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Hydrogel glycan microarrays

V.I. Dyukova [a], E.I. Dementieva [a], D.A. Zubtsov [a], O.E. Galanina [b], N.V. Bovin [b], A.Yu. Rubina [a,*]

[a] *Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, 119991 Moscow, Russia*
[b] *Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, 117997 Moscow, Russia*

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Abstract

The technology of hydrogel microchips manufacturing, which was developed previously for covalent immobilization of DNA and proteins, was applied for the preparation of glycochips and combined glyco/protein chips. Microchips consist of hydrogel drops separated with hydrophobic surface. Spacered amino-saccharides and polyacrylamide glycoconjugates were used for immobilization. Gel elements were ~1 nl in volume (150 μm in diameter and 25 μm in height), and the amount of covalently immobilized saccharide in the glycoarray was 0.4–1.7 pmol per gel element. Hydrogel glycan microchips were used for quantitative assay of antibodies against blood group antigens and assay of lectins with fluorescent detection. In all cases, only specific interaction with chip-immobilized saccharides was observed, whereas the background signal was very low. The detection limit of on-chip assays was comparable to that of the standard 96-well plate assays. Mixing of reaction solution allowed us to decrease the duration of the assays significantly: 2–3 h for incubation and development steps and 10 min for washing. A method for determination of association constants for binding of compounds with chip-immobilized ligands from the kinetics of their binding is proposed. Combined microchips containing different types of biomolecules can be designed and used for simultaneous detection of different compounds.

Keywords: Gel microchips; Glycan microarrays; Hydrogel glycochips; Ricin; Lectins; Antibodies; Combined glyco/protein chip; Carbohydrates

Biological microchips, universal multifunctional biotechnological tools, are increasingly used in modern genomic and proteomic studies to determine various compounds as well as to investigate their interactions in vitro [1–5]. A biochip is a matrix carrying up to several thousand immobilized biological probes (e.g., DNA, proteins). Most microchips are two-dimensional; that is, probes are immobilized on the surface of glass, plastic, or membrane. Three-dimensional hydrogel-based microchips, developed by Mirzabekov and his coworkers at the Engelhardt Institute of Molecular Biology of the Russian Academy of Sciences, consist of hydrogel drops several nanoliters in volume separated from each other with hydrophobic glass or plastic surface.

Immobilization of biological compounds within three-dimensional porous polymer gels occurs simultaneously with the gel formation. It involves covalent binding of biological compounds containing active groups with the components of growing polymer chains during photopolymerization or chemically initiated polymerization in the presence of a crosslinking bifunctional agent. For the immobilization of probes, such as DNA, proteins, and other biological compounds, within the volume of the polymer carrier, their active groups should be able to enter into addition or substitution reaction with fragments of the forming polymer during its photo-initiated or chemically-initiated synthesis. Amino and sulfhydryl groups can serve as active groups for in-gel immobilization. The total amount of the monomer and the crosslinker and their ratio in the gel composition can be varied to achieve optimal porosity of the gel for different assays [6,7].

* Corresponding author. Fax: +7 095 135 14 05.
E-mail address: arubina@biochip.ru (A.Yu. Rubina).

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The advantages of immobilization in gel drops over immobilization on two-dimensional surfaces for microchips are increased immobilization capacity, homogeneous water surrounding of immobilized molecules, absence of contact of immobilized molecules with hydrophobic support, and (consequently) increased stability of chip-immobilized probes. The technology for three-dimensional gel microchips manufacturing was used successfully for the preparation of both DNA [7] and protein [8] microchips. Oligonucleotide microchips are currently used for the diagnostics of drug-resistant strains of tuberculosis [9], orthopox viruses [10], and leukemia [11]. Protein hydrogel microchips are used for studies of protein–protein and protein–ligand interactions, enzymatic reactions, and different immunoassays [8,12,13]. Microchip-based immunoassays provide a basis for the development of test systems for simultaneous quantitative determination of a series of tumor-associated markers in human sera [12] and biological toxins [13], with the sensitivity of the assays comparable to or higher than standard immunological methods. The current article is a continuation of a series of articles devoted to hydrogel drop microchips [7,8,13] and describes hydrogel-based glycochips and their potential applications in diagnostics and studies of carbohydrate-binding proteins.

According to the latest studies summarized in the Swiss–Prot database, more than 50% of all known proteins are glycosylated. Cells of living organisms are covered with diverse glycans that participate in various biological processes, particularly viral entry, signal transduction, cell–cell and cell–bacteria interactions, and inflammation (for a review, see Ref. [14] and the references therein). The glycome, the entire collection of carbohydrates of a cell, is more complex than the genome or the proteome. The functions of carbohydrates are not studied nearly as well as those of proteins, and there is a significant increase in the interest for glycomics boosted by improved methods for carbohydrate synthesis and analysis [15].

The majority of modern studies in the field of functional glycomics are aimed at the creation of glycochips because biochip technology allows one to register simultaneously hundreds of protein–carbohydrate and DNA–carbohydrate interactions using a minimal amount of analyzed compound. Several approaches to the manufacturing of glycan microarrays through noncovalent or covalent immobilization of carbohydrates have been described previously. The technologies developed for the manufacturing of DNA microarrays are also used for preparation of glycan arrays, and commercially available slides for DNA arrays are often used as matrices for immobilization of glycans. Noncovalent immobilization involves adsorption of polysaccharides on nitrocellulose-coated slides [16] or on polystyrene microtiter plates [17], adsorption of glycans as glycolipids on nitrocellulose [18], or immobilization using streptavidin–biotin interactions, for example, binding of biotinylated polyacrylamide glycoconjugates with streptavidin-coated support [19,20]. Glycoarrays for the studies of lectins and substrate specificity of galactosyltransferase were manufactured on the basis of self-assembled monolayers of alkanethiolates on gold surface [21].

To create arrays, glycans can be covalently immobilized on different surfaces such as glass and plastic. Commercially available amine-reactive *N*-hydroxysuccinimidyl (NHS)[1]-activated glass slides were used for the construction of glycan array consisting of 200 major glycan structures of glycoproteins and glycolipids [22]; in this work, glycans or glycoconjugates contained amino-functionalized linkers. The same chemistry was employed for the covalent binding of aminopropyl-terminated glycosides on NHS glass, and the array was used for the screening of 172 glycan structures for their ability to bind siglec-8 (i.e., sialic acid binding immunoglobulin-like lectin 8) [20]. An alternative covalent binding is based on thiol chemistry; carbohydrate and glycoprotein microarrays prepared by this method were used to study glycan-dependent gp120–protein interactions [23]. Covalent immobilization of glycans, glycoconjugates, and lectins on microarrays was also carried out on thin film-coated photoactivatable surfaces (photochips and OptoDex biotin platforms) [24].

