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Brief Communication

# High-Throughput Screening of Glycan-Binding Proteins Using Miniature Pig Kidney *N*-Glycan-Immobilized Beads

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## SUMMARY

**Glycan recognition leading to cell-cell interactions, signaling, and immune responses is mediated by various glycan-binding proteins (GBPs) showing highly diverse ligand specificities. We describe here a rapid glycan immobilization technique via 4-hydrazinobenzoic acid (HBA)-functionalized beads and its application to high-throughput screening of miniature pig kidney *N*-glycan-binding proteins by using a mass-spectrometric approach. Without any derivatization steps, the purified pig kidney *N*-glycans were directly immobilized on to HBA-functionalized beads and subsequently used to identify GBPs from human serum. This screening method showed remarkable performance for identifying potential GBPs closely involved in pig-to-human xenograft rejection mediated by human serum, including antibodies, cytokines, complement components, siglec, and CD antigens. Thus, these results demonstrate that the GBP screening method was firmly established by one-step immobilization of the *N*-glycans on to microsphere and highly sensitive mass-spectrometric analysis.**

## INTRODUCTION

Eukaryotic cell surfaces are very rich in glycoconjugates, such as complex *N*- and *O*-glycans, on glycoproteins or glycolipids (Varki, 1999). The expressed glycans not only affect the stability and solubility of proteins, but also protect proteins from proteolysis (Taylor and Drickamer, 2003). In addition, the extracellular glycosylation is mainly involved in cell-protein and cell-cell interaction followed by various immunological events (Rudd et al., 2001; Paulson et al., 2006). In xenotransplantation, the interaction between a nonhuman glycan highly expressed on pig endothelial cells (e.g., Gal$\alpha$1,3Gal, abbreviated to Gal) and human xenoreactive natural antibodies or lectins induces hyperacute immune response resulting in rapid graft failure (Oriol et al., 1993). In spite of production of $\alpha$1,3-galactosyltransferase gene-knockout (GT-KO) pigs that do not express Gal, specific interactions between non-Gal antigens on the pig cells and their glycan-binding proteins (GBPs) from human serum still induce acute humoral xenograft rejection (Kuwaki et al., 2005). The identification of GBPs in human serum other than natural antibodies that are responsible for cell-surface sugar recognition will provide information that may resolve the problems of the innate and adaptive immune responses to xenografts and will provide insight into the development of improved strategies to achieve successful xenotransplantation.

The development of efficient analytical GBP screening methods toward specific glycan motifs, however, has been difficult owing to a very low affinity (in the range of $\mu$M to mM) of GBP-glycan binding (Collins and Paulson, 2004) and the lack of well-characterized glycan libraries. In addition, highly-sensitive analytical tools are required to identify low-abundant GBPs in real biological systems. Recently, several attempts to use glycan array formats (Blixt et al., 2004; Xia et al., 2005; Adams et al., 2004; Fazio et al., 2002; Houseman and Mrksich, 2002; Park and Shin, 2002; Ratner et al., 2004) to determine glycan specificities and candidate ligands of the GBPs have been reported, but these microarray-based approaches are not suitable for screening of unknown GBPs from diverse protein sources, such as human serum. In the microarray format, relevant information anticipating the matches between specific GBPs and glycans is required for detection of the screened GBPs by using GBP-specific primary and secondary antibodies (Blixt et al., 2004; Adams et al., 2004).

To covalently immobilize glycans on to polymeric solid supports, glycan can be derivatized to appropriately activated

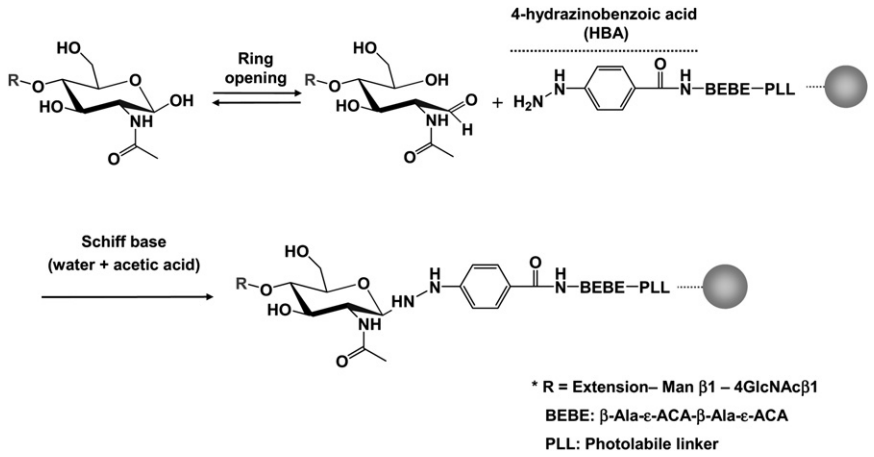


**Figure 1. One-Step Conjugation of the Free *N*-Glycans with 4-Hydrazinobenzoic Acid-Functionalized Beads**

forms, such as an amino group (Blixt et al., 2004), azido group (Fazio et al., 2002), and cyclopentadiene (Houseman and Mrksich, 2002). However, the conventional glycan-immobilization methods inevitably require laborious and time-consuming purification steps to remove unreacted glycans, chemicals, and byproducts during glycan derivatization. Moreover, they are limited to the immobilization of monosaccharides and short oligosaccharides because of their restrictive site specificity and poor reactivity. Renaudet et al. reported resin-based carbohydrate immobilization method for high-throughput screening of multivalent protein-carbohydrate interactions (Renaudet and Dumy, 2005, 2006). Although the resin-bound carbohydrate probes are quite applicable to the rapid discovery of new selective carbohydrate-based ligands, the chemoselective oxime ligation with solid supports inevitably requires the aminooxy-derivatized carbohydrates.

Herein, we developed a versatile one-step glycan immobilization method to covalently immobilize the free reducing part of the glycan site specifically. The method was directly applied to rapid and high-throughput GBP screening with highly sensitive mass spectrometry. Without any derivatization steps, a 4-hydrazinobenzoic acid (HBA)-containing hydrazide group was introduced on to core-shell-type beads prior to the immobilization of the underivatized free glycans (Figure 1). The glycan-HBA beads that present the immobilized glycans might mimic a cell-surface covered by glycoconjugates, allowing direct interactions with other cells or proteins. Furthermore, the direct immobilization of unmodified oligosaccharides gives significant advantages over the other conventional methods from an operational point of view, especially when working with complex glycans available from natural sources. The directional conjugations of linear oligosaccharides and chicken ovalbumin *N*-glycans on to HBA-functionalized beads were carried out as model systems, and their correct attachments to the bead surface were demonstrated by using MALDI-TOF MS and specific lectins. Finally, the specific pathogen-free Chicago Medical School miniature pig kidney *N*-glycan-immobilized beads were applied to high-throughput screening of specific GBPs from human serum followed by mass spectrometry. In this study, a LTQ Orbitrap hybrid Fourier Transform Mass Spectrometer (LTQ Orbitrap FT MS) that provides high mass accuracy and sensitivity was used to identify low-abundant GBPs in human serum. To the best of our knowledge, heteromixtures of completely natural glycan conjugates have not been used for screening of GBPs from human serum previously.

## RESULTS AND DISCUSSION

To conjugate free reducing glycans through a one-step reaction on to beads, a β-Ala-ε-aminocaproic acid-β-Ala-ε-aminocaproic acid (BEBE)-photolabile linker (PLL) was built up by using a 9-fluorenylmethyloxycarbonyl(Fmoc)/*t*Bu strategy on to the HiCore resin (see the Supplemental Data available with this article online; Lee et al., 2007), which structure is a core-shell-type and shows a low level of nonspecific binding of proteins (Kim et al., 2004, 2006a). *N*-*t*-butyloxycarbonyl (*N*-Boc)-protected HBA (*N*-Boc-HBA), i.e., a chemoselective anchoring agent, was directly conjugated with BEBE-PLL-resin, and trifluoroacetic acid (TFA) was used for the deprotection of the Boc group. Various glycans were immobilized on the functionalized beads via Schiff base reaction between the aldehyde group at the reducing end of the *N*-glycans and the hydrazide group of HBA-conjugated beads under mild acidic conditions and subsequent cyclization reaction (Lee and Shin, 2005).

To validate the immobilization of free *N*-glycans on to HBA-functionalized beads, a commercially available tetrasaccharide, N,N′,N″,N‴-tetraacetylchitotetraose ($GlcNAc_4$, GlcNAcβ1-4GlcNAcβ1-4GlcNAcβ1-4GlcNAc), was used, which has the same reducing *N*-acetylhexosamine residue as free *N*-glycans. Every individual step of the synthesis of glycan-HBA bead construction was directly confirmed by the expected mass spectra of the released synthetic product by using MALDI-TOF MS and PLL linker (Figure 2A; Kim et al., 2007). The major peaks at *m/z* 408.2 (BEBE) and 642.0 (Boc-HBA-BEBE) in the form of $[M+Na]^+$ indicated the sequential order of HBA-functionalized bead synthesis. After deprotection with TFA, the $GlcNAc_4$ was coupled with HBA-functionalized beads under a mild acidic condition, and the intact mass at *m/z* 1354.7 ($GlcNAc_4$-HBA-BEBE) exactly matched our calculated value. To our knowledge, this is the first report on the application of MALDI-TOF MS and PLL linker to demonstrate stepwise coupling of glycans on to beads. The complete coupling (*m/z* 986.8) between $GlcNAc_4$ (*m/z* 852.9) and HBA was also demonstrated in soluble phase (Supplemental Data). Thus, these results directly prove that HBA-functionalized beads are well suited for the one-step conjugation of free *N*-glycans without any derivatization. Furthermore, the HBA-conjugated beads allowing UV photocleavage via PLL linker are surely applicable to facile purification and analysis of glycans from the crude proteolytic digests. Recently, Nishimura et al. demonstrated the glycoblotting method for the purification of glycans from the crude proteolytic digests using aminooxy-functionalized solid particles (Nishimura et al., 2004), but the method has not been applied to GBPs screening yet.

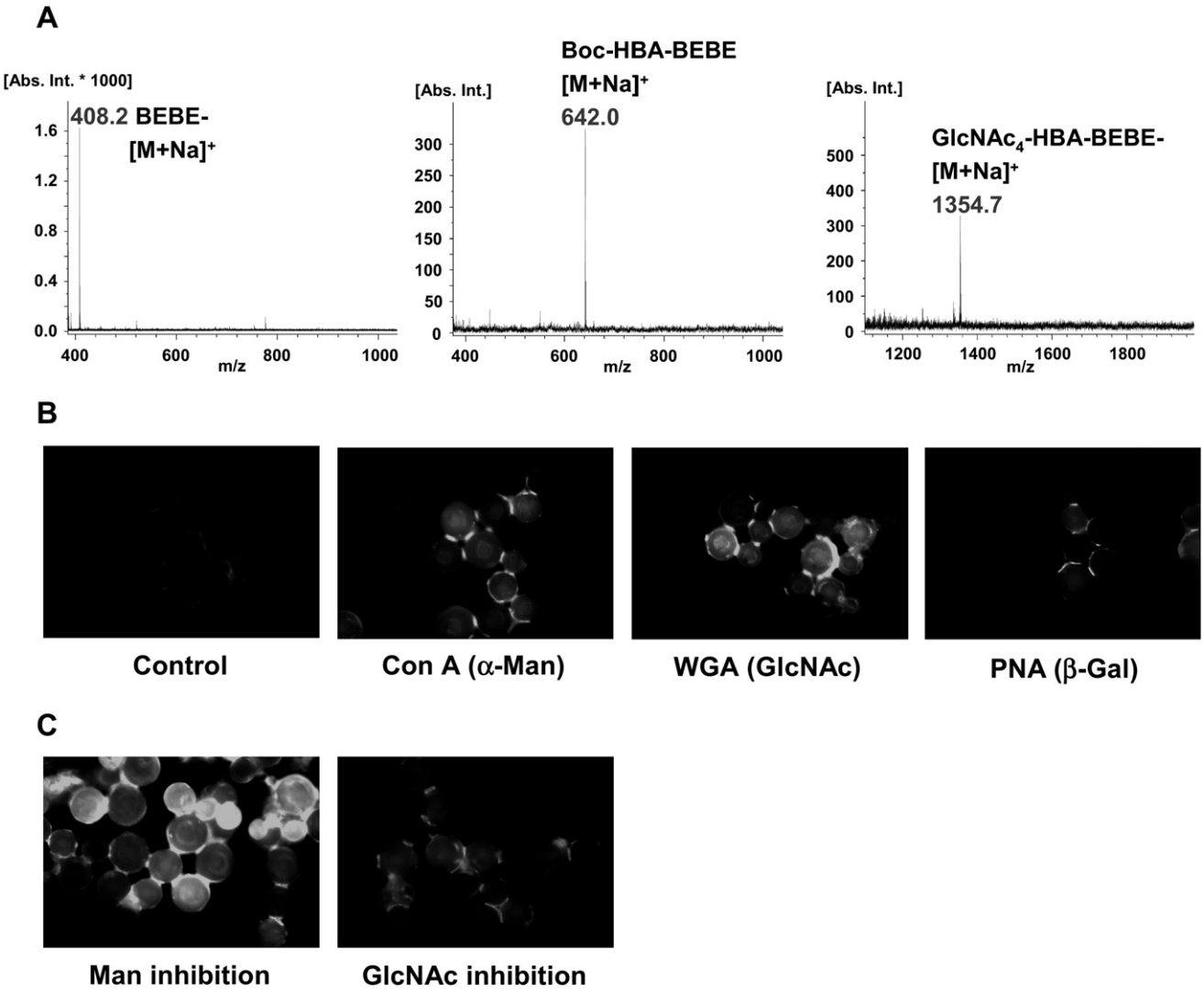


**Figure 2. Demonstration of Conjugation between Free Glycans and HBA-Functionalized Beads**

(A) MALDI spectra of BEBE, *N*-Boc-HBA-BEBE, and $GlcNAc_4$-HBA-BEBE obtained from after UV photocleavage of photolabile linker (PLL) introduced on to core-shell-type beads.

(B) Specific fluorescent-conjugated lectin binding image on glycan (ovalbumin)-HBA beads. Concanavalin A (ConA, for terminal α-mannose); wheat germ agglutinin (WGA, for β-*N*-acetyl-glucosamine); *Arachis hyphgaea* lectin (PNA, for terminal β-galactose).

(C) Inhibition test against glycan (ovalbumin)-HBA beads using appropriate monosaccharides (noninhibitory Man and inhibitory GlcNAc) and WGA.

To demonstrate the orientation of the immobilized glycans, specific lectin-binding and monosaccharide inhibition assays with lectins and monosaccharides were performed. Well-characterized chicken ovalbumin, which predominantly contains terminal-GlcNAc, Gal, and Man and lacks NeuAc (Harvey et al., 2000), was chosen to demonstrate the oriented immobilization of the branched *N*-glycan on to HBA-functionalized beads. The conjugation between free ovalbumin *N*-glycans and HBA was also identified via MALDI-TOF MS analysis, indicating complete conjugation of the highly branched *N*-glycans to HBA (Supplemental Data). Tetramethylrhodamine-conjugated concanavalin A (ConA), tetramethylrhodamine-conjugated wheat germ agglutinin (WGA), and Alexa Fluor-conjugated lectin PNA from *Arachis hypogaea* specific to branched terminal Man, β-GlcNAc, and β-Gal, respectively, were incubated with glycan (ovalbumin)-HBA bead. As expected from the previous studies, the *N*-glycan (ovalbumin)-HBA beads showed the highest fluorescence signal with WGA, which was significantly higher than that with ConA or PNA (Figure 2B). In addition, the fluorescence signals with WGA against terminal-GlcNAc of glycan (ovalbumin)-HBA beads decreased with the addition of the *N*-acetyl-D-glucosamine (GlcNAc), while D-Mannose did not inhibit the interaction between WGA and glycan (ovalbumin)-HBA beads (Figure 2C). Recently, Grün et al. reported a one-step biotinylation procedure via hydrazide group to reducing end of carbohydrates (Grun et al., 2006). They directly demonstrated that sialic acids were not degraded during glycan derivatization via hydrazide group at elevated temperature. Thus, the reducing parts of the highly branched *N*-glycans were

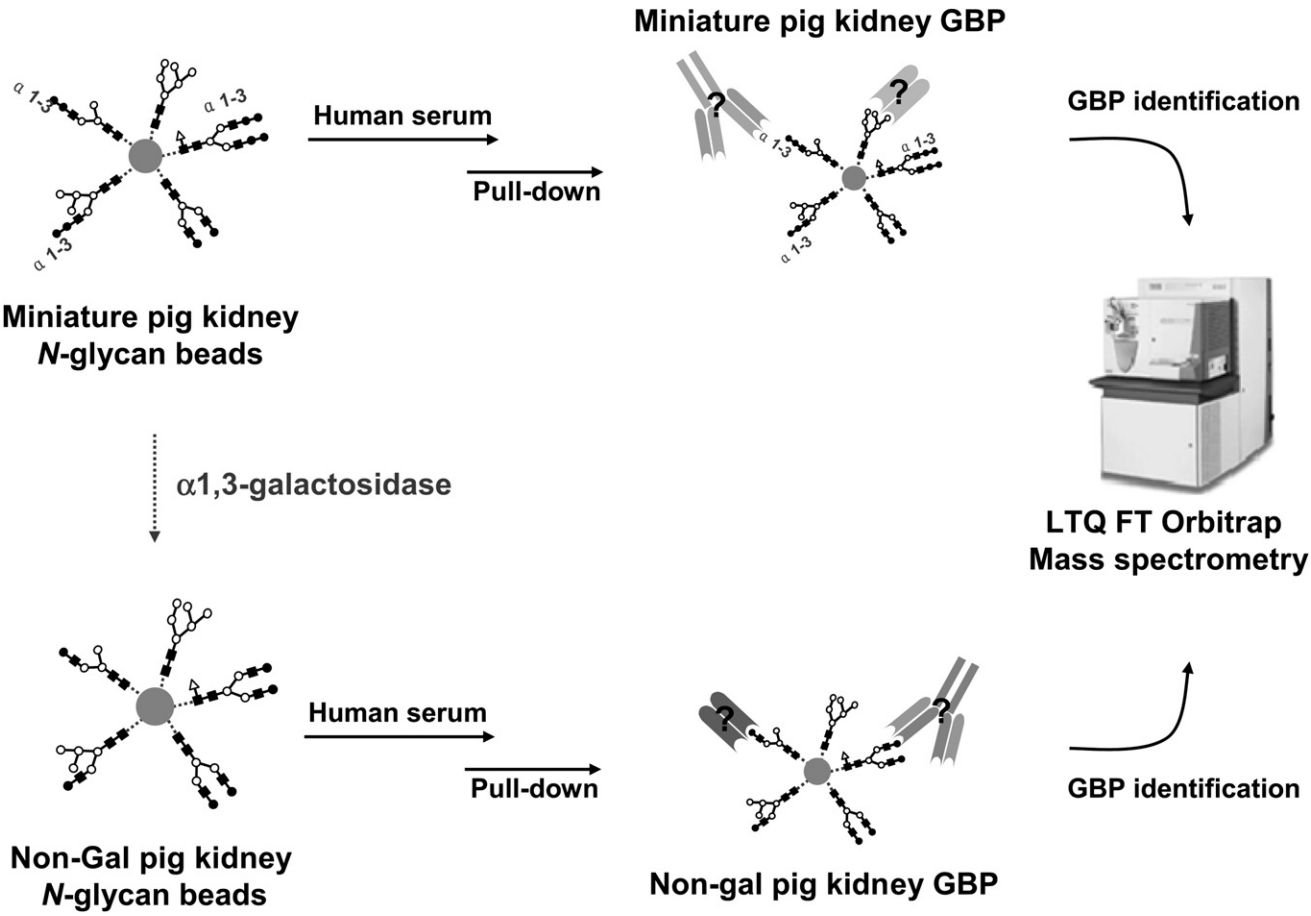


**Figure 3. General Procedure for the Sreening of Glycan-Binding Proteins by Miniature Pig Kidney *N*-Glycan-Immobilized Beads and Mass Spectrometry**

Glycan-HBA bead was applied to the identification of miniature pig *N*-glycan (natural/non-Gal type)-binding proteins from human serum by using the glycan-binding protein (GBP) screening method.

directionally immobilized on the beads, and the lectins specifically recognized the apex of the immobilized ovalbumin *N*-glycans.

Next, the glycan-HBA beads were applied for screening of pig kidney *N*-glycan-binding proteins from human serum that might trigger antigenic reactions during xenotransplantation as described in Figure 3. Although GT-KO pigs have been produced to overcome hyperacute rejection in pig-to-baboon organ transplantation, there still ensue unknown associations between degalactosylated (non-Gal) antigens and human proteins that are involved in many immune-system functions that are believed to contribute to acute humoral xenograft rejection and to the adaptive immune response (Kuwaki et al., 2005). To release free *N*-glycans from a pig kidney, miniature pig kidney membrane was prepared, as described (Kim et al., 2006b). The membrane fraction was denatured and subsequently deglycosylated by PNGase F. The released free *N*-glycans were purified with a porous graphitic carbon cartridge, qualified (over 100 *N*-glycans) and quantified (around 30% contained α-Gal) by using various mass-spectrometric strategies (Y.-G.K., G.-C.G., David J. Harvey, and B.-G.K., unpublished data). To prepare non-Gal *N*-glycans, pig natural *N*-glycans were treated with α-galactosidase in vitro. The total pig *N*-glycan and the non-Gal *N*-glycan were individually immobilized on to HBA-functionalized beads. The albumin- and IgG-depleted human serum was first incubated with HBA-functionalized beads to remove nonspecific-binding proteins, and subsequently the unbound fraction was transferred to the glycan immobilized beads. After washing with PBS buffer, the bound proteins were digested overnight with sequencing-grade trypsin. The tryptic-digested peptides were directly applied to the LTQ Orbitrap FT MS, and the pig kidney *N*-glycan-binding proteins in human serum were identified with SEQUEST software. In addition, the nonspecific binding fraction was also tryptic digested and analyzed by LTQ Orbitrap FT MS. As we expected, most of the nonspecific bound proteins were high-abundant proteins in human serum such as apolipoproteins, α-2 macroglobin, and prealbumin (data not shown).

Our sensitive method enabled us to identify several lectin-like pig natural and non-Gal GBPs, which are known to play a role in xenograft rejection, and several uncharacterized candidate GBPs (Table 1 and Figure 4). First, the identification of IgM in both the natural and non-Gal beads was certainly in good agreement with previous reports (Rood et al., 2005), suggesting that

**Table 1. Identification of Miniature Pig Kidney Natural/Non-Gal Type *N*-Glycan-Binding Proteins Using LTQ Orbitrap FT MS**

| | Natural Pig Kidney *N*-Glycan | | | Non-Gal Pig Kidney *N*-Glycan | | |
|---|---|---|---|---|---|---|
| Protein | Max. XCorr (Charge) | Del CN | Abundance of the Detected Peptide[b] | Max. XCorr (Charge) | Del CN | Abundance of the Detected Peptide |
| Immunoglobulin mu Chain[a] | | | | | | |
| KQVGSGVTTDQVQAEAKE | 4.72(2) | 0.55 | 7 | 4.66(2) | 0.40 | 3 |
| KGVALHRPDVYLLPPARE | | | | 4.65(3) | 0.49 | 5 |
| KYVTSAPMPEPQAPGRY | | | | 2.74(2) | 0.47 | 5 |
| KVSVFVPPRD | 2.58(2) | 0.49 | 3 | | | |
| CD40 Ligand: Tumor Necrosis Factor (Ligand) Superfamily Member 5 | | | | | | |
| RQGFYYIYTQVTFCSNRE | 3.06(2) | 0.42 | 21 | 3.08(2) | 0.40 | 19 |
| Complement Component 3 | | | | | | |
| REGVQKEDIPPADLSDQVPDTESETRI | 5.92(3) | 0.59 | 3 | 6.26(3) | 0.56 | 3 |
| RILLQGTPVAQMTEDAVDAERL | 6.38(2) | 0.59 | 31 | 6.32(2) | 0.57 | 33 |
| RSEETKENEGFTVTAEGKG | 5.15(2) | 0.61 | 1 | 5.31(2) | 0.61 | 14 |
| KLSINTHPSQKPLSITVRT | 3.99(2) | 0.55 | 8 | | | |
| RIPIEDGSGEVVLSRK | 3.73(2) | 0.49 | 2 | | | |
| Complement Component 4B | | | | | | |
| RVTASDPLDTLGSEGALSPGGVASLLRL | | | | 5.50(2) | 0.73 | 2 |
| KMRPSTDTITVMVENSHGLRV | 4.82(3) | 0.49 | 1 | 4.80(2) | 0.45 | 3 |
| KVLSLAQEQVGGSPEKL | 4.60(2) | 0.55 | 3 | | | |
| RHLVPGAPFLLQALVRE | | | | 3.98(3) | 0.39 | 3 |
| RGPEVQLVAHSPWLKD | | | | 3.17(2) | 0.43 | 2 |
| RVGDTLNLNLRA | 3.44(2) | 0.33 | 2 | 3.61(2) | 0.32 | 2 |
| Clusterin Isoform 1: Complement-Associated Protein | | | | | | |
| RVTTVASHTSDSDVPSGVTEVVVKL | 6.31(2) | 0.66 | 25 | 6.32(2) | 0.65 | 14 |
| KLFDSDPITVTVPVEVSRK | 5.26(2) | 0.54 | 51 | 5.12(2) | 0.54 | 40 |
| KTLLSNLEEAKK | 3.65(2) | 0.31 | 25 | 3.11(2) | 0.32 | 19 |
| RIDSLLENDRQ | 3.15(2) | 0.32 | 3 | 3.18(2) | 0.26 | 3 |
| Chemokine (C-X-C Motif) Ligand 2: GRO2 Oncogene | | | | | | |
| RLLRVALLLLLLVAASRRA | 3.68(3) | 0.26 | 14 | 3.68(3) | 0.27 | 14 |
| Heparin Cofactor II[a] | | | | | | |
| KHQGTITVNEEGTQATTVTTVGFMPLSTQVRF | | | | 3.63(3) | 0.41 | 1 |
| REYYFAEAQIADFSDPAFISKT | | | | 3.56(2) | 0.57 | 1 |
| KTLEAQLTPRV | 3.00(2) | 0.35 | 3 | 2.98(2) | 0.20 | 2 |
| RSVNDLYIQKQ | | | | 2.67(2) | 0.18 | 1 |
| Coagulation Factor II (Prothrombin) | | | | | | |
| KSLEDKTERELLESYIDGRI | 6.03(3) | 0.61 | 7 | 5.14(3) | 0.62 | 1 |
| RELLESYIDGRI | 2.76(2) | 0.37 | 1 | 3.32(2) | 0.37 | 3 |
| Platelet Factor 4 Chemokine (C-X-C Motif) Ligand 4[a] | | | | | | |
| RHITSLEVIKA | 2.93(2) | 0.48 | 3 | 2.66(2) | 0.36 | 5 |
| Proplatelet Basic Protein (CXCL7) | | | | | | |
| KGKEESLDSDLYAELRC | 3.34(2) | 0.52 | 4 | 3.74(2) | 0.58 | 3 |
| CD72[a] | | | | | | |
| KLSNMENRLKP | 1.74(1) | 0.16 | 1 | | | |
| CD209 (DC-SIGN)[a] | | | | | | |
| KFWICKKSAASCSRD | | | | 2.73(2) | 0.10 | 1 |
| CD14[a] | | | | | | |
| RSTLSVGVSGTLVLLQGARG | | | | 3.08(2) | 0.30 | 2 |
| Sialic Acid Binding Ig-like Lectin 7[a] | | | | | | |
| KSARPAADVGDIGMKD | | | | 2.64(2) | 0.54 | 1 |

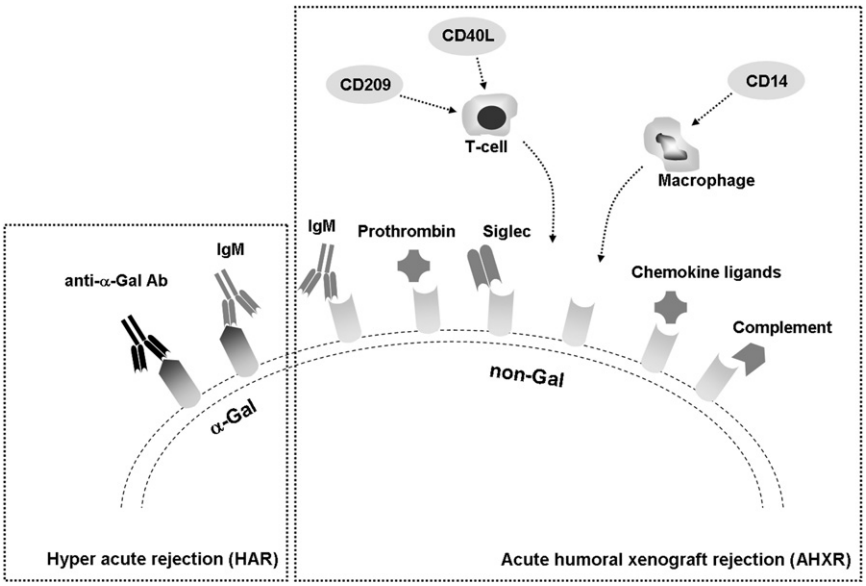


**Figure 4. Summary of Miniature Pig Kidney *N*-Glycan-Binding Proteins Potentially Triggering Xenograft Rejection Mechanism**

Natural anti-α-Gal antibodies binding to Gal epitopes predominantly initiate hyperacute rejection of a pig xenograft. In this study, non-Gal-binding proteins that may play important roles in the acute humoral xenograft rejection and acute cellular rejection are identified by highly sensitive GBP screening method.

IgM-related xenograft rejection would be caused by the direct interaction between IgM and α-Gal/non-Gal antigens. In addition, the identified cytokines and complement components are clearly involved in acute-phase inflammatory responses (Baldwin et al., 2003). Interestingly, although the lectin-like activities of prothrombin and proplatelet basic protein have not been characterized yet, key proteins in the coagulation cascade of human blood have been shown to be involved in xenograft rejection (Robson et al., 2000), for example in the immediate blood-mediated inflammatory reaction (IBMIR) that follows pancreatic islet transplantation into the portal vein. Finally, the finding of CD antigens and sialic acid-binding Ig-like lectin (Siglec) as non-Gal-binding proteins suggests a possible role of these antigens in rejection of GT-KO pig organs. Notably, CD209 and CD40 ligand are costimulatory molecules that activate T cells (van Kooyk and Geijtenbeek, 2002; Grewal and Flavell, 1996), and CD14 is known to be a major receptor in the activation of macrophages (Tobias and Ulevitch, 1993). As shown in Table 1, several proteins binding to total pig *N*-glycan (including α-Gal epitopes) were also found to have affinity to the *N*-glycan pool without α-Gal antigens. These data may indicate that the proteins found in both the *N*-glycan pools are non-Gal-binding proteins, which can be competing with anti-α-Gal antibodies for xenograft rejection. However, the human immune responses triggered by the non-Gal-binding proteins may be later than those by anti-α-Gal antibodies causing hyperacute rejection.

Taken together, the Gal/non-Gal antigens can activate both the innate and adaptive immune responses, so the targeting of these antigens will be an attractive avenue toward control of graft rejection using GT-KO pigs as organ donors. Thus, using this GBP screening system, we could discover promising candidates of pig kidney *N*-glycan-binding proteins whose specific roles and significances in xenotransplantation rejection remain to be fully determined.

## SIGNIFICANCE

**This study demonstrated that glycan-HBA beads could be applied to the screening of GBPs in complex body fluid samples. Our approach has several important advantages, such as (1) one-step covalent conjugation of the free reducing part of a *N*-glycan without any derivatization after isolating the glycan from a biological source, (2) a dominant ring-closed reducing end of *N*-glycans, (3) identification of low-abundant proteins by using highly sensitive mass spectrometry, and (4) high-throughput screening of GBPs for the development of tissue- or cell-specific glycan library beads. In addition, because we obtained glycans from natural sources released by endoglycosidases, the chemical synthesis of branched complex glycans was unnecessary to cover the diversity of the glycan library. The introduction of highly sensitive mass spectrometries such as MALDI-TOF MS and LTQ Orbitrap FT MS enabled us to validate stepwise coupling of glycan on to beads and to identify low-abundant pig glycan-binding proteins existing in human serum. The identified miniature pig kidney *N*-glycan-binding proteins will provide a clue for understanding and overcoming additional barriers to the transplantation of GT-KO pig organs into humans. Although in this method, the identified GBPs could not exactly pair with diverse glycan structures immobilized on to beads, this weakness might be overcome by a technique such as elution with specific monosaccharide-containing buffer. Therefore, this high-throughput GBP-screening method pioneers the investigation of the biological roles of GBP-ligand interactions in situ and should allow the rapid detection of microbial or viral pathogens and the screening of glycan-specific GBPs as diagnostic markers of various diseases.**

Max. XCorr is the highest XCorr value (>1.7, 2.5, 3.0 for singly, doubly, and triply charged peaks, respectively) of the identified peptide. DelCN is the difference in magnitude between the peptide fit with the highest Xcorr and the peptide fit with the second best Xcorr.

[a] This marked protein has lectin-like activity.

[b] The experiment was triplicated to evaluate an abundance of the detected peptide.

## EXPERIMENTAL PROCEDURES

### Preparation of *N*-Glycans from Miniature Pig Kidney Membrane Proteins and Chicken Ovalbumin

The Institutional Animal Care and Use Committee of the Seoul National University Hospital approved all animal procedures. The specific pathogen-free (SPF) Chicago Medical School (CMS) miniature pigs were donated from Dr. Yoon Berm Kim of Rosalind Franklin University of Medicine and Science, CMS (North Chicago, IL, USA; Setcavage and Kim, 1976). The miniature pig kidney membrane proteins (36.4 mg $ml^{-1}$) were prepared as described (Kim et al., 2006b). The fractionated membrane proteins and chicken ovalbumin (20 mg/ml, Sigma, St. Louis, MO) were denatured at 90°C for 10 min for efficient glycosidase cleavage of the *N*-glycans. After cooling at room temperature, Peptide *N*-glycosidase F (PNGase-F, Roche, Germany) was treated at 37°C for 16 hr. The proteins were precipitated by adding four volumes of cold ethanol and the mixture was kept in ice for 2 hr. The *N*-deglycosylated proteins were centrifuged down to a pellet and the supernatant containing the oligosaccharides was transferred to new eppendorf tubes. The ethanol was evaporated, and the remaining *N*-glycans were dissolved by ultrapure water. The released *N*-glycans were purified by solid-phase extraction with a porous graphitic carbon cartridge (PGC cartridge, Alltech. Associates, Deerfield, IL). First, the cartridge was washed with 0.1% (v/v) TFA in 80% ACN/$H_2O$ (v/v) followed by $H_2O$. The solution of *N*-glycans was applied to the PGC cartridge. Subsequently, the cartridge was washed with pure water to remove salts and contaminants in the sample. *N*-glycans were eluted twice with 40% ACN in 0.05% (v/v) TFA in $H_2O$. Each fraction was collected and concentrated by Speed Vac-concentrator (Ecospin 3180C, Hanil Research and Development, Korea) prior to glycan immobilization on to the functionalized bead. Before the purified pig kidney *N*-glycans were used, they were verified by mass spectrometric approaches (Y.-G.K., G.-C.G., David J. Harvey, and B.-G.K., unpublished data). The half of the purified pig kidney *N*-glycan was used for non-Gal bead production in vitro by a digestion of Green coffee bean α-galactosidase (five unit/ml, EC 3.2.1.22, specificity for α1-3/4/6-linked galactose, Glyko, Inc., Upper Heyford, UK) as described (Kim et al., 2006b).

### Preparation of HBA-Functionalized Bead and Coupling Procedure of *N*-Glycan on to the Bead

The linker and spacer bound resin was synthesized by conventional method of Fmoc solid phase synthesis as follows. HiCore resin (0.3 mmol $g^{-1}$, 300 mg, 0.09 mmol) was swollen in NMP (N-methyl pyrrolidone, 10 ml, Junsei Chemical, Co., Tokyo, Japan) and then added to mixture of Fmoc-photolabile linker (Fmoc-4-[4-(1-aminoethyl)-2-methoxy-5-nitrophenoxy]butyric acid; 94 mg, 0.18 mmol, ChemTech, Louisville, KY), BOP (benzotriazol-1-yloxytris(dimethylamino) phosphonium hexafluorophosphate; 80 mg, 0.18 mmol), HOBt (1-hydroxybenzotriazole; 24 mg, 0.18 mmol), and DIEA (diisopropylethylamine; 63 μl, 0.36 mmol) (Kim et al., 2004). The mixture was stirred at 25°C for 2 hr in a shaking incubator, and the solution containing excess reagents was filtered out with nitrogen gas. The resin was washed with NMP, DCM (dichloromethane, Junsei Chemical, Co., Tokyo, Japan) and MeOH. Fmoc was removed at each cycle with 20% piperidine/NMP (3+17 min), and the resin was washed with NMP, DCM, and MeOH. Successively, Fmoc-ε-aminocaproic acid (Fmoc-ε-ACA, BeadTech; 64 mg, 0.18 mmol, Seoul, Korea) and Fmoc-β-alanine (Fmoc-β-Ala; 56 mg, 0.18 mmol, BeadTech, Seoul, Korea) were alternately coupled on the Fmoc-photolabile linker in the same manner by two times. And then Boc-4-hydrazinobenzoic acid (Boc-HBA; 56 mg, 0.18 mmol, NeoMPS, San Diego, CA) was introduced on the photolabile linker and spacer coupled resin by BOP mediated coupling. Finally, Boc group was removed by adding neat trifluoroacetic acid (TFA, Sigma, St. Louis, MO) at −20°C with gently shaking for 2 hr (Uematsu et al., 2005). The resin was washed with DCM and MeOH after the reagent was filtered out. The prepared miniature pig kidney *N*-glycans (natural 4.5 mg, non-Gal 4.5 mg), ovalbumin glycans (5 mg) and N,N′,N″,N‴-tetraacetylchitotetraose (2 mg, GlcNAc1-β-4-GlcNAc1-β-4-GlcNAc1-β-4GlcNAc, Toronto Research Chemicals, Inc., North York, ON, Canada) were respectively dissolved by ultrapure water (350 μl) and acetic acid (150 μl). The each glycan solution was mixed with HBA-resin and incubated at 25°C for 4 hr. After the *N*-glycan coupling reaction, the supernatant was discarded, and the *N*-glycan beads were washed with $dH_2O$ five times. The completion of each coupling reaction was also confirmed through Kaiser's ninhydrin test (Supplemental Data; Kaiser et al., 1970).

### MALDI-TOF MS

Samples were prepared by mixing an aqueous solution of the sample (0.5 μl) with a saturated solution of 2,5-dihydroxybenzoic acid (DHB, Sigma, St. Louis, MO) in acetonitrile (0.3 μl) on the stainless-steel MALDI target and allowing the mixture to dry under ambient conditions. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) for the *N*-glycan coupling validation was performed with Biflex IV equipped with a 337 nm nitrogen laser (Bruker, Bremen, Germany). The analyte ions were accelerated at 20 kV under delayed extraction conditions in the positive reflectron mode. The analytical range of laser power was adjusted to obtain a good resolution and signal-to-noise ratio. MALDI-TOF spectra were acquired from *m/z* 500 to 3500 with an average of 200 shots. Data acquisition and processing were performed with Bruker X-TOF 5.1.1 and Biotools 2.1 program (Bruker, Bremen, Germany).

### Binding Study of Fluorophore-Labeled Lectin on to Chicken Ovalbumin *N*-Glycan Bead

The prepared chicken ovalbumin *N*-glycan bead was incubated with tetramethylrhodamine-conjugated concanavalin A (Con A, 1 μg $ml^{-1}$, Molecular Probes, Eugene, OR), tetramethylrhodamine-conjugated wheat germ agglutinin (WGA, 1 μg $ml^{-1}$, Molecular Probes, Eugene, OR), and Alexa Fluor-conjugated lectin PNA from *Arachis hypogaea* (1 μg $ml^{-1}$, Molecular Probes, Eugene, OR) at room temperature for 1 hr. To verify further the *N*-glycan coupling on to HBA-resin, the inhibition study was carried out through the coincubation with each monosaccharide (D-Mannose and N-acetyl-D-glucosamine, 200 mM, Sigma, St. Louis, MO) and tetramethylrhodamine-conjugated WGA. After the incubation, the beads were gently washed with PBS buffer three times. The binding was analyzed by using a confocal laser scanning microscope (LSM5 Pascal, Carl Zeiss, Germany).

### Miniature Pig Kidney *N*-Glycan-Binding Protein Screening from Human Serum

Albumin and the major subclasses of gamma-globulin (IgG) of human serum (40 μl, obtained from a healthy male donor, 41 ages) were simultaneously removed by using ProteoSeek Albumin/IgG Removal Kit (Pierce, Rockford, IL, USA) according to the manufacturer's instruction. The human serum was first incubated with glycan unconjugated beads at 37°C for 1 hr, and the unbound fraction was transferred to glycan immobilized beads. Each glycan bead (20 mg) and serum (100 μl) was incubated at 37°C for 1 hr, and the supernatant was discarded. After mild washing with PBS buffer three times, the bound proteins were eluted from the beads by elution buffer (0.2 M glycine-HCl [pH 2.2]) for 10 min and neutralized by neutralizing buffer (1 M Tris[hydroxymethyl]aminomethane-HCl [pH 9.1]). The eluted proteins were denatured at 90°C for 10 min. After cooling at room temperature, tryptic digestion was proceeded by adding 10 μl of trypsin (1 μg $μl^{-1}$, sequencing grade, Promega, Madison, WI) into each sample, which was then incubated at 37°C for 16 hr. The tryptic digested peptides were dried and dissolved by ultrapure water for further LTQ mass spectrometric analysis process.

### LTQ ESI-MS/MS Analysis

The mass spectrometric analysis of ESI-MS/MS was performed on LTQ Orbitrap hybrid Fourier Transform Mass Spectrometer (Thermo Electron Corp, USA) with nanospray source in positive ion mode at the spray voltage of 1.50 kV. The heated capillary was maintained at a temperature of 200°C. For the MS/MS fragmentation, 35% of normalized collision energy and 2 Da of isolation width were used. Five scan events were performed. The first full scan with LTQ was followed by the second full scan with FT orbitrap, and then three data-dependent MS/MS analyses were performed. The spray tip for nanospray ion source was made following the method of Gatlin et al. (1998) with P-2000 laser puller (Shutter Instrument, Novato, CA, USA) to create a 5 micron tip. Samples were infused to C18 (Agilent, USA)-packed spray tip by using homemade high-pressure bomb with a pressure of 1 MPa of nitrogen gas. The loaded sample was eluted with gradient from 0% B buffer to 100% B buffer at a flow rate of 0.3 μl $min^{-1}$ by using Ultimate 3000 2D-nano LC (LC packing, Netherland). Buffer A was prepared with water/acetonitrile (98/2 [v/v]) and 0.1% formic acid, and buffer B was prepared with water/acetonitrile (20/80 [v/v]) and

0.1% formic acid. The maximum ion collection time was set to 10 ms, and three microscans were averaged per scan.

## SUPPLEMENTAL DATA

Supplement Data include overall immobilization procedure of glycans on to beads, MALDI spectra of coupling between N,N′,N″,N‴-tetraacetylchitotetraose ($GlcNAc_4$) and 4-hydrazinobenzoic acid, MALDI spectrum and mass list of coupling between *N*-glycans from chicken ovalbumin gradeV and 4-hydrazinobenzoic acid, exoglycosidase digestion of pig kidney *N*-glycans, and MS/MS spectra of the single peptide-based identified CD40 ligand and Chemokine (C-X-C motif) ligand 2 annotated with masses as well as fragment assignments, and can be found with this article online at http://www.chembiol.com/cgi/content/full/15/3/215/DC1/.

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## REFERENCES

Adams, E.W., Ratner, D.M., Bokesch, H.R., McMahon, J.B., O'Keefe, B.R., and Seeberger, P.H. (2004). Oligosaccharide and glycoprotein microarrays as tools in HIV glycobiology; glycan-dependent gp120/protein interactions. Chem. Biol. *11*, 875–881.

Baldwin, W.M., Ota, H., and Rodriguez, E.R. (2003). Complement in transplant rejection: diagnostic and mechanistic considerations. Springer Semin. Immunopathol. *25*, 181–197.

Blixt, O., Head, S., Mondala, T., Scanlan, C., Huflejt, M.E., Alvarez, R., Bryan, M.C., Fazio, F., Calarese, D., Stevens, J., et al. (2004). Printed covalent glycan array for ligand profiling of diverse glycan binding proteins. Proc. Natl. Acad. Sci. USA *101*, 17033–17038.

Collins, B.E., and Paulson, J.C. (2004). Cell surface biology mediated by low affinity multivalent protein-glycan interactions. Curr. Opin. Chem. Biol. *8*, 617–625.

Fazio, F., Bryan, M.C., Blixt, O., Paulson, J.C., and Wong, C.H. (2002). Synthesis of sugar arrays in microtiter plate. J. Am. Chem. Soc. *124*, 14397–14402.

Gatlin, C.L., Kleemann, G.R., Hays, L.G., Link, A.J., and Yates, J.R., 3rd. (1998). Protein identification at the low femtomole level from silver-stained gels using a new fritless electrospray interface for liquid chromatography-microspray and nanospray mass spectrometry. Anal. Biochem. *263*, 93–101.

Grewal, I.S., and Flavell, R.A. (1996). The role of CD40 ligand in costimulation and T-cell activation. Immunol. Rev. *153*, 85–106.

Grun, C.H., van Vliet, S.J., Schiphorst, W.E., Bank, C.M., Meyer, S., van Die, I., and van Kooyk, Y. (2006). One-step biotinylation procedure for carbohydrates to study carbohydrate-protein interactions. Anal. Biochem. *354*, 54–63.

Harvey, D.J., Wing, D.R., Kuster, B., and Wilson, I.B. (2000). Composition of N-linked carbohydrates from ovalbumin and co-purified glycoproteins. J. Am. Soc. Mass Spectrom. *11*, 564–571.

Houseman, B.T., and Mrksich, M. (2002). Carbohydrate arrays for the evaluation of protein binding and enzymatic modification. Chem. Biol. *9*, 443–454.

Kaiser, E., Colescott, R.L., Bossinger, C.D., and Cook, P.I. (1970). Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. Anal. Biochem. *34*, 595–598.

Kim, H., Cho, J.K., Chung, W.J., and Lee, Y.S. (2004). Core-shell-type resins for solid-phase peptide synthesis: comparison with gel-type resins in solid-phase photolytic cleavage reaction. Org. Lett. *6*, 3273–3276.

Kim, D.H., Lee, H.Y., Kim, H., Kim, H., Lee, Y.S., and Park, S.B. (2006a). Quantitative evaluation of HiCore resin for the nonspecific binding of proteins by on-bead colorimetric assay. J. Comb. Chem. *8*, 280–285.

Kim, Y.G., Kim, S.Y., Hur, Y.M., Joo, H.S., Chung, J., Lee, D.S., Royle, L., Rudd, P.M., Dwek, R.A., Harvey, D.J., et al. (2006b). The identification and characterization of xenoantigenic nonhuman carbohydrate sequences in membrane proteins from porcine kidney. Proteomics *6*, 1133–1142.

Kim, Y.G., Shin, D.S., Kim, E.M., Park, H.Y., Lee, C.S., Kim, J.H., Lee, B.S., Lee, Y.S., and Kim, B.G. (2007). High-throughput identification of substrate specificity for protein kinase by using an improved one-bead-one-compound library approach. Angew. Chem. Int. Ed. Engl. *46*, 5408–5411.

Kuwaki, K., Tseng, Y.L., Dor, F.J., Shimizu, A., Houser, S.L., Sanderson, T.M., Lancos, C.J., Prabharasuth, D.D., Cheng, J., Moran, K., et al. (2005). Heart transplantation in baboons using α1,3-galactosyltransferase gene-knockout pigs as donors: initial experience. Nat. Med. *11*, 29–31.

Lee, M.R., and Shin, I. (2005). Facile preparation of carbohydrate microarrays by site-specific, covalent immobilization of unmodified carbohydrates on hydrazide-coated glass slides. Org. Lett. *7*, 4269–4272.

Lee, T.K., Ryoo, S.J., and Lee, Y.S. (2007). A new method for the preparation of 2-chlorotrityl resin and its application to solid-phase peptide synthesis. Tetrahedron Lett. *48*, 389–391.

Nishimura, S., Niikura, K., Kurogochi, M., Matsushita, T., Fumoto, M., Hinou, H., Kamitani, R., Nakagawa, H., Deguchi, K., Miura, N., et al. (2004). High-throughput protein glycomics: combined use of chemoselective glycoblotting and MALDI-TOF/TOF mass spectrometry. Angew. Chem. Int. Ed. Engl. *44*, 91–96.

Oriol, R., Ye, Y., Koren, E., and Cooper, D.K. (1993). Carbohydrate antigens of pig tissues reacting with human natural antibodies as potential targets for hyperacute vascular rejection in pig-to-man organ xenotransplantation. Transplantation *56*, 1433–1442.

Park, S., and Shin, I. (2002). Fabrication of carbohydrate chips for studying protein-carbohydrate interactions. Angew. Chem. Int. Ed. Engl. *41*, 3180–3182.

Paulson, J.C., Blixt, O., and Collins, B.E. (2006). Sweet spots in functional glycomics. Nat. Chem. Biol. *2*, 238–248.

Ratner, D.M., Adams, E.W., Su, J., O'Keefe, B.R., Mrksich, M., and Seeberger, P.H. (2004). Probing protein-carbohydrate interactions with microarrays of synthetic oligosaccharides. ChemBioChem *5*, 379–382.

Renaudet, O., and Dumy, P. (2005). Synthesis of multitopic neoglycopeptides displaying recognition and detection motifs. Bioorg. Med. Chem. Lett. *15*, 3619–3622.

Renaudet, O., and Dumy, P. (2006). On-bead synthesis and binding assay of chemoselectively template-assembled multivalent neoglycopeptides. Org. Biomol. Chem. *4*, 2628–2636.

Robson, S.C., Cooper, D.K., and d'Apice, A.J. (2000). Disordered regulation of coagulation and platelet activation in xenotransplantation. Xenotransplantation *7*, 166–176.

Rood, P.P., Hara, H., Ezzelarab, M., Busch, J., Zhu, X., Ibrahim, Z., Ball, S., Ayares, D., Awwad, M., and Cooper, D.K. (2005). Preformed antibodies to alpha1,3-galactosyltransferase gene-knockout (GT-KO) pig cells in humans, baboons, and monkeys: implications for xenotransplantation. Transplant. Proc. *37*, 3514–3515.

Rudd, P.M., Elliott, T., Cresswell, P., Wilson, I.A., and Dwek, R.A. (2001). Glycosylation and the immune system. Science *291*, 2370–2376.

Setcavage, T.M., and Kim, Y.B. (1976). Variability of the immunological state of germfree colostrum-deprived Minnesota miniature piglets. Infect. Immun. *13*, 600–607.

Taylor, M.E., and Drickamer, K. (2003). Introduction to Glycobiology (Oxford, New York: Oxford University Press).

Tobias, P.S., and Ulevitch, R.J. (1993). Lipopolysaccharide binding protein and CD14 in LPS dependent macrophage activation. Immunobiology *187*, 227–232.

Uematsu, R., Furukawa, J., Nakagawa, H., Shinohara, Y., Deguchi, K., Monde, K., and Nishimura, S. (2005). High throughput quantitative glycomics and

 

glycoform-focused proteomics of murine dermis and epidermis. Mol. Cell. Proteomics *4*, 1977–1989.

van Kooyk, Y., and Geijtenbeek, T.B. (2002). A novel adhesion pathway that regulates dendritic cell trafficking and T cell interactions. Immunol. Rev. *186*, 47–56.

Varki, A. (1999). Essentials of Glycobiology (New York: Cold Spring Harber Laboratory Press).

Xia, B., Kawar, Z.S., Ju, T., Alvarez, R.A., Sachdev, G.P., and Cummings, R.D. (2005). Versatile fluorescent derivatization of glycans for glycomic analysis. Nat. Methods *2*, 845–850.

  