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**A sulfated glycosaminoglycan array for molecular interactions between
glycosaminoglycans and growth factors or anti-glycosaminoglycan antibodies**

Short title

Sulfated GAG array for interaction analyses

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Abstract

Glycosaminoglycans (GAGs) take part in numerous biological processes by binding to protein molecules and functionally regulating protein-ligand interactions; therefore, molecular interactions of GAGs have been studied by several methods including surface plasmon resonance, enzyme-linked immunosorbent assays, and GAG microarrays. To achieve rapid, sensitive and high-throughput screening of GAG interactions, we have developed a novel microarray, in which GAGs including chondroitin sulfate, heparan sulfate, and heparin were immobilized. The microarray is made from cyclic polyolefin substrate coated with metacrylate polymers, which have phospholipid groups as side chains. The polymer has aminooxy groups also, which specifically react with aldehyde groups at the reducing termini of GAG chains, whereas the phospholipid groups prevent non-specific adsorption of proteins. Thus, minute amounts of GAGs can be chemically immobilized on the surface with low non-specific binding of proteins. Using this array, interactions between GAGs and antibodies against chondroitin or heparan sulfate and heparin-binding growth factors were examined. The results were in agreement with previously reported specificities, suggesting that the GAG array is useful for high-throughput interaction analyses between GAGs and functional proteins in miniscule amounts, and can be applied to both basic studies of GAGs and the development of diagnostic methods for metabolic diseases involving GAGs.

Keywords: Antibodies; Glycosaminoglycans; Growth factors; Microarray; Molecular interactions

Introduction

Glycosaminoglycans (GAGs¹) are very long linear polysaccharides that are present in almost all tissues in the animal kingdom, but are not found in plants. GAGs are extremely diverse in molecular weight and the degree and position of sulfation, among other properties. On the basis of their diverse structure, GAGs are involved in various biological processes such as cell proliferation, cell differentiation, cancer metastasis, viral infection, nerve regeneration and differentiation of stem cells through intermolecular interactions with different functional proteins. Therefore, it is important to investigate the molecular mechanisms of interactions between GAGs and specific proteins under various physiological and pathological conditions.

To analyze the interactions of GAGs, by ELISA for example, the target GAGs are immobilized on microtiter plates and allowed to react with a specimen. Generally, however, these analyses are time-consuming and need large sample volumes; therefore, rapid high-throughput analytical techniques with high sensitivity for small amounts of samples are desirable.

As a high-throughput analysis technology, microarray has become popular in recent years. In microarray analysis, a number of ligands are immobilized on a substrate, enabling the analysis of multiple interactions at one time. Microarray is superior in that a comprehensive analysis of an interaction can be carried out at one time with minute amounts of samples. In addition, microarrays can be used for various materials, although development has mainly progressed in DNA and protein microarray so far.

In preparing a microarray, highly reactive functional groups such as amino groups and thiol groups are generally used to immobilize the ligand. However, sugar chains have many hydroxyl groups that are less reactive than their functional groups;

thus, it is difficult to ensure that only the specific hydroxyl group is selectively immobilized. Many of the sugar chain microarrays reported so far have been generated using an immobilization method similar to established microarrays, such as DNA and protein microarrays. That is, linkers with a reactive functional group are introduced at the reducing end of a sugar chain, which is then chemically immobilized on the activated glass substrate.

For example, Consortium for Functional Glycomics adopted a method for glycans that were aminoethylated at the reducing end for immobilization on a glass surface activated with *N*-hydroxysuccinimide. This array is called the “Printed glycan array” [1]. Similarly, Shin et al. reported a method of introducing a maleimide group to glycans via an alkyl chain spacer and bonding with a substrate presenting a thiol group [2]. Furthermore, Wong et al. constructed a microarray by reaction of an azide group introduced via a spacer at the reducing end of glycans with an alkyne on the surface of substrate [3]. In each of these methods, however, it is necessary to introduce linkers to all glycans; thus, they require considerable man-hours and cost. Furthermore, as the numbers of glycans increase, it becomes more difficult to obtain the glycan library itself that is required to prepare a microarray.

In this study, we have combined surface treatment techniques [4, 5] and a “Glycoblottting method” [6] to develop a novel plastic GAG microarray, in which free GAG chains are immobilized easily and efficiently by trapping their reducing end. In addition, we demonstrate that this microarray can be applied to the analysis of interactions between GAGs and growth factors or antibodies.

In the Glycoblottting method [6], glycans released from glycoproteins and glycolipids have a hemiacetal group equivalent to an aldehyde group at their reducing

end in contrast to biomolecules such as nucleotides, peptides, amino acids, and lipids, which have no aldehyde group. Therefore, as shown in Fig.1, if the hemiacetal group can be selectively captured, then glycans can be recovered specifically, differentiating them chemically from other biomolecules. For example, Furukawa et al. synthesized functional polymers with hydrazide groups (hydrazide beads) by suspension polymerization and made a specific collection of glycans [6].

Using the above-described method, a special treatment was applied to the surface of a plastic substrate (polyolephin). Aminoxy groups were introduced because they bind more strongly to aldehyde groups present at the reducing termini of the glycans than hydrazide groups [6]. Thus, after contacting the substrate surface, glycans with a reducing end could be easily immobilized by applying heat (Fig. 2).

Furthermore, another feature incorporated in the surface treatment of the substrate is the suppression of non-specific adsorption of biomolecules such as proteins. When analyzing the interaction, non-specific adsorption of the analyte to the substrate generates background noise and results in a decrease in detection sensitivity. Therefore, a blocking treatment is generally used to suppress non-specific adsorption by a solution such as BSA or skim milk; in such an approach, however, verification of the blocking solution may be needed to check that it responds sufficiently.

As shown in Supplementary Fig. S1, the coating with methacrylic polymer prevented efficiently non-specific adsorption of hydrophobic, basic or glycoproteins. The polymer that we have developed contains phospholipid groups, which are superior at preventing non-specific adsorption of proteins as compared with general blocking solution. In this study a glass-like substrate made of cyclic polyolefin was molded into a slide by Sumitomo Bakelite as the base of the GAG microarray, and was coated by

methacrylic polymer [8, 9] for immobilization of glycans. As a result, surface treatment by this polymer has enabled the detection of interactions via glycans at a high S/N ratio without a blocking step. Generally, the interaction between a glycan and a protein is much weaker than that between an antigen and antibody reaction; thus, suppressing the background noise is especially significant in the analysis of interactions of glycans. The utility of this GAG microarray as a tool for interaction analysis is described below.

Materials and Methods

Materials

Chondroitin sulfate A (CS-A), CS-B (dermatan sulfate), CS-C, CS-D, CS-E, heparan sulfate (derived from bovine kidney; HS), heparin (derived from pig intestines; Hep), and chondroitin (desulfated CS-A; Chn) were purchased from Seikagaku Corp. (Tokyo, Japan). The hyaluronan oligosaccharide preparation (HA-oligo; molecular mass of less than 10,000 Da) was obtained from Kewpie Corp. (Tokyo, Japan). Cy3-maltose, which was used as a grid marker in preparing the GAG microarray, was synthesized by NARD Institute, Ltd. (Hyogo, Japan).

Recombinant human midkine (hMK), pleiotrophin (hPTN), FGF-1 (acidic FGF, aa 16-155; hFGF-1), VEGF₁₆₅ (hVEGF), and HGF (hHGF) were purchased from R&D Systems (Minneapolis, MN, USA). Recombinant human FGF-2 (basic FGF; hFGF-2) was purchased from PEPROTECH (Rocky Hill, NJ, USA).

Anti-CS monoclonal antibody (anti-CS mAb; clone CS-56), anti-CS mAb (clone MO-225), anti-HS mAb (clone F58-10E4) were purchased from Seikagaku Corp. Anti-hMK mAb, anti-hPTN mAb, and anti-hFGF-1 mAb were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-FGF-2 mAb was purchased from

PEPROTECH. Anti-hVEGF mAb and anti-hHGF mAb were purchased from R&D Systems.

Cy3-AffiniPure F(ab')₂ fragment of goat anti-mouse IgM was purchased from Jackson ImmunoResearch (West Grove, PA, USA). Cy3-labeled anti-mouse IgG was purchased from GE Healthcare UK Ltd. (Little Chalfont, UK). In addition, mouse monoclonal Ab WF6 (mAb WF6) was prepared by a previously described method [10].

Preparation of the GAG microarray

A glass-like substrate made of cyclic polyolefin was molded into a slide by Sumitomo Bakelite as the base of the GAG microarray. The size of the slide was 75-mm long, 25-mm wide and 1-mm thick, and was coated by methacrylic polymer [8, 9] for immobilization of glycan. The slide was then immersed in 2 M HCl for 4 h at 37 °C, so that aminooxy groups were present on the surface of the substrate. The slide was washed twice with water, and dried by centrifugation.

Nine kinds of GAG were immobilized on the substrate: CS-A, CS-B, CS-C, CS-D, CS-E, HS, Hep, Chn (desulfated CS-A), and HA-oligo. They were prepared at a concentration of 0.9 mg/mL in spotting buffer (100 mM acetate buffer, pH 5.0, containing 0.01% Triton X-100 and 0.01% polyvinyl alcohol [M_w = 1,500] at a final concentration). Next, the GAG solutions were spotted (n = 3) on the substrate using a BioChip Arrayer (Filgen, Aichi, Japan) [11]. About 1 ~ 10 nL of GAG solution was applied per spot, corresponding to a GAG weight of 0.9 ~ 9 ng. This meant that, for a GAG with an average molecular mass of 20,000 Da, the amount on the array would be 0.05 ~ 0.5 pmol. After spotting, the substrate was placed in an oven for 1 h at 80 °C to immobilize the GAGs. After the reaction, it was washed once with water, and then

immersed in an aqueous solution of 10 mg/mL succinic anhydride for 1 h at room temperature to cap unreacted aminoxy groups. Finally, it was washed twice with water, and dried by centrifugation.

Assay with commercial reagents

Binding assays for anti-GAG antibodies

Binding assays for anti-GAG antibodies were performed as follows: (1) A hybridization cover (Sumitomo Bakelite, Tokyo, Japan) was mounted onto a slide, and defined concentrations of anti-GAG antibody in a reaction buffer (50 mM Tris-HCl, pH 7.4, containing 100 mM NaCl, 1 mM CaCl₂, 1 mM MnCl₂, 1 mM MgCl₂, 0.05% Tween 20) (70 µL) were added. (2) The slide was incubated at room temperature for 2 h. (3) The hybridization cover was removed and the slide was washed with washing buffer (50 mM Tris-HCl, pH 7.4, containing 100 mM NaCl, 1 mM CaCl₂, 1 mM MnCl₂, 1 mM MgCl₂) for 2 min (once) and twice with water for 1 min. (4) The slide was dried by centrifugation (2,000 rpm, 2 min, room temperature). (5) A new hybridization cover was mounted onto the slide and 5 µg/mL Cy3-labeled goat anti-mouse IgM (70 µL) was added. (6) The slide was incubated at room temperature for 1 h. (7) The hybridization cover was removed, and the slide was washed with washing buffer for 2 min (once) and twice with water for 1 min. (8) The slide was dried by centrifugation (2,000 rpm, 2 min, room temperature), and the fluorescent intensity of the spots was measured.

Binding assays for growth factors

Binding assays for growth factors were performed as described for anti-GAG antibodies except that 5 µg/mL mouse anti-human growth factor antibody (70 µL) was

used instead of anti-GAG antibody, and 5 $\mu\text{g/mL}$ Cy3-labeled goat anti-mouse IgG (70 μL) was used instead of Cy3-labeled goat anti-mouse IgM.

Results

Interaction with commercially available antibodies and growth factors using GAG microarray

In order to investigate the performance of the newly developed GAG microarray, we examined its substrate specificity for commercially available antibodies (3 kinds) and growth factors (6 kinds). As described in the Materials and Methods, the amount of GAG on the microarray was 0.05 ~ 0.5 pmol. The concentrations of antibodies and growth factors were varied as described in the legend of Table 1. Interactions between the antibodies or growth factors and GAGs are indicated in Table 1 (observed interactions are described as “+”), and the main results for each sample are summarized below.

Antibodies

Anti-CS mAb (clone CS-56) reacted with CS-A, CS-C and CS-D (Fig. 3-A). The detection limit varied between 1 and 100 ng/mL of antibody for CS-C and CS-D, and between 0.1 and 10 $\mu\text{g/mL}$ for CS-A (Fig. 4-A). Anti-CS mAb (clone MO-225) reacted with CS-C, CS-D and CS-E (Fig. 3-B). The detection limit varied between 0.1 and 1 $\mu\text{g/mL}$ of antibody for CS-C and CS-D, and between 1 and 10 $\mu\text{g/mL}$ for CS-E (Fig. 4-B). Anti-HS mAb (clone F58-10E4) reacted with HS and Hep (Fig. 3-C) with detection limits of 0.1 ~ 1 $\mu\text{g/mL}$ of antibody for both GAGs (Fig. 4-C).

Growth factors

The growth factor hMK reacted with CS-E and Hep (Fig. 5-A) with a detection limit of 0.4 ~ 2 $\mu\text{g/mL}$ of growth factor for Hep, and 2 ~ 10 $\mu\text{g/mL}$ for CS-E (Fig. 6-A). For hPTN, interactions were observed with CS-E and Hep (Fig. 5-B). In this assay, signals were observed for Hep even at a concentration of 0.4 $\mu\text{g/mL}$, which was the minimum growth factor concentration tested. By contrast, the detection limit for CS-E was 10 ~ 50 $\mu\text{g/mL}$ of growth factor.

hFGF-1 reacted with CS-E and Hep (Fig. 5-C). The detection limit varied between 1 and 10 $\mu\text{g/mL}$ of growth factor for Hep and between 10 ~ 50 $\mu\text{g/mL}$ for CS-E (Fig. 6-B). The growth factor hFGF-2 reacted with CS-B, CS-E, HS and Hep (Fig. 5-D). In this assay, the detection limit varied between 0.01 and 0.1 $\mu\text{g/mL}$ of growth factor for Hep, and between 0.1 and 1 $\mu\text{g/mL}$ for CS-B, CS-E and HS. For CS-A, signals were observed at a growth factor concentration of 10 $\mu\text{g/mL}$, which was the maximum concentration tested, but it was judged that this was a nonspecific reaction.

hVEGF reacted with CS-E and Hep (Fig. 5-E) with detection limits of 0.4 ~ 2 $\mu\text{g/mL}$ of growth factor for Hep and 2 ~ 10 $\mu\text{g/mL}$ for CS-E (Fig. 6-C). For hHGF, interactions were observed with CS-B, CS-E, HS and Hep (Fig. 5-F). In this assay, signals were observed for Hep even at a growth factor concentration of 0.4 $\mu\text{g/mL}$, which was the minimum concentration tested. In addition, signal levels were higher at 10 $\mu\text{g/mL}$ than at 50 $\mu\text{g/mL}$. For the other GAGs that interacted, the detection limit was around 10 $\mu\text{g/mL}$ of growth factor.

Interaction with mAb WF6 using GAG microarray

For mAb WF6, which was recently generated for screening of diseases including osteoarthritis, rheumatoid arthritis and ovarian cancer [10, 12], the assay was carried out on the GAG microarray in the same manner as described above. Specific reactions with CS-C and CS-D were observed for mAb WF6 as shown in Fig. 7(A). In addition, these interactions demonstrated a concentration dependence as shown in Fig. 7(B).

Discussion

Growth factors function in the regulation of various physiological and cytological processes, and act as signals between cells by specifically binding to a receptor protein on the surface of a target cell. In general, most growth factors have specific interactions with GAGs, and we therefore considered that the utility of the GAG microarray as an analytical tool would be shown if we could confirm these specific interactions on the microarray.

With regard to the antibodies tested, anti-CS mAb (clone CS-56) is specific for CS and is widely used in immunohistochemical studies of various tissues [13, 14]. It mainly recognizes CS-A and CS-C [15], and has subsequently been found to recognize sequences containing the A-D sequence [16]. Given these facts, the present results (in which it reacted with CS-A, CS-C and CS-D) are reasonable.

Similarly, anti-CS mAb (clone MO-225) is also specific for CS and is widely used in immunohistochemical studies of various tissues. It recognizes the determinant on the CS (dual structure of D-GlcA-2-O-sulfate(β 1-3)GalNAc-6-O-sulfate: D-unit) [17], and has also been found to recognize sequences containing the A-D sequence [16]. Therefore, the present results (in which it reacted with CS-C, CS-D and CS-E) are also

reasonable. Anti-HS mAb (clone F58-10E4) recognizes GlcNS in HS [18, 19]. It does not bind to HA, CS, CS-B, or keratan sulfate. Consistent with this, clone F58-10E4 reacted with HS and Hep in the present study.

Regarding the growth factors tested, hMK is an ~13-kDa protein with an abundance of cysteine and basic amino acids, and consists of two kringle domains at the C-terminus and N-terminus. It is mainly the C-terminal kringle domain that plays a role in the activity of hMK, and this domain contains two Hep-binding sites [20-22]. hMK binds strongly to regions of over-sulfated HS, as well as the tri-sulfated structure and E-type structure of CS [23-26]. Given these observations, the present results (in which hMK reacted with CS-E and Hep) are reasonable. hPTN is a protein forms a unique gene family with hMK, and about 50% of amino acid sequence is shared between hPTN and hMK [20]. In keeping with this, hPTN reacted with CS-E and Hep on the present GAG microarray.

The FGF family comprises polypeptides of 17 ~ 26 kDa, such as FGF-1 and FGF-2; at least 22 kinds of FGFs have been confirmed in human. It is considered that most FGF proteins that are secreted outside the cell bind HS chains that exist in the extracellular matrix [27, 28]. FGF-1 is an ~17-kDa Hep-binding growth factor, and the presence of many 6-*O*-sulfate groups is important to interact specifically with Hep/HS. By contrast, FGF-2 does not need these groups [29]. In particular, a size of more than 10 residues and a structure rich in iduronic acid(2-*O*-sulfate)-GlcNS(6-*O*-sulfate) are required for the specific interaction between FGF-1 and HS [30]. In addition, FGF-1 does not bind to CS-E [31]. In the present results, however, the observed binding between hFGF-1 and CS-E differs from previous reports and will be the subject of future investigation. Other results (in which hFGF-1 reacted with Hep) are reasonable.

FGF-2 is an ~18-kDa Hep-binding growth factor of 154 amino acids [32]. FGF-2 binds HS as strong as Hep if the HS has more than 10 sugar residues and has a structure rich in iduronic acid(2-O-sulfate)-GlcNS [33, 34]. In addition, FGF-2 binds to CS-B containing iduronic acid, and highly sulfated CS-E [31, 35]. Therefore, the present results (in which FGF-2 reacted with CE-B, CS-E, HS and Hep) are sound.

VEGF is an ~22-kDa Hep-binding growth factor, which has a dimer structure consisting of subunits and a Hep-binding site in the C-terminus. VEGF has subfamilies termed A to E, and it binds one of 3 kinds of VEGF receptors (VEGF-R1, -R2, -R3) to transfer its signal [36]. VEGF exists as an isoform of 5 different sizes (VEGF₁₂₁, VEGF₁₄₅, VEGF₁₆₅, VEGF₁₈₉, VEGF₂₀₆, where the subscript indicates the number of constituent amino acids) [37]. VEGF-A₁₆₅, which exists most in vivo, binds to VEGF-R1 and -R2, but not to VEGF-R3. It is known that VEGF specifically binds to CS-E [38]. In keeping with these observations, hVEGF reacted with CS-E and Hep in the present study. HGF is a Hep-binding growth factor. It is heterodimer, in which an ~69-kDa α -chain (heavy chain) and ~34-kDa β -chain (light chain) are bound by an S-S bond [39]. It binds to CS-B [40, 41]. In the present results, hHGF was found to react with CS-B, CS-E, HS and Hep.

Because the above data confirmed that the GAG microarray that we developed was able to detect interactions properly, we examined the specific interaction of an antibody that is commercially unavailable. WF6 is a useful antibody that can be used to diagnose ovarian cancer, osteoarthritis, and rheumatoid arthritis. It has reaction specificity with CS-C and CS-D, and it recognizes their special 8-sugar sequence [42]. The present results, in which WF6 interacted with CS-C and CS-D, were consistent with previously published characteristics [42]. Thus, these results collectively showed that

the GAG microarray that we have developed can be used as a tool for the analysis of the interactions between GAGs and protein.

As described above, we immobilized GAGs chemically on the substrate by first applying a surface treatment (presenting aminooxy groups) and then applying the Glycoblotting method. This means that we achieved not only the specific immobilization of glycans such as GAGs but also position-selective immobilization by binding GAGs at their reducing end. In addition, because there was no need to make modifications to the GAGs, this method has many benefits. For example, it would be possible to immobilize non-modified GAGs extracted from a biological sample after treatment with weak alkali such as 0.5 M LiOH to release GAGs from the core protein or peptide [43, 44]. In addition, by making this microarray, the interaction analysis could be carried out with a very small amount of GAGs and proteins. Because GAGs extracted from natural products are often available in only tiny amounts or are expensive, it is a considerable advantage that a small quantity of sample is used.

The present microarray is based on a plastic substrate. Plastic has the advantage that it can be processed into various shapes such as a slide glasses, microplates, and microfluidic formats with a fine flow channel. As a result, a plastic substrate can be provided in a format suitable for the measurement needs and can be easily mass-produced at low cost. In the future, when use of microarrays for diagnostic purposes becomes widespread, we think that the plastic-based microarray will become mainstream because it is inexpensive and disposable.

On the basis of the present screening results, a more detailed analysis using the same type of GAGs with different degrees of sulfation and molecular weight would bring deeper knowledge of molecular biology. In the future, we will also develop such

GAG microarrays as a variation of the technique.

As mentioned above, using the newly developed GAG microarray enabled us to detect multiple interactions with a small amount of sample and without modification of the GAGs, and to expedite the interaction analysis. As a result, this microarray will contribute to future studies of the biological functions of GAGs.

Conclusions

To achieve rapid, sensitive and high-throughput screening for molecular interactions of GAGs, we have developed a novel plastic microarray that can be immobilized with various GAGs at their reducing end without modification. Using this microarray, we confirmed specific interactions between GAGs and commercially available growth factors and antibodies. Furthermore, we demonstrated the specificity toward CS-C and CS-D of an antibody (WF6) that can be applied to the diagnosis of ovarian cancer, osteoarthritis, and rheumatoid arthritis [10, 12].

This microarray will be useful for developing diagnostic methods for diseases, in which growth factors, cytokines, and/or chemokines are secreted, because only minute amounts of serum samples are required. It can be expected that this microarray will accelerate molecular biological research and drug discovery involving GAGs for a variety of pathological conditions.

Conflict of Interest

The authors declare no conflict of interest.

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Abbreviations:

GAG, glycosaminoglycan; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; S/N, signal/noise; CS, chondroitin sulfate; HS, heparan sulfate; Hep, heparin; Chn, chondroitin; HA, hyaluronan; MK, midkine; PTN, pleiotrophin; FGF, fibroblast growth factor; VEGF, vascular endothelial growth factor; HGF, hepatocyte growth factor; mAb, monoclonal antibody; Cy3, cyanine 3; Ig, immunoglobulin; Mw, molecular weight; GlcA, D-glucuronic acid; GalNAc, *N*-acetyl-D-galactosamine; GlcNS, *N*-sulfated-D-glucosamine

functional protein	immobilized GAGs								
	CS-A	CS-B	CS-C	CS-D	CS-E	HS	HP	Chn	HA-Oligo
anti-CS mAb (Clone:CS-56)	++		++	++					
anti-CS mAb (Clone:MO-225)			++	++	+				
anti-HS mAb (Clone:F58-10E4)						++	++		
hMK					++		++		
hPTN					++		++		
hFGF-1					++		++		
hFGF-2		++			++	++	++		
hVEGF					++		++		
hHGF					+	++	++		

Table 1. The reaction specificity between the GAG microarray and various commercial samples. The assays were performed as described in Materials and Methods. The amount of GAG solution (0.9 mg/mL) spotted on the microarray was 1 ~ 10 nL; therefore, the GAG weight was 0.9 ~ 9 ng. Thus, for example, for a GAG with an average molecular mass of 20,000 Da, the quantity of GAG was 0.05 ~ 0.5 pmol. The concentration of antibodies and growth factors were as follows: anti-CS mAb (clone CS-56): 10, 0.1, 0.001 µg/mL, anti-CS mAb (clone MO-225): 10, 1, 0.1, 0.01, 0.001 µg/mL, anti-HS mAb (clone F58-10E4): 10, 1, 0.1, 0.01, 0.001 µg/mL, hMK: 50, 10, 2, 0.4 µg/mL, hPTN: 50, 10, 2, 0.4 µg/mL, hFGF-1: 50, 10, 1, 0.1, 0.01, 0.001 µg/mL, hFGF-2: 10, 1, 0.1, 0.01, 0.001 µg/mL, hVEGF: 50, 10, 2, 0.4 µg/mL, hHGF: 50, 10, 2, 0.4 µg/mL.

Figure Legends

Fig. 1. Using their aldehyde group at the reducing terminus, GAGs were covalently and chemoselectively linked to the NH₂ of an aminooxy group on a solid surface via oxime bond formation under weak acidic conditions [6, 7].

Fig. 2. Patterns of a GAG microarray based on the glycoblotting method. The aminooxy group present on the surface of the substrate reacts chemically and selectively with the hemiacetal group at the reducing end of the sugar chain. 1, HA-oligo; 2, CS-A; 3, CS-B; 4, Hep; 5, CS-E; 6, HS, GlcNAc, *N*-acetyl-D-glucosamine; GalNAc, *N*-acetyl-D-galactosamine; GlcA, D-glucuronic acid; IdoA, L-iduronic acid.

Fig. 3. Images of the fluorescence scanning of slides. The slides were imaged at 10-mm resolution on a Typhoon TRIO+ variable mode imager (GE Healthcare UK Ltd., Little Chalfont, UK) with a green (532 nm) laser and a 580 BP 30 filter at a PMT voltage of 600V and normal sensitivity. Lane 1, CS-A; lane 2, CS-B; lane 3, CS-C; lane 4, CS-D; lane 5, CS-E; lane 6, HS; lane 7, Hep; lane 8, Chn; lane 9, HA-oligo; lane 10, buffer only; lane G, grid marker (Cy3-maltose).

Fig. 4. The correlation between antibody concentration and signal intensity. The slides were imaged at 10-mm resolution on a Typhoon TRIO+ variable mode imager (GE Healthcare) with a green (532 nm) laser and a 580 BP 30 filter at a PMT voltage of 600V and normal sensitivity. Lane 1, CS-A; lane 2, CS-B; lane 3, CS-C; lane 4, CS-D; lane 5, CS-E; lane 6, HS; lane 7, Hep; lane 8, Chn; lane 9, HA-oligo; lane 10, buffer only; lane G, grid marker (Cy3-maltose).

Fig. 5. Images of the fluorescence scanning of slides. The slides were imaged at 10-mm resolution on a Typhoon TRIO+ variable mode imager (GE Healthcare) with a green (532 nm) laser and a 580 BP 30 filter at a PMT voltage of 600V and normal sensitivity. Lane 1, CS-A; lane 2, CS-B; lane 3, CS-C; lane 4, CS-D; lane 5, CS-E; lane 6, HS; lane 7, Hep; lane 8, Chn; lane 9, HA-oligo; lane 10, buffer only; lane G, grid marker (Cy3-maltose).

Fig. 6. The correlation between each growth factor concentration and signal intensity. Representative examples are shown. The slides were imaged at 10-mm resolution on a Typhoon TRIO+ variable mode imager (GE Healthcare) with a green (532 nm) laser and a 580 BP 30 filter at a PMT voltage of 600V and normal sensitivity. Lane 1, CS-A; lane 2, CS-B; lane 3, CS-C; lane 4, CS-D; lane 5, CS-E; lane 6, HS; lane 7, Hep; lane 8, Chn; lane 9, HA-oligo; lane 10, buffer only; lane G, grid marker (Cy3-maltose).

Fig. 7. The specificity and concentration dependency of mAb WF6. (A) Images of the fluorescence scanning of slides. (B) The correlation between antibody concentration and signal intensity. The slides were imaged at 10-mm resolution on a Typhoon TRIO+ variable mode imager (GE Healthcare) with a green (532 nm) laser and a 580 BP 30 filter at a PMT voltage of 600V and normal sensitivity. Lane 1, CS-A; lane 2, CS-B; lane 3, CS-C; lane 4, CS-D; lane 5, CS-E; lane 6, HS; lane 7, Hep; lane 8, Chn; lane 9, HA-oligo; lane 10, buffer only; lane G, grid marker (Cy3-maltose).

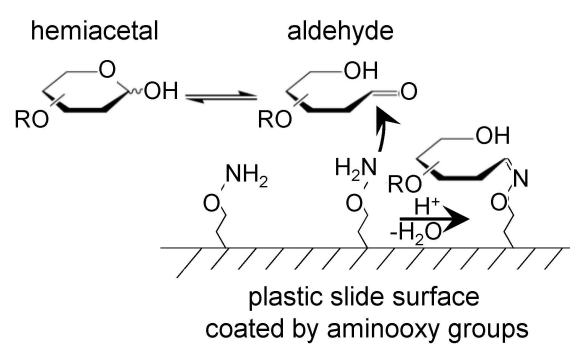


Fig. 1

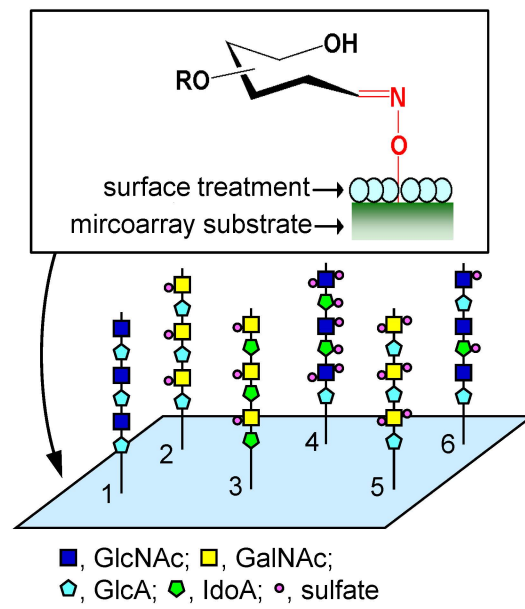
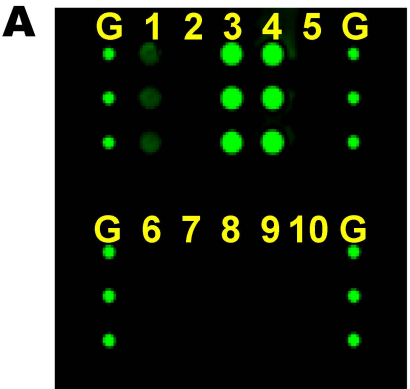
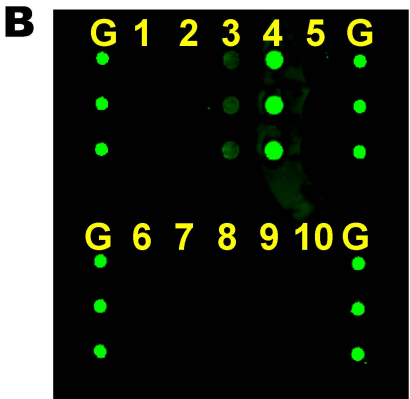


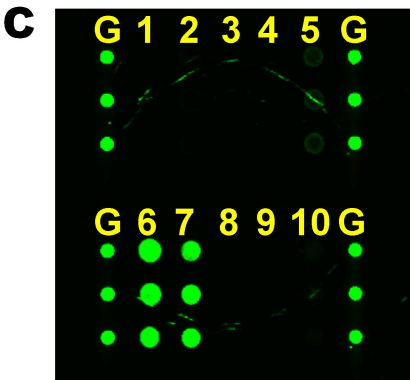
Fig. 2



Anti CS antibody (CS-56)
10 µg/mL



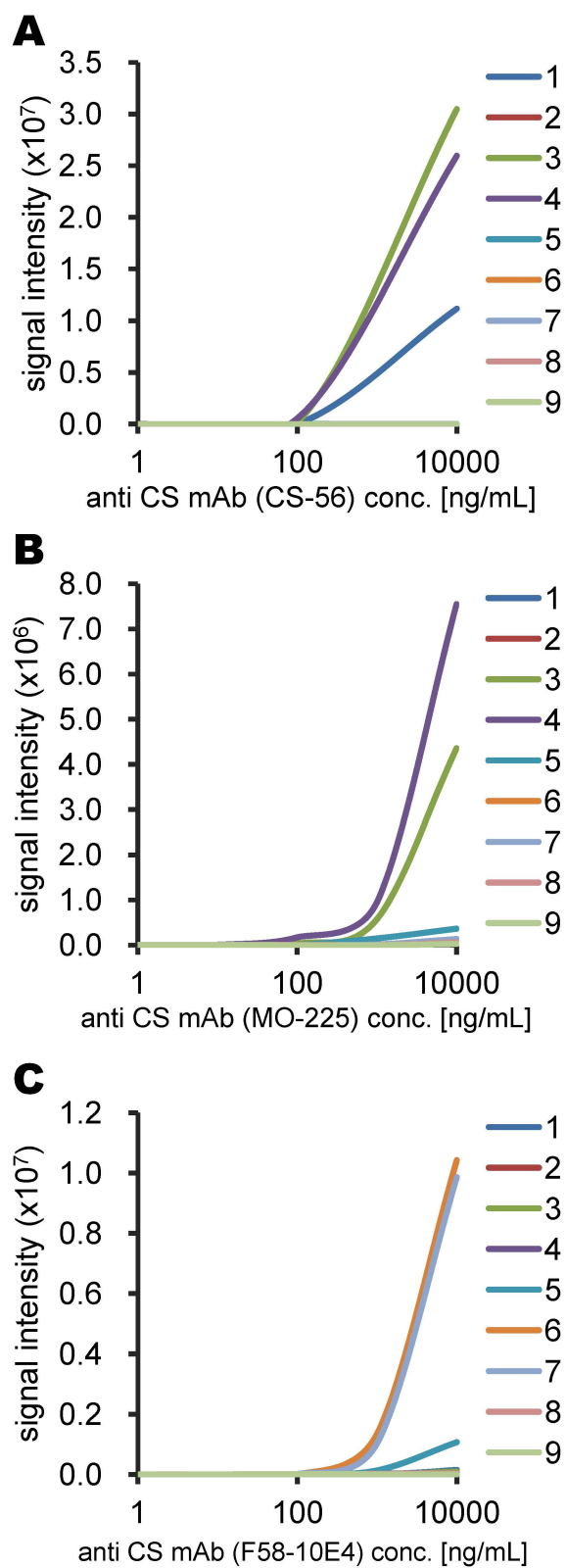
Anti CS-D antibody (MO-225)
10 µg/mL



Anti HS antibody (F58-10E4)
10 µg/mL

Fig. 3

Fig. 4



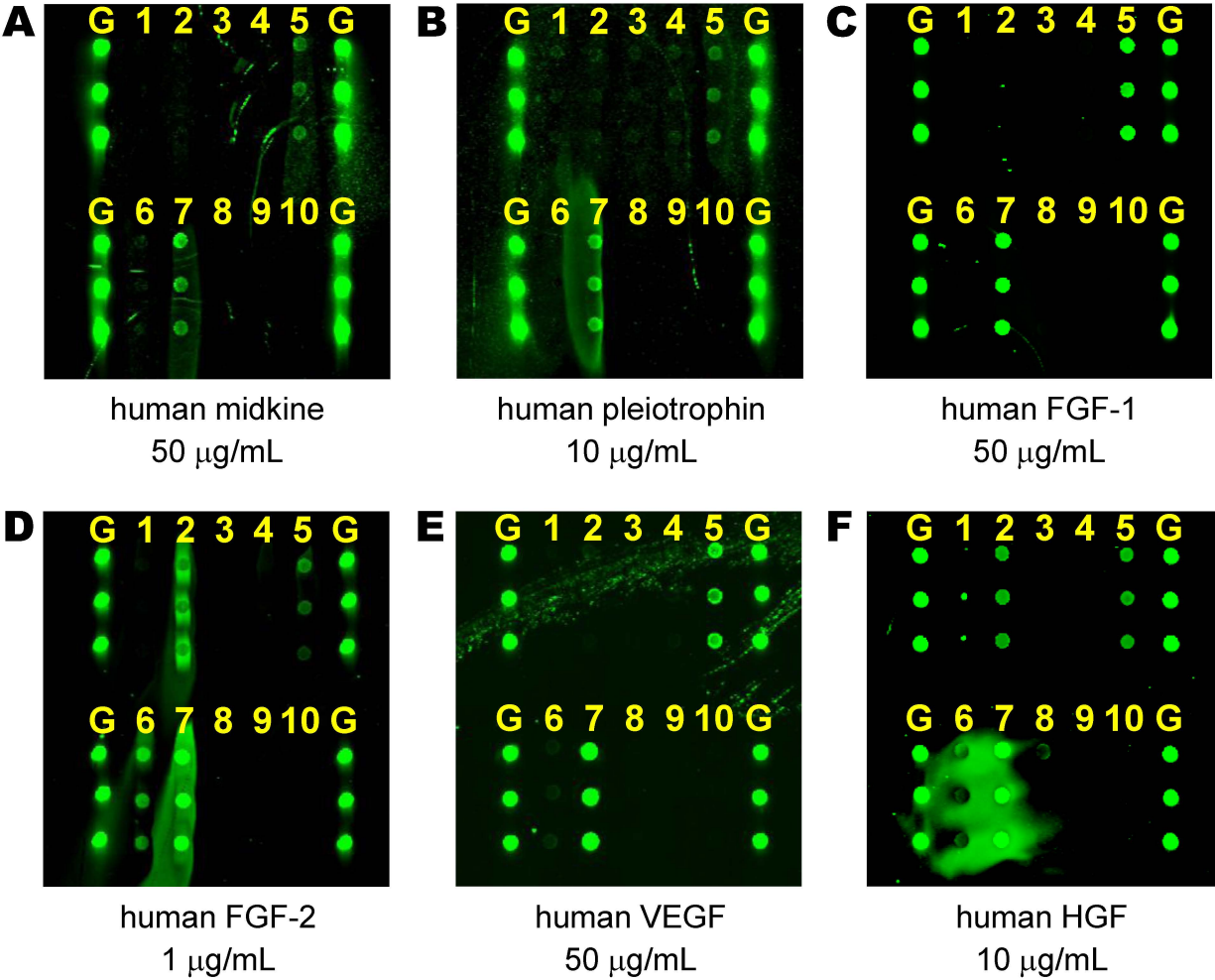
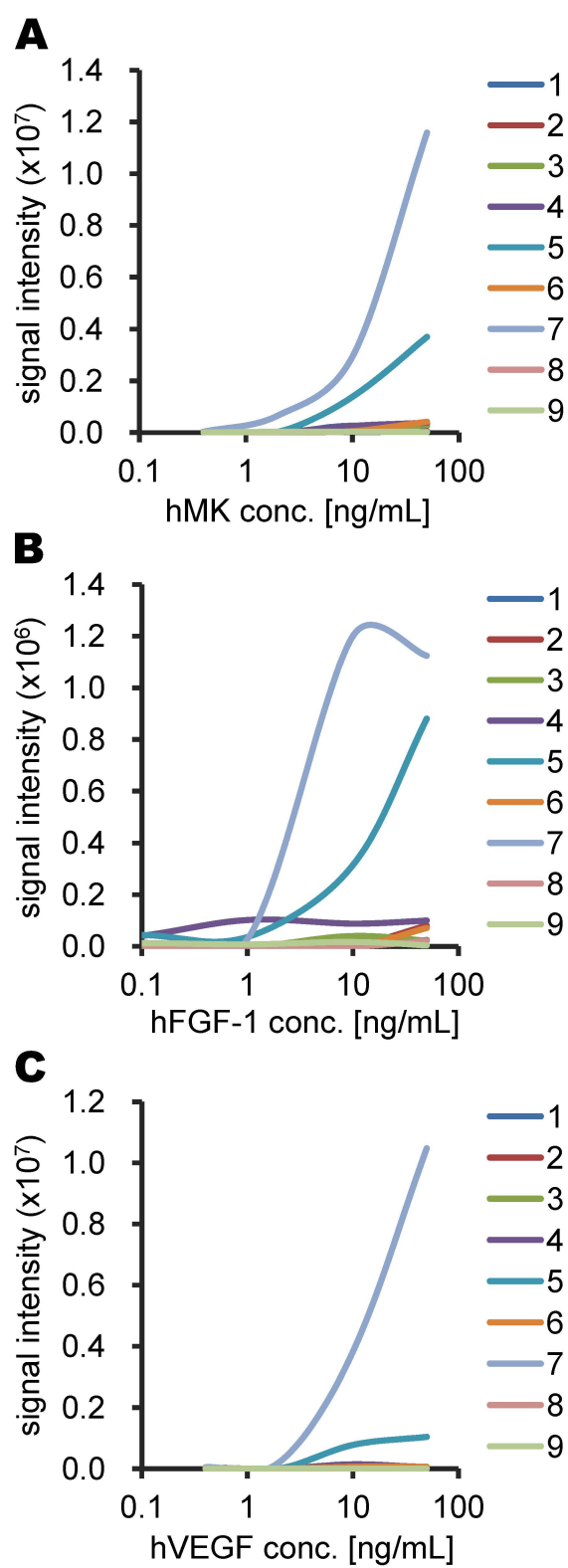


Fig. 5

Fig. 6



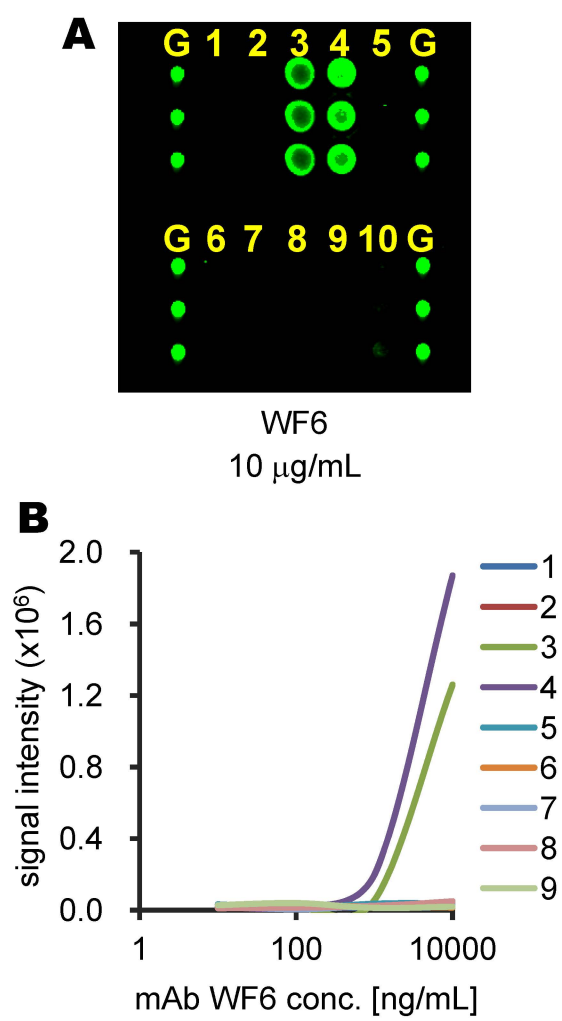


Fig. 7