The goal of the current work was to apply the technology for the manufacturing of three-dimensional hydrogel microchips that was developed for immobilization of DNA and proteins for the preparation of glycoarrays and combined glyco/protein arrays. These microchips were then used to study the binding of antibodies and lectins to immobilized ligands.

Materials and methods

Glass slides for the fabrication of microarrrays were purchased from Corning Glass Works (Corning 2947 Micro Slides, Corning, NY, USA), and 96-well black Maxisorp plates were purchased from Nunc (Denmark). Micro Bio-Spin chromatography columns were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Methacrylamide, *N*,*N*′-methylenebisacrylamide, glycerol, phosphate-buffered saline (PBS) tablets, polyvinyl alcohol (50,000 Da) (PVA), polyvinyl pyrrolidone (360,000 Da) (PVP), Tween 20, and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA). Cy5-labeled anti-mouse IgG antibodies, Cy3 fluorescence dye as mono-succinimide ester, Sephadex G-25 coarse, and anti-mouse IgG–biotin conjugate were purchased from Amersham Biosciences (Piscataway, NJ, USA). Anti-human IgG–biotin conjugate, streptavidin–alkaline phosphatase (AP) conjugate, and 4-methylumbelliferyl phosphate were

[1] *Abbreviations used:* NHS, *N*-hydroxysuccinimidyl; PBS, phosphate-buffered saline; PVA, polyvinyl alcohol; PVP, polyvinyl pyrrolidone; BSA, bovine serum albumin; AP, alkaline phosphatase; PAA, polyacrylamide; mAb, monoclonal antibody; R60, ricin; RTA, ricin toxic subunit; RCA, ricin agglutinin; PBST, phosphate-buffered saline containing 0.1% Tween 20; CCD, charge-coupled device; ELISA, enzyme-linked immunosorbent assay.

purchased from Southern Biotech (Birmingham, AL, USA).

Spacered blood group trisaccharides A and B (A_{tri}, Ga1NAcα1-3(Fucα1-2)Gal; B_{tri}, Ga1α1-3(Fucα1-2)Gal), lactose, α-mannose, disaccharide B (spacer = $-OCH_2CH_2CH_2NH_2$), and A_{tri} and B_{tri} as polyacrylamide (PAA) glycoconjugates (soluble, 30–40 kDa, molar content of saccharide ligand 20%) were obtained from Lectinity (Moscow, Russia). Mouse monoclonal antibodies (mAbs) A16 against A_{tri} and B8 against B_{tri} as cultural fluid were obtained from the All-Russian Hematology Research Center (Moscow, Russia).

Other chemical reagents were obtained from commercial suppliers and used without further purification.

Ricin (R60), ricin toxic subunit (RTA), ricin agglutinin (RCA), and mAbs against R60 (1RK1 and Rch1), against RTA (ARK1), against RCA (1RAK4), and against viscumin (TAS and MnA9) were kindly provided by M.M. Moisenovich and A.G. Tonevitskii (Department of Biology, Lomonosov Moscow State University).

Antibodies and ricin were labeled with Cy3 dye according to the manufacturer's instructions.

Fabrication of microchips

Hydrogel drop microchips with covalently immobilized saccharides were fabricated by a polymerization-mediated immobilization procedure as described previously for DNA microchips [6,7]. Solutions for the preparation of microchips contained methacrylamide (4.75%, w/v), *N*,*N*′-methylene bisacrylamide (0.25%, w/v), and glycerol (50%, v/v) in PBS and were titrated with Temed to pH 10.5. Saccharides modified with amino groups, PAA conjugates of saccharides, or antibodies were added to gel solution, and the mixtures were put into the wells of a 384-well microtitration plate and spotted onto bind silane-treated glass slides using a QArray pin robot (Genetix, UK). Polymerization of gel arrays was carried out under UV light with a maximum wavelength of 350 nm, and irradiation intensity 0.06 μW/cm^2, for 50 min at 20 °C. Microchips were washed in PBS containing 0.1% Tween 20 (PBST) for 20 min at 20 °C with shaking. After polymerization and washing, an array of identical gel drops attached to the glass surface was formed (Fig. 1A). Standard gel elements were approximately 1 nl in volume (25 μm high and 150 μm in diameter). Before the assays, all microchips were checked for quality, and microchips with deviations in positions and radii of gel elements within 5% of the average values were taken for the experiments. Microchips can be stored in the presence of 50% glycerol at 2–8 °C for at least 6 months without loss of ligand-binding properties.

On-chip fluorescence measurements

Quantitative fluorescence measurements were carried out using a custom-built research fluorescence microscope equipped with a cooled charge-coupled device (CCD) camera, a Peltier thermotable, and a temperature controller or a portable fluorescence biochip analyzer with laser excitation source (Engelhardt Institute of Molecular Biology

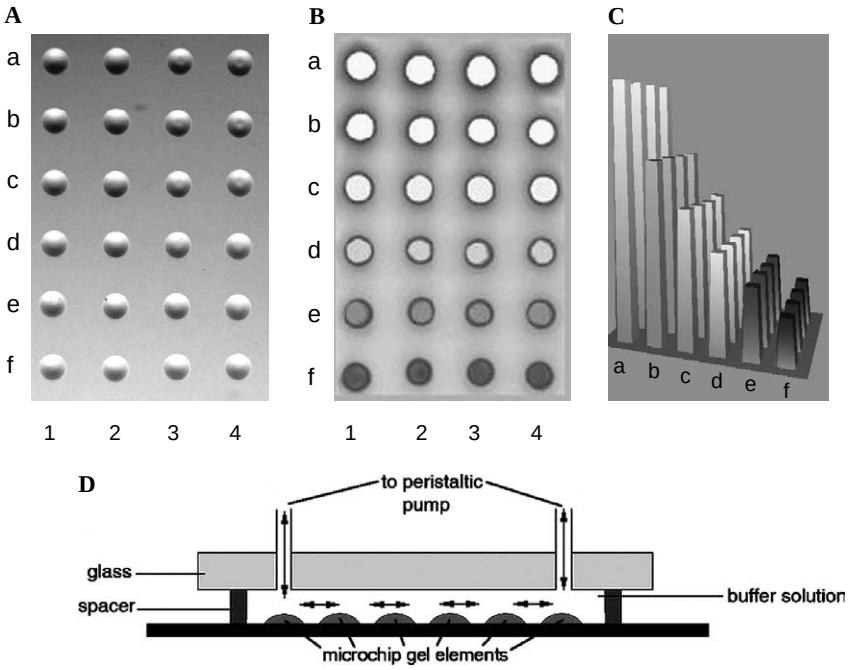

Fig. 1. (A) Photograph of microchip gel elements in transmitted light. Diameter of gel drops was 150 μM. (B) Fluorescence image of microchip with immobilized lactose after interaction with ricin (500 ng/ml) and development with Cy5-labeled anti-ricin Rch1 antibodies (7 μg/ml). Concentrations of chip-immobilized lactose were 3.1 (elements a1–a4), 2 (b1–b4), 1.4 (c1–c4), 0.9 (d1–d4), 0.6 (e1–e4), and 0.4 (f1–f4) pmol per gel element. (C) Diagram of fluorescent intensity of gel elements with different concentrations of chip-immobilized lactose (microchip shown in A and B). (D) Schematic view of the chamber with inlet/outlet tubes for experiments with mixing with peristaltic pump.

[EIMB], Moscow, Russia) [25]. Both instruments were equipped with computers with a data acquisition board. Measurements with Cy3 and Cy5 fluorescence dyes were carried out using 535/590 nm and 650/670 excitation/emission filters, respectively. All measurements were performed at 20 °C.

The ImaGel Research program developed in our laboratory allows one to obtain and process fluorescence signals from each individual gel element (Figs. 1B and C). The fluorescence intensity from a gel element was calculated according to the following formula:

$$F = \frac{S - B_{dc}}{B_{rg} - B_{dc}}, \quad (1)$$

where S is the integral fluorescence calculated for the image area occupied by the element, B_{dc} is the noise signal produced by dark current at zero illumination intensity, and B_{rg} is the integral fluorescence intensity of a control slide of red glass at the position occupied by the same gel pad. Red glass slide was used to take into account possible spatial nonhomogeneity of the illumination source. The fluorescence intensity was calculated as a median value for the set of four elements.

Estimation of efficiency of immobilization of saccharides in microchip gel elements

The efficiency of immobilization was estimated using fluorescently labeled oligonucleotide modified with the amino-terminal group NH_2-$(CH_2)_6$-ATGC-Cy3. The oligonucleotide was immobilized within microchip gel elements under the conditions described above for the immobilization of saccharides. The chip was washed with PBST until the fluorescence signal from the chip remained constant. The immobilization efficiency was calculated as the ratio of the fluorescence signals before and after the washing.

Assays on glycochips

Before proceeding to the assays, microchips with immobilized glycans were incubated for 2 h at 20 °C in the blocking buffer (PBS containing 1% PVA) and rinsed briefly with water. The solution for sample dilution was PBS containing 0.15% PVA and 0.15% PVP. The assays were carried out either without mixing or with mixing using a peristaltic pump. The assays without mixing were carried out in transparent reaction chambers (in situ frames, Eppendorf Scientific, Westbury, NY, USA), 50–100 μl in volume. The assays with mixing were carried out in a specially constructed reaction chamber (Fig. 1D) made of quartz glass, $15 \times 5 \times 0.25$ mm, with inlet/outlet tubing connectors 1 mm in outer diameter. The chamber was fixed on a chip by silicon spacers. The reaction mixture (total volume 80 μl) was injected into the chamber through the tubing connectors using a micropipette. The working volume of the reaction chamber was 20 μl, and the remaining 60 μl was distributed between the two tubes. To generate a mixing flow in the reaction chamber, the inlet/outlet tubes were connected to a Minipulse 2 peristaltic pump (Gilson, France) that was modified to function in oscillation mode. The reaction solution was pumped through the chamber. Mixing was achieved by switching the flow direction every 2 s; the flow rate was 6 ml/min. After completion of the assay, the chip was washed with PBST for 10 min with mixing in the same chamber; the flow rate for the washing procedure was 3 ml/min. After washing, the chamber was removed and the chip was rinsed with water, air-dried, and used for fluorescent measurements.

Interaction of mAbs against A_{tri} and B_{tri} blood group antigens with chip-immobilized trisaccharides

Microchips with immobilized A_{tri} containing amino group and in the form of PAA glycoconjugate

Trisaccharide A with terminal amino group and A_{tri} in the form of PAA glycoconjugate were immobilized in different gel elements of the microchip in concentrations from 1.7 to 0.07 μmol/ml with respect to A_{tri}, corresponding to the quantity of immobilized compound from 1.7 to 0.07 pmol per gel element. Cultural fluid containing mAbs against A_{tri} (A16, 80 μl/chip) was diluted with PBS and applied on microchips, and the chips were kept overnight at 10 °C. The dilution ratio was between 1:10 and 1:10,000. After washing with PBST for 15 min, a solution of Cy5-labeled anti-mouse antibodies (1 μg/ml in PBS, 80 μl) was added to each microchip and the chips were kept for 3 h at 20 °C. After washing the chips, fluorescence signals from gel elements were measured.

Microchips with immobilized saccharides for studies of binding of antibodies

Microchips with gel elements containing immobilized A_{tri} and B_{tri} in concentrations from 7.1 to 0.6 μmol/ml, corresponding to the quantity of immobilized carbohydrate from 7.1 to 0.6 pmol/gel element, were manufactured. Spacered trisaccharides with amino terminal group were used for immobilization. The assays were carried out using mixing with a peristaltic pump. Then 80 μl of mAbs against A_{tri} (A16), or mAbs against B_{tri} (B8) diluted with PBS (dilution ratio from 1:10 to 1:10,000), was added to the microchip in the mixing chamber, and the solution in the chamber was mixed with a peristaltic pump for 3 h at 20 °C. The solution was changed for PBST, and the microchip was washed for another 10 min. After washing, 80 μl of Cy5-labeled anti-mouse antibodies (1 μg/ml in PBS) was added to each microchip, and the mixing was continued for 2 h at 20 °C. After washing with PBST for 10 min, the chamber was removed and the microchip was rinsed with water, air-dried, and used for fluorescence measurements.

Analysis of human blood sera on microchips with chip-immobilized blood group trisaccharides

Amino-modified A_{tri} and B_{tri} were immobilized in gel elements at 7.1 μmol/ml of gel. Human blood serum, III

group (80 μl), undiluted or as twofold serial dilutions with PBS, was added to the microchips in mixing chambers, and the reaction solutions were mixed for 3 h at 20 °C. The microchips were washed with PBST for 10 min, 80 μl of mouse antibodies against human IgG (1 μg/ml in PBS) was added to each microchip, and mixing was carried out for 1 h at 20 °C. After washing with PBST for 10 min, 80 μl of Cy5-labeled anti-mouse antibodies (1 μg/ml in PBS) was added to each microchip and the chips were incubated with mixing for 1 h at 20 °C. After washing with PBST for 10 min, the chambers were removed and microchips were rinsed with water, air-dried, and used for fluorescence measurements.

Specificity of binding of ricin with chip-immobilized saccharides

Microchips with immobilized lactose, disaccharide B, α-mannose, A_{tri}, B_{tri} (all at 2.5 mg/ml), antibodies against ricin 1RK1 (0.05 mg/ml), and antibodies against viscumin TAS and MnA9 (both 0.25 mg/ml) were manufactured. The antibodies against viscumin were used as controls for nonspecific binding. Solutions of ricin R60, RTA, and RCA in concentrations from 30 to 1000 ng/ml were applied on microchips, and the chips were incubated for 16 h at 20 °C. After washing, solutions of mAbs against ricin (Rch1), RTA (ARK1), and RCA (1RAK4), 7 μg/ml in PBS, were applied, and the chips were incubated for 3 h at 20 °C. After washing with PBST, the chips were developed with Cy5-labeled anti-mouse antibodies (1 μg/ml in PBS) for 3 h at 20 °C and used for fluorescence measurements.

On-chip kinetic measurements

Solution of Cy3-labeled ricin was applied on microchips with immobilized saccharides and/or antibodies in the reaction chamber designed for on-chip mixing. The reaction was carried out at 20 °C with mixing using a peristaltic pump (flow rate 6 ml/min) or without mixing. Fluorescence images were obtained from each gel element of the chip every 2 min for 20 h, and the results were processed using the ImaGel Research program to obtain the dependencies of fluorescence intensity versus time for each gel element.

Determination of association constants for binding of ricin with chip-immobilized ligands

Our previous studies of kinetics of interaction of oligonucleotides and proteins with ligands immobilized in hydrogel elements of microchips [26,27] showed that the kinetics of binding (i.e., the time course of fluorescence signal) is a saturation curve and that the corresponding saturation time τ_B may be approximated as follows:

$$\tau_B = \tau_D[A]_{imm}\frac{K_{ass}}{1+K_{ass}[B]_{sol}}, \quad (2)$$

where $\tau_D \cong R^2/D_{gel}$ is the diffusion time corresponding to the diffusion of the ligand in a gel element with radius R without immobilized probe, D_{gel} is the diffusion coefficient, $[A]_{imm}$ is the concentration of immobilized probe (which in our case is an immobilized saccharide or antibody), $[B]_{sol}$ is the concentration of ricin in solution, and K_{ass} (M^{-1}) is the association constant for the following reaction of ricin with the immobilized probe:

$$A_{imm} + B_{sol} \leftrightarrows AB. \quad (3)$$

The time to reach saturation, τ_B, is determined from the kinetic curve of the interaction of Cy3-labeled ricin with chip-immobilized probe. The saturation time depends on the concentration of immobilized probe and on the concentration of ricin in solution. For a given constant concentration of immobilized compound and two concentrations of ricin in solution, a set of two equations can be deduced:

$$\begin{cases} \tau_{1B} = \tau_D[A]_{imm}\frac{K_{ass}}{1+K_{ass}[ricin]_{1sol}} \\ \tau_{2B} = \tau_D[A]_{imm}\frac{K_{ass}}{1+K_{ass}[ricin]_{2sol}} \end{cases}, \quad (4)$$

where τ_{1B} and τ_{2B} are saturation times determined for two concentrations of ricin in solution: $[ricin]_{1sol}$ and $[ricin]_{2sol}$, respectively. From Eq. (4), the association constant can be determined:

$$K_{ass} = \frac{\tau_{2B}-\tau_{1B}}{\tau_{1B}[ricin]_{1sol} - \tau_{2B}[ricin]_{2sol}}. \quad (5)$$

For the determination of association constants, microchips with immobilized lactose, disaccharide B (all at 5 mg/ml), and antibodies against ricin 1RK1 (0.1 mg/ml) were manufactured. A solution of Cy3-labeled ricin, 1.5×10^{-8}, or 1.5×10^{-9} M in PBS was applied on the microchip. Fluorescence images were obtained from each gel element of the chip every 2 min for 20 h, and the results were processed to obtain the dependencies of fluorescence intensity versus time for each gel element. Saturation times for all concentrations of ricin were determined, and the association constants for the reaction of ricin with lactose, disaccharide B, and anti-ricin antibodies 1RK1 were calculated using Eq. (5).

Enzyme-linked immunosorbent assay on 96-well plates

Plates were coated with A_{tri}–PAA and B_{tri}–PAA conjugates, twofold dilutions in 0.05 M Na–carbonate buffer (pH 9.6), for 1 h at 37 °C, beginning with the concentration of carbohydrate ligand in the coating buffer 9 μM. Coated plates were blocked with 3% BSA in PBS for 1 h at 37 °C and washed three times with PBST. Then 100 μl of mAbs A16 and B8 at different dilutions starting from 1:100 was added and the plates were incubated for 1 h at 37 °C, washed three times with PBST, incubated with biotinylated anti-mouse IgG (1:2000 in PBS containing 0.3 % BSA), and washed. Finally, the plates were incubated with streptavidin–AP (1:2500 in PBS containing 0.3% BSA) and washed. Fluorescent AP substrate, 4-methylumbelliferyl phosphate,

10^{-4} M in 0.03 M diethanolamine buffer (pH 9.8) containing 10^{-5} M $MgCl_2$ was added to the plates, and they were incubated for 30 min at 20 °C. Fluorescence was measured on a Victor 2 multilabel counter (PerkinElmer, Boston, MA, USA). Each assay was done in duplicate. Blank reaction was performed without the mAbs, and blank readings were subtracted from the final fluorescence data to provide the corrected values.

Results

Manufacturing of hydrogel-based glycan microchips: assay of mAbs

The technology developed earlier for the covalent immobilization of DNA and proteins in hydrogel elements of microchip [6–8] was used for immobilization of saccharides. Methacrylamide and methylene bisacrylamide were used as basic gel-forming monomers. Trisaccharides A_{tri} and B_{tri} as —$OCH_2CH_2CH_2NH_2$ glycosides and as 30- to 40-kDa soluble PAA conjugates [28] were taken for microchip manufacturing. The same PAA conjugates were used for coating of 96-well polystyrene microplates in the standard enzyme-linked immunosorbent assay (ELISA) procedure.

At the first step of the manufacturing of hydrogel microchips, gel-forming monomers containing multiple bonds, in our case methacrylamide and methylene bisacrylamide, are mixed with the compounds to be immobilized to obtain the polymerization mixture. The polymer is formed during photo-initiated radical polymerization; other types of initiation (e.g., chemical initiation) may also be used. A growing polymer chain of the gel contains radicals and double bonds that readily react with amino, sulfhydryl, or other active groups of immobilized compounds such as oligonucleotides, proteins, nucleic acids, and saccharides, which can thus be incorporated into the polymer structure [29,30]. At alkaline pH (10.5), amino groups are in active unprotonated form and enter into the reaction with bifunctional methylene bisacrylamide during the methacrylamide gel polymerization. Methacrylamide is taken as a gel-forming monomer instead of acrylamide because it is inactive in the reaction of nucleophilic addition of amino group at its double bond, whereas acrylamide enters readily into this addition reaction, blocks amino groups, and removes them from the polymerization process.

PAA conjugates of saccharides contain terminal multiple bonds. It is known that compounds containing such bonds may be used for the synthesis of polymers by radical polymerization irrespective of the manner of its initiation (photochemical or chemical), and the reaction proceeds through the steps of initiation, growth, and interruption of the polymer chain [31]. If a chain interruption occurs during the polymerization process, the resulting compound can be subjected to further polymerization at multiple bonds. PAA conjugates of saccharides take part in the radical polymerization reaction at the acrylamide double bond together with the other gel-forming monomers.

After polymerization, an array of individual three-dimensional gel drops 150 μm in diameter and 25 μm in height was formed. The drops were separated from each other by hydrophobic glass surface (Fig. 1A). An array of 4×6 gel drops occupies 0.4 mm^2 of chip surface.

Trisaccharides as –$OCH_2CH_2CH_2NH_2$ glycosides were immobilized in microchip gel elements in concentrations from 1.7 to 0.15 μmol/ml. In parallel, the same trisaccharides as PAA conjugates were immobilized in the same concentrations (1.7–0.15 pmol/gel element). Because we had no saccharides containing fluorescence label, the immobilization efficiency was estimated indirectly using the oligonucleotide containing the amino-terminal group and Cy3 fluorescence label. In an earlier work, we demonstrated that the immobilization of amino-modified oligonucleotides within gel elements by copolymerization with gel-forming monomers occurs at their NH_2 group rather than at the heterocyclic moiety and that immobilization efficiency does not depend on the oligonucleotide structure [7]. Immobilization of the oligonucleotide was carried out under the same conditions as immobilization of saccharides (see Materials and methods) and within the same concentration range. The immobilization efficiency (i.e., percentage of immobilized compound of its total amount taken for immobilization) for the NH_2-$(CH_2)_6$-ATGC-Cy3 oligonucleotide was 50% within the concentration range of 0.1–2 μmol/ml.

Chip-immobilized trisaccharides were tested for their interaction with mAbs A16 against A_{tri}. Fig. 2A shows the dependence of fluorescence signals of gel elements containing immobilized trisaccharide A after their interaction with A16 antibodies at different dilutions. The fluorescence signals from gel elements with immobilized A_{tri} with the amino-terminal group were 1.5 to 2.0 times higher than those from immobilized A_{tri}–PAA at the same concentration of A_{tri} for a series of antibody dilutions. The concentration of the carbohydrate ligand in the experiments shown in Fig. 2A was 1.7 μmol/ml.

Fig. 2B demonstrates the dependence of fluorescence signal on the concentration of immobilized A_{tri}. As one can see, only specific signals were observed. Saturation of the curve was not achieved at the used concentrations of immobilized saccharide. Taking into account these results, aminated saccharides were chosen as probes for further in-gel immobilization for the manufacturing of glycochips.

For comparison, the results of conventional ELISA obtained on microplates coated with polyacrylamide conjugates of A_{tri} and B_{tri} are given in Fig. 3. After the incubation with anti-A_{tri} A16 antibodies, the plates were incubated with anti-mouse IgG–biotin followed by incubation with streptavidin–AP and developed with fluorescent AP substrate. The concentration dependence obtained on microchips and 96-well plates (cf. Figs. 2 and 3) and the minimal concentration of antibodies (maximum dilution of antibodies) that can be reliably detected in the ELISA and by chip-immobilized saccharides are similar (Figs. 2 and 3A). In the on-chip experiments, the minimal concentration (detection limit) was determined as the concentra-

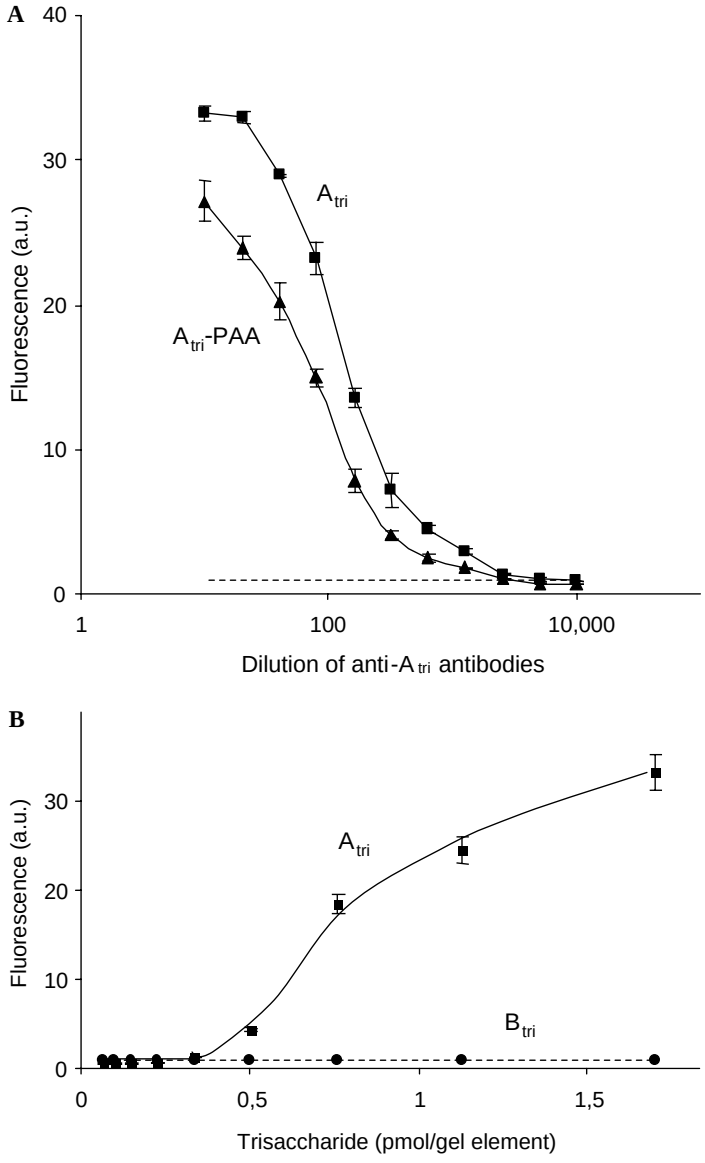

Fig. 2. (A) Dependence of fluorescence signals from gel elements with immobilized A_{tri}–$OCH_2CH_2CH_2NH_2$ (■) and A_{tri}–PAA (▲) (1.7 pmol carbohydrate/gel element for both series) on dilution of anti-A_{tri} mAbs A16. Microchips were incubated with A16 mAbs and developed with Cy5-labeled anti-mouse antibodies. Dotted line indicates background signal from gel elements without immobilized trisaccharide. (B) Dependence of fluorescence signal on concentration of immobilized A_{tri} (■) and B_{tri} (●) in microchip gel elements. Spacered saccharides with the amino-terminal group were taken for immobilization. Microchips were incubated with cultural fluid containing anti-A_{tri} A16 antibodies (dilution 1:10) and developed with Cy5-labeled anti-mouse antibodies. a.u., arbitrary units.

tion of antibodies at which specific signal was twice as high as the background signal, that is, the signals from gel elements with immobilized B_{tri}.

The main difference between the microchips and the plates was the amount of immobilized carbohydrate ligand. In the case of microchips, the maximum amount of immobilized saccharide (Fig. 2A) was 1.7 pmol per gel element. Taking into account 50% immobilization efficiency, it corresponded to 3.4 pmol of saccharide per gel element added originally to the polymerization mixture. For microplate ELISA, the standard amount of saccharide per microplate well used for antibody assay was 1 nmol (Fig. 3A).

Kinetics of interaction of ricin with chip-immobilized carbohydrate probes: effect of mixing

The reaction diffusion kinetics for hydrogel-based microchips with immobilized oligonucleotides and antibodies was studied previously [26,27]. Here, we investigated the kinetics of binding of ligands with chip-immobilized saccharides using the interaction of fluorescently labeled ricin with chip-immobilized lactose and disaccharide B as model interactions. Our software registers fluorescence images from each gel element of the chip every 2 min and plots the dependencies of fluorescence intensity versus time for

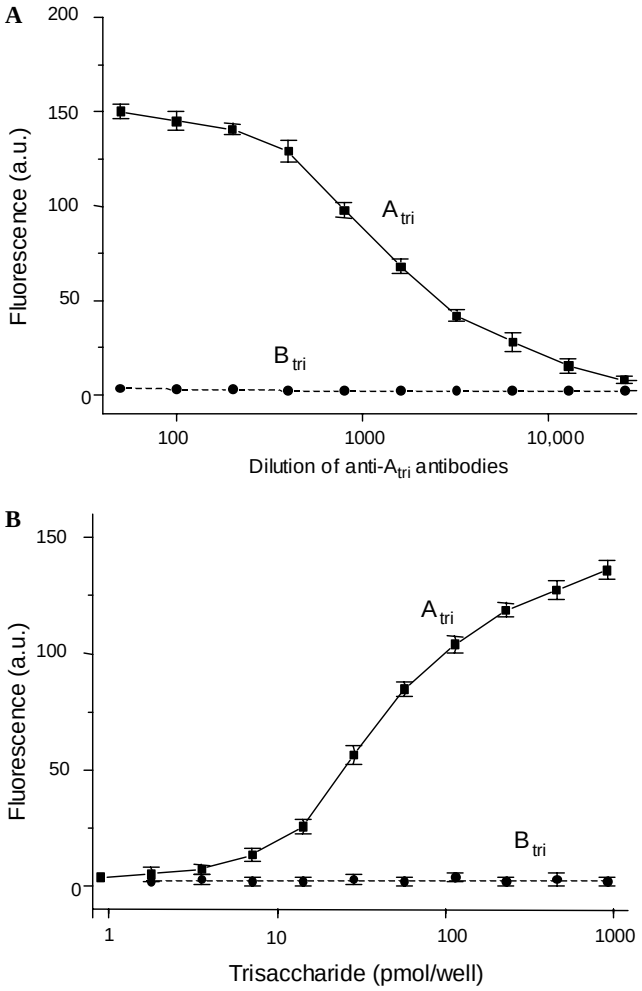

Fig. 3. Microplate ELISA of anti-A_{tri} antibodies with fluorescence detection. (A) Dependence of signal on dilution of antibodies. Microplate wells were coated with A_{tri}–PAA (■) and B_{tri}–PAA (●) (1 nmol/well). The plates were incubated with anti-A_{tri} A16 mAbs at different dilutions and developed with IgG–biotin, conjugate of streptavidin with AP, and fluorescent AP substrate (see Materials and methods). (B) Dependence of signals on concentration of carbohydrate ligands. Microplate wells were coated with A_{tri}–PAA (■) and B_{tri}–PAA (●) at different dilutions. The plates were incubated with anti-A_{tri} mAb A16 (dilution 1:100) and developed with IgG–biotin, conjugate of streptavidin with AP, and fluorescent AP substrate.

each gel element. The kinetic curves for binding of ricin with chip-immobilized saccharides were obtained with and without mixing (see Materials and methods) (Fig. 4). The binding kinetics is a saturation curve, and the time to reach 90% of saturation without mixing varies from 7 to 20 h. It should be noted that the saturation time depends on the concentration of ligand in solution and on the concentration of chip-immobilized carbohydrate in accordance with Eq. (4).

To carry out on-chip reactions with mixing by switching the direction of the flow generated by peristaltic pump, a special reaction chamber was constructed (Fig. 1D). Mixing of the reaction solution during the incubation of ligands with chip-immobilized saccharides accelerates the binding significantly. At the concentration of ricin $1.5\times$

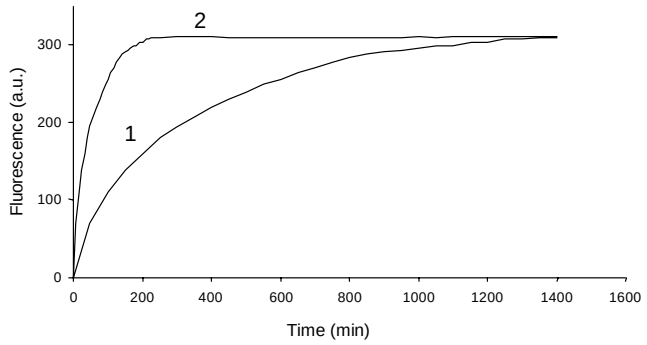

Fig. 4. Kinetic curves for binding of ricin with chip-immobilized lactose obtained without mixing (1) and with mixing with peristaltic pump (2). Concentration of ricin-Cy3 in solution was 1.5×10^{-9} M, concentration of immobilized lactose was 6.5 pmol/gel element.

10^{-9} M, the saturation of signal is achieved in 17 h without mixing and in 3 h with mixing.

Determination of association constants for binding of ricin with chip-immobilized ligands

Previous studies of the reaction diffusion kinetics for hydrogel-based microchips [26,27] showed that association constants for binding of ligands with chip-immobilized probes can be calculated from the saturation times of kinetic curves Eqs. (4), (5). Saturation times for binding of ricin with chip-immobilized anti-ricin antibodies, disaccharide B, and lactose were determined at two concentrations of ricin, and association constants were calculated using Eq. (5) (Table 1). The obtained values of association constants for binding of ricin with disaccharide B and lactose (1.7×10^7 and 1.5×10^7 M^{-1}, respectively) are close to literature data for binding of ricin with different saccharides ($[0.77–1.44]\times10^7$ M^{-1}) [32].

Assay of antibodies on hydrogel glycochips

Assay of antibodies against blood group antigens was carried out on microchips with immobilized blood group trisaccharides. Spacered trisaccharides A and B with the amino-terminal group were taken into polymerization. To accelerate the assay, all procedures—incubation of microchips with hybridoma cultural fluids or blood serum containing specific antibodies, development, and washing of the microchip—were carried out in mixing mode. The signals after 3 h of initial incubation with mixing were 2.5 to 4.0 times higher than those without mixing (Fig. 5A). Indeed, Fig. 4 clearly shows that without mixing, saturation of the signal is not achieved in 3 h.

The results shown in Fig. 5 indicate that microchips with immobilized trisaccharides can be used for the study of antibodies in human sera by the sandwich immunoassay procedure. In particular, fluorescence signals from the immobilized A_{tri} were two times higher than the background at 1:6500 dilution of serum.

Table 1
Association constants (K_{ass}) of ricin binding to chip-immobilized ligands

Concentration of ricin in solution (M)	Chip-immobilized ligands					
	Anti-ricin mAb 1RK1		Disaccharide B		Lactose	
	Saturation time (min)	K_{ass} (M^{-1})	Saturation time (min)	K_{ass} (M^{-1})	Saturation time (min)	K_{ass} (M^{-1})
1.5×10^{-8}	440 ± 5	$(1.20 \pm 0.02) \times 10^{8}$	550 ± 5	$(1.5 \pm 0.1) \times 10^{7}$	850 ± 5	$(1.70 \pm 0.1) \times 10^{7}$
1.5×10^{-9}	1060 ± 5	$(1.20 \pm 0.02) \times 10^{8}$	660 ± 5	$(1.5 \pm 0.1) \times 10^{7}$	1040 ± 5	$(1.70 \pm 0.1) \times 10^{7}$

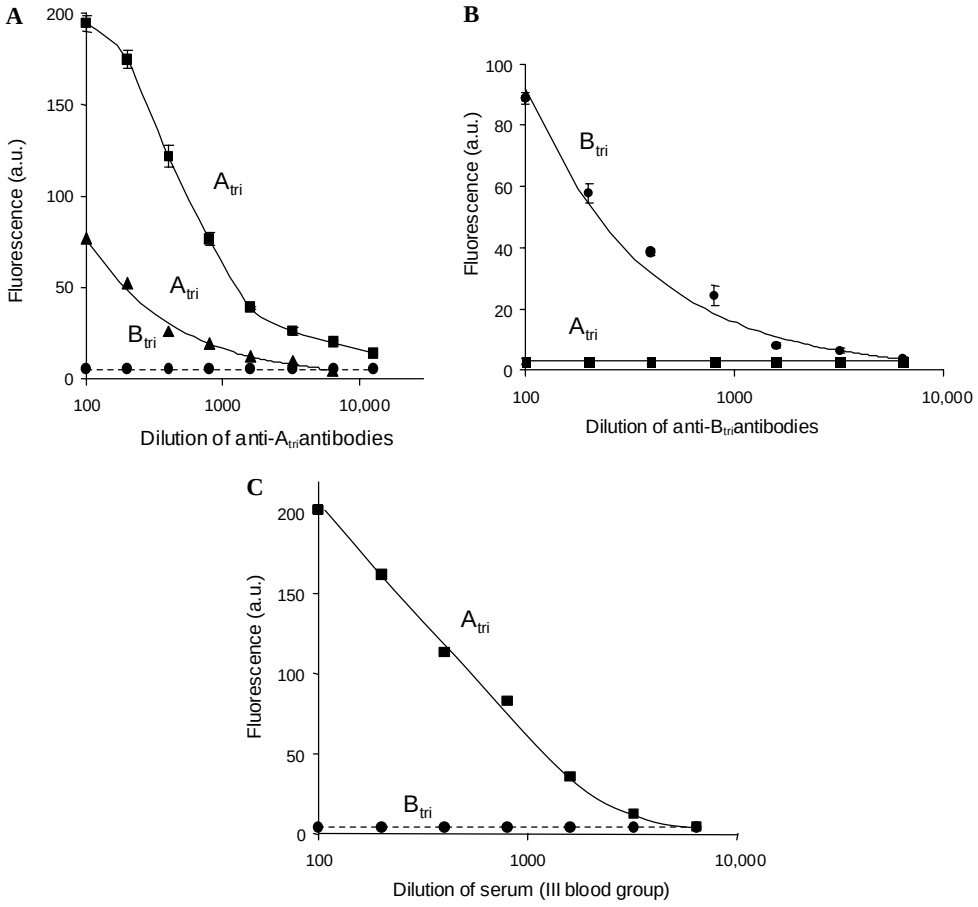

Fig. 5. Assay of anti-A_{tri} and anti-B_{tri} antibodies on microchips with immobilized blood group trisaccharides. A_{tri} (■, ▲) and B_{tri} (●) were immobilized in microchip gel elements (7.1 pmol/gel element). (A) Dependence of fluorescence signal from chip-immobilized trisaccharides on dilution of anti-A_{tri} A16 antibodies. The assays were carried out with (■) and without (▲) on-chip mixing. (B) Dependence of fluorescence signal from chip-immobilized trisaccharides on dilution of anti-B_{tri} B8 antibodies. The assays were carried out with mixing. (C) Dependence of fluorescence signal from chip-immobilized trisaccharides on dilution of serum, blood group III. The assay was carried out with mixing.

Combined chip with immobilized saccharides and antibodies

The specificity of binding of ricin, RTA, and RCA with different carbohydrate ligands was studied using hydrogel-based combined glycan/antibody microchips. Hydrogel microchips containing immobilized saccharides (lactose, disaccharide B, α-mannose, A_{tri}, and B_{tri}) and anti-ricin 1RK1 antibodies were manufactured. Gel elements containing antibodies against viscumin were included as a control for nonspecific binding.

Ricin, a 66-kDa glycoprotein, consists of two subunits, RTA and RTB, connected by a disulfide bridge. RTA is responsible for the cytotoxicity of ricin, whereas RTB is a galactose-binding lectin that is responsible for the penetration of the toxin into cells [33]. Different concentrations of ricin, RTA, and RCA were applied on microchips with immobilized saccharides and antibodies. After incubation, microchips were developed with specific mAbs against these proteins (1Rch1 mAb against ricin, ARK1 mAb against RTA, and 1RAK4 mAb against RCA) followed by Cy5-labeled anti-mouse antibodies.

In addition to specific signals from chip-immobilized anti-ricin and anti-RCA antibodies, specific binding of ricin and RCA with chip-immobilized lactose and disaccharide B was observed (Figs. 6A and B). The signals from ricin toxin subunit RTA were close to background; that

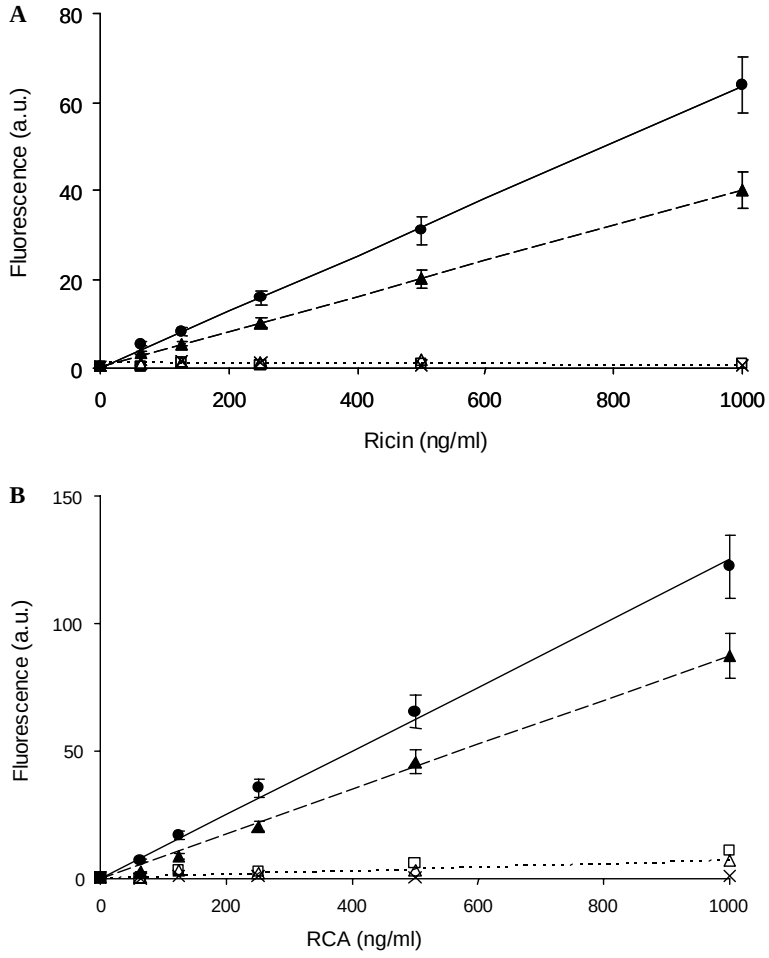

Fig. 6. Binding specificity of ricin (A) and RCA (B) with microchip-immobilized saccharides: lactose (●), disaccharide B (▲), A_{tri} (△), B_{tri} (□), and mannose (x) (2.5 mg/ml gel).

is, no binding of RTA with immobilized saccharides was observed (data not shown). The results obtained on hydrogel glycan microchips agree with existing data on glycan specificity of ricin subunits [32]. The detection limit of the on-chip ricin sandwich assay (i.e., the concentration of ricin at which the fluorescence signal was twice as high as the background) was 30 ng/ml.

Fluorescence signals were directly proportional to the concentration of immobilized saccharide ligand (Figs. 1B and C). The minimum amount of immobilized saccharide (lactose) that is necessary for the assay of ricin was determined using microchips with gel elements containing different concentrations of immobilized lactose from 3.1 to 0.4 pmol per gel element. At a high concentration of ricin in solution (500–1000 ng/ml), fluorescence signals can be clearly seen at the lowest concentration of immobilized lactose. At 50 ng/ml of ricin, fluorescence signals can be seen from gel elements containing 0.9 pmol of immobilized lactose.

Discussion

The goal of this work was to apply the previously developed technology of hydrogel microchips manufacturing [6–8,12,13], which was used for DNA and protein microchips, to the manufacturing of microchips with immobilized saccharides. The results presented in this article indicate that the proposed technology is rather universal given that it allows one to prepare biochips containing different biologically active molecules, including covalently immobilized saccharides. For covalent immobilization in hydrogels, different probes containing amino, sulfhydryl, or other groups, which can enter into the addition or substitution reaction during gel polymerization, can be used [6].

The majority of existing arrays, be they DNA arrays, protein arrays, or glycoarrays, are two-dimensional; that is, probes are immobilized on the surface of microchip support. The main technological limitation of the two-dimensional chips is a relatively low amount of immobilized compounds. To increase the immobilization capacity of two-dimensional chips, additional chemical treatment of microchip supports is sometimes used, for instance, coating with poly-L lysine [34]. Slides with gel coatings are commercially available from PerkinElmer Life Sciences (polyacrylamide gel, 20–30 μm thick) and XanTec Bioanalytics (polysaccharide gel). In these cases, probes for immobilization are dispensed onto the gel layer, leading to uneven distribution of probes and leaving most material immobilized

on the surface of the gel. Gel-coated slides have been shown to reach better detection limits as compared with two-dimensional microchips, but with greater intra- and interfield variation [4,5]. In first-generation gel microchips developed at EIMB, the probes to be immobilized were applied on the surface of a preformed gