S T A P P A H G V T S A P D T	44	G V T S A P D T R P A P G S **T** A P P A H G V T	Tn
5	P A H G V T S A P D	25	G S T A P P A H G V T S A P D	45	G V T S A P D **T** R P A P G S T A P P A H G V T	T
6	P P A H G V T S A P	26	P G S T A P P A H G V T S A P	46	G V T S A P D T R P A P G **S** T A P P A H G V T	T
7	A P P A H G V T S A	27	A P G S T A P P A H G V T S A	47	G V T S A P D T R P A P G S **T** A P P A H G V T	T
8	T A P P A H G V T S	28	P A P G S T A P P A H G V T S	48	G V T S A P D **T** R P A P G S T A P P A H G V T	Sialyl-T
9	S T A P P A H G V T	29	R P A P G S T A P P A H G V T	49	G V T S A P D T R P A P G **S** T A P P A H G V T	Sialyl-T
10	G S T A P P A H G V	30	T R P A P G S T A P P A H G V	50	G V T S A P D T R P A P G S **T** A P P A H G V T	Sialyl-T
11	P G S T A P P A H G	31	D T R P A P G S T A P P A H G	51	S T A P P A H G V T S A P D T R P A P G S T A	
12	A P G S T A P P A H	32	P D T R P A P G S T A P P A H	52	S T A P P A H G V **T** S A P D T R P A P G S T A	Tn
13	P A P G S T A P P A	33	A P D T R P A P G S T A P P A	53	S T A P P A H G V T **S** A P D T R P A P G S T A	Tn
14	R P A P G S T A P P	34	S A P D T R P A P G S T A P P	54	S T A P P A H G V T S A P D **T** R P A P G S T A	Tn
15	T R P A P G S T A P	35	T S A P D T R P A P G S T A P	55	S T A P P A H G V **T** S A P D T R P A P G S T A	T
16	D T R P A P G S T A	36	V T S A P D T R P A P G S T A	56	S T A P P A H G V T **S** A P D T R P A P G S T A	T
17	P D T R P A P G S T	37	G V T S A P D T R P A P G S T	57	S T A P P A H G V T S A P D **T** R P A P G S T A	T
18	A P D T R P A P G S	38	H G V T S A P D T R P A P G S	58	S T A P P A H G V **T** S A P D T R P A P G S T A	Sialyl-T
19	S A P D T R P A P G	39	A H G V T S A P D T R P A P G	59	S T A P P A H G V T **S** A P D T R P A P G S T A	Sialyl-T
20	T S A P D T R P A P	40	P A H G V T S A P D T R P A P	60	S T A P P A H G V T S A P D **T** R P A P G S T A	Sialyl-T

3.4. Autoantibodies reacting with MUC1 glycopeptides in normal human sera

Autoantibodies against autologous tumor-associated antigens are known to be detected in the asymptomatic stage of cancer and can thus serve as highly sensitive biomarkers for early cancer diagnosis [34,35]. Recent pioneering works that uncovered the occurrence of human serum autoantibodies to glycopeptides in various cancer patients [31,33,61] greatly motivated us to test our glycopeptide microarray for highly sensitive and selective detection of anti-MUC1 autoantibodies in the circulating bloodstream. It is considered that the repertoire of autoantibodies to MUC1 fragments is a molecular "fingerprint" of antibodies produced by the dynamic interplay between host's individual immune system and pre-cancerous/cancer cells and may hold promise in detecting early stages malignant processes [93]. Given that the immunosurveillance by such anti-cancer autoantibodies can identify and destroy cancer precursor cells in most healthy cases [94–96], it is not surprising that we can observe significant differences in the serum level of such autoantibodies even in cases for healthy human controls. In fact, it can be seen that a few serum samples from healthy controls also showed IgG autoantibodies to some MUC1 peptides with Tn- or T-antigens, whereas the serum levels were apparently lower than those detected in sera from patients with breast, ovarian, and prostate cancer [31,33]. We hypothesized that autoantibody signature to cancer-relevant MUC1 glycopeptides in the serum of "normal" individuals is fundamental to assess dynamic immune response to pre-cancerous cells. Small populations of pre-malignant cells expressing MUC1 displaying aberrant *O*-glycans that generate IgG autoantibodies may not proceed to malignancy at very early stages of tumor development, and are hence eliminated. The immune response to a tumor as it progresses from early to late stage might be updated and renewed, and result in autoantibodies against progressively more cancer dominant MUC1 epitopes. It is likely that lack or defect of the host immune system to eliminate pre-malignant cells would lead to malignancy at later stage. Therefore, it seems that the late stage anti-MUC1 autoantibody profile could be comprised of an accumulation of antibodies generated at the earlier stages. Our interest was focused on the feasibility of our microarray format in the quantitative analysis of anti-MUC1 IgG autoantibodies in the healthy human sera.

As a preliminary pilot study, human sera collected from six healthy volunteers (male n = 3, female n = 3) were incubated with MUC1 microarray displaying compounds **41**–**60** at 100-fold dilutions. The levels of IgG autoantibodies were monitored by probing with biotin-labeled goat anti-human IgG and Cy™3-labled streptavidin. As anticipated, the result clearly indicated that sera from 6 healthy individuals have significant levels of IgG autoantibodies to various MUC1 fragments carrying Tn, T, and sialyl-T antigens, which showed apparent preference for the peptide sequence (Fig. 5). Surprisingly, our array format elicited for the first time the significant level of IgG autoantibodies recognizing MUC1 fragments bearing sialyl-T antigen (sialylated core 1) in healthy human sera. The levels of IgG autoantibodies to MUC1 peptides with sialyl-T antigen were found to be higher than those to peptides with other glycoforms and 6 out of 6 healthy individuals had IgG autoantibodies to MUC1 glycopeptides **50** (GS̲TA), **59** (VTS̲A), and **60** (PDT̲R) bearing this trisaccharide antigen (Fig. 5B). The IgG autoantibody reacting with glycopeptide **60** shares the minimal epitope structure for anti-KL6 mAb, clinically important biomarker for diagnosing interstitial pneumonia [47,48]. More strikingly, it should be noted that high level KL-6 can be often detected by this mAb in sera from various cancer patients due to lower level autoantibody to MUC1 fragments containing an epitope structure shared with anti-KL-6 mAb [50–54]. In other words, circulating IgG autoantibody in normal human sera reacting with this unique epitope may have an essential role for identifying cellular malignancy and preventing disease progression. However, 5 out of

Table 2
Anti-MUC1 monoclonal antibodies used in this study.

Clone	Isotype	Immunogen
VU-3D1	IgG1	ZR-75-1 breast cancer cell line
VU-12E1	IgG1	ZR-75-1 breast cancer cell line
VU-11E2	IgG1	ZR-75-1 breast cancer cell line
Ma552	IgG1	ZR-75-1 breast cancer cell line
VU-3C6	IgG1	ZR-75-1 breast cancer cell line
SM3	IgG1	Deglycosylated purified milk mucin
DF3	IgG1	Membrane enriched fractions of human metastatic breast carcinoma
KL-6	IgG1	Human lung adenocarcinoma-derived cell line (VMRC-LCR)

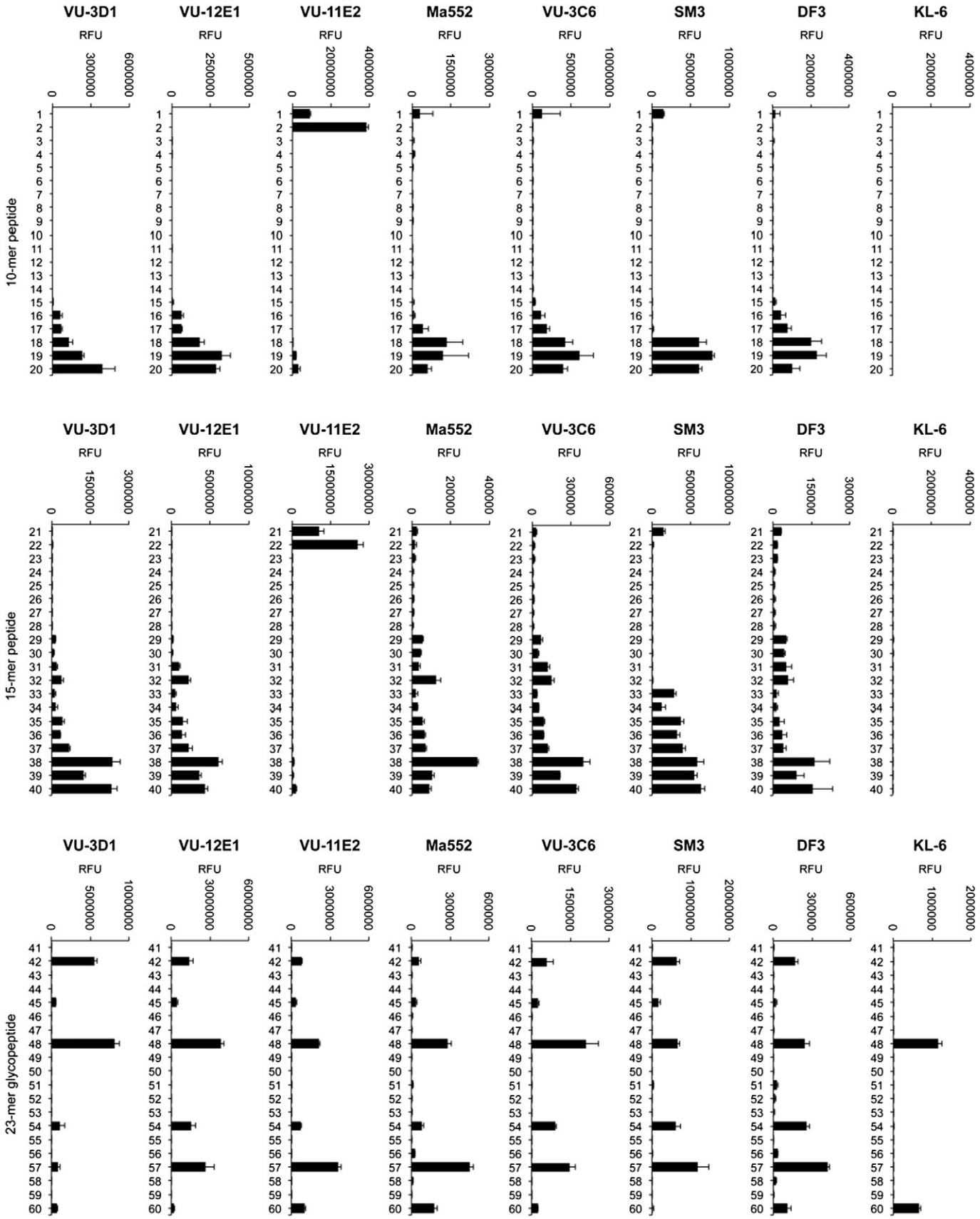

Fig. 3. Epitope mapping analysis of anti-MUC1 mAbs. Fluorescent responses of anti-MUC1 monoclonal antibodies against 10-mer peptides (top), 15-mer peptides (mddle), and 23-mer peptides/glycopeptides. Each column represents the average fluorescence intensity detected in four spots including standard deviation (see also, Figure S3). An ideal concentration of peptides/glycopeptides for the immobilization, the dynamic range and the detection limit of IgG (100 pg ml^{-1} – 50 ng ml^{-1}) were investigated by means of anti-MUC1 mAb (VU3C6) as an example (Figures S1 and S2).

Table 3
Comparison of peptide epitopes for anti-MUC1 mAbs revealed in this study and the results reported in TD-4 workshop.

Clone	Peptide epitope	
	TD-4 workshop findings[(a)]	Results in this study[(b)]
VU-3D1	SAP**DTRPAP**	GVTSA**PDTRPAP**
VU-12E1	P**DTRPAP**	TSAP**DTRPAP**
VU-11E2	TS**APDTRP**	PPAHG**VTSAPDTRP**
Ma552	GVTSA**PDTRPAP**	TSA**PDTRPAP**
VU-3C6	GVTSA**PDTRPAP**	TSA**PDTRPAP**
SM3	**APDTRP**	**APDTRPA**
DF3	AP**DTRPAP**	A**PDTRPAP**
KL-6	Not-tested	Not-detected

The peptides corresponding to the positive spots with equal to or more than 20% relative intensities of the spot with maximal intensity detected in individual microarray were used for identifying overlapped epitope sequences.
[(a)]Data reported by Price et al. [14] 7-mer (underline), 9-mer (bold), and 20-mer (plain) peptides were respectively used to epitope mapping using different assay procedures by three different groups.
[(b)]10-mer results shown in bold type; 15-mer results shown in plain type; overlapped sequences of both results shown underlined.

6 donors (except donor 2) exhibited the circulation of IgG autoantibodies to **49** (GSTA) and **58** (VTSA) involving sialyl-T antigen, suggesting that the antibody might have an anti-carbohydrate reactivity rather than against "glycopeptides". It was also revealed that only 2–4 out of 6 donors had IgG autoantibodies to MUC1 glycopeptides having Tn or T antigen [42–46,53] as well as naked MUC1 peptides **41** or **51**. There might be a considerable difference in the serum levels of the MUC1 autoantibodies between healthy human subjects. These results indicate significantly that the immune response to cancer-related MUC1 fragments might be amplified, edited and accumulated during the individual immune surveillance processes in the presence of pre-malignant cells at very early stages of tumor development. This may be a reason why some patients develop these anti-MUC1 autoantibodies while others do not, although further investigation using large number of serum samples of health volunteers is necessary to test this hypothesis.

High levels of IgG autoantibodies to some MUC1 glycopeptides in the sera of various cancer patients may become potential biomarkers for early detection of cancer [31–33]. However, it is considered that autoantibody signature of the cancer patients is a result of an accumulation of anti-MUC1 autoantibodies generated at the earlier stages and the serum level of such IgG autoantibodies may also be influenced by the individual depending on whether their immune system is compromised through many factors such as genetic background, other diseases, stress-associated factors, and immunosuppression [97]. On the other hand, it was also reported that high levels of a subset of IgG autoantibodies to MUC1 glycopeptides in early stage breast cancer patients are associated significantly with reduced incidence and increased time to metastasis [31]. This exciting finding suggests that such anti-MUC1 autoantibodies may have a crucial function in the identification and inhibition of cancer progression.

Importantly, the finding of a difference in the serum autoantibody signature of healthy volunteers was achievable by means of the present microarray format that can reduce background noise caused by non-specific protein adsorption. This allowed for highly sensitive and accurate detection of the amplified interaction between low level serum autoantibodies and densely clustered MUC1 glycopeptides depending on the strict glycoform difference at the individual glycosylation sites. Judging from the dynamic range of the array estimated by using VU3C6 (SI, Figures S1 and S2), the levels of above IgG autoantibodies circulating sera appeared to be in a range from 100 pg/ml to 50 ng/ml (0.67 pM–335 pM).

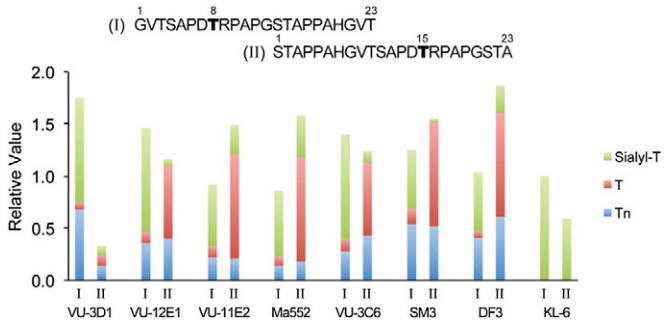

Fig. 4. The effect of microenvironment of the DTR motif on the binding affinity and glycan specificity of anti-MUC1 mAbs. A stacked bar graph summarizes relative glycan-binding specificities for 23-mer glycopeptides carrying three different *O*-glycans (Tn, T, and sialyl-T) at Thr [8] of fragment I [42,45,48] and Thr [15] of fragment II [54,57,60], respectively.

4. Conclusion

We established a standardized method for the preparation of new class microarray platform that allows highly sensitive and accurate characterization of IgG antibodies to MUC1 peptides/glycopeptides. The cyclic polyolefin plastic slides functionalized with a coating copolymer having reactive aminooxy- and biocompatible phospholipid-like groups enabled not only chemoselective and spontaneous tethering of compounds carrying a ketone/aldehyde group through an oxime bond formation but minimizing background noise caused by non-specific protein adsorption. Advantage of this array format was demonstrated by rapid and precise epitope mapping analysis of seven anti-MUC1 mAbs among mAbs tested, in which anti-KL-6 mAb showed the highest binding specificity toward the combination glycopeptide epitope carrying sialyl T antigen (Neu5Acα2,3Galα1,3GalNAcα1→) at Pro-Asp-Thr-Arg motif. On the contrary, other six anti-MUC1 mAbs could not discriminate the modification with three cancer related carbohydrate antigens such as Tn, T, and sialyl T at this motif while the binding affinity with the peptide epitope was enhanced significantly by the glycosylation at this region. It should be emphasized that this assay permitted highly sensitive detection of serum IgG autoantibodies to MUC1 glycopeptides at a wide dynamic range from 100 pg ml^{-1} to 50 ng ml^{-1} (0.67 pM–335 pM). The present microarray uncovered for the first time the occurrence of IgG autoantibodies to the MUC1 fragments carrying sialyl T-antigen at five potential *O*-glycosylation sites in the healthy human sera at 100-fold dilutions. Intriguingly, the array also showed the significant level circulation of IgG autoantibody to MUC1 fragments having T antigen at PDTR motif, although antibody toward other MUC1 epitopes with T antigen at a different glycosylation site was not detected. However, the present microarray slides do not cover MUC1 peptides with sialyl-Tn [92,98] and a variety of *O*-glycoforms involved in core 2 [49], core 3 [31–33], and core 6 series having other important roles in the regulation of cancer cell differentiation [99]. It is noteworthy that human breast carcinoma cell lines MCF-7 and ZR75-1 produce dominantly MUC1 with core 2-based *O*-glycans (83% in MCF-7 and 75% in ZR75-1, respectively), while T47D expresses endogenous MUC1 bearing core 1-based sialyl T antigen as one of its major glycoforms (45.9%) [100,101].

Merit of the present peptides/glycopeptides microarray platform is evident because the autoantibody signature may be a potent biomarker both for early diagnosis of malignancy and cancer vaccine development, while the levels of serum IgG autoantibodies to cancer relevant glycopeptide epitopes seem to depend strongly on the individual's immune response generated during the intricate immunosurveillance processes. To scrutinize the difference in the serum levels of autoantibodies to combining glycopeptide epitopes as highly potential biomarkers, advent of high performance array format has been strongly needed. The present straightforward strategy would greatly accelerate the discovery research of new class autoantibodies as well as the development of therapeutic mAbs reacting specifically with disease-relevant glycopeptide epitopes [102].

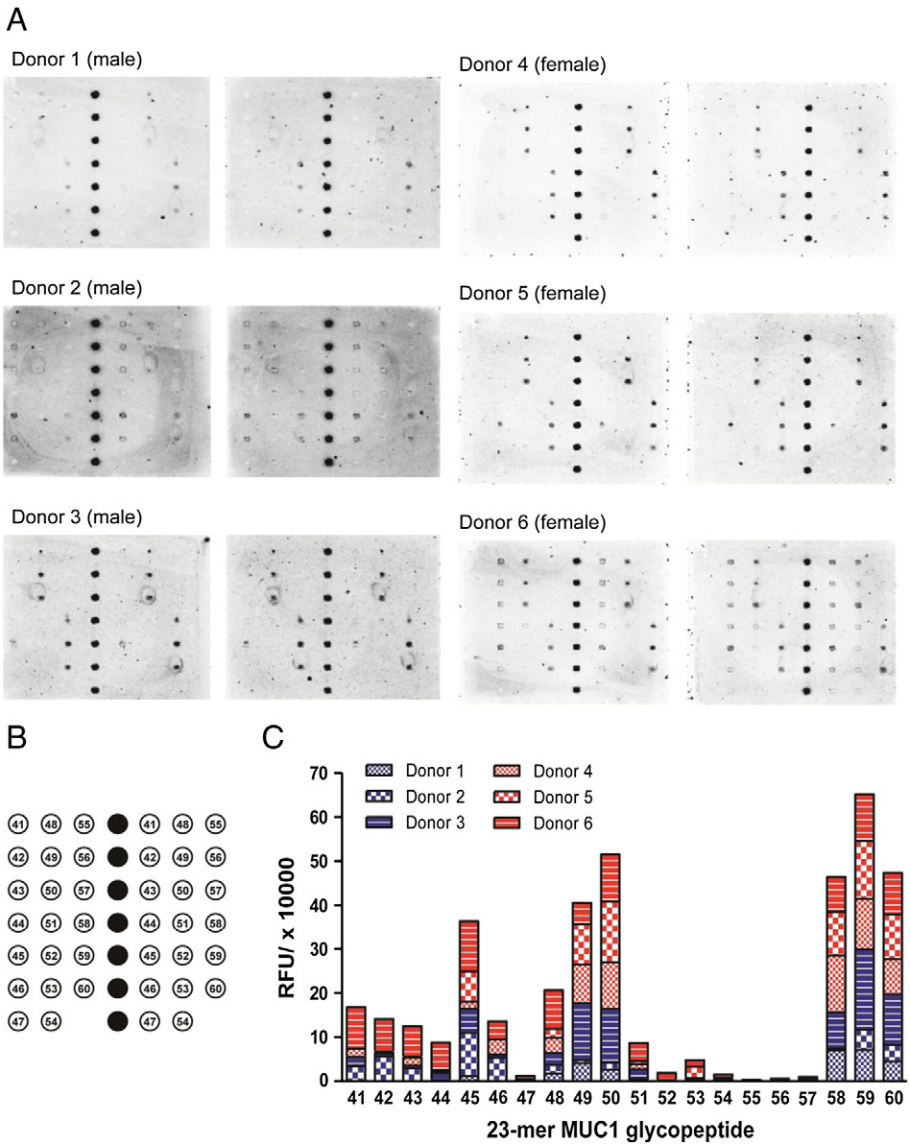

Fig. 5. IgG autoantibody signature revealed by glycopeptide microarray. (A) Fluorescent scanner images in gray scale-coloration of binding assays of six human sera specimens using 23-mer (glyco)peptide microarrays. (B) Layout of the compounds (The seven spots with strong fluorescence at center in each well are grid markers corresponding to Cy3 mono-functional dye). (C) A stacked bar graph of 6 healthy human sera. The results were represented as the mean of the fluorescent response from duplicate determinations of two independent experiments. The blue and red bars indicate the response from male and female sera, respectively.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2013.11.009.

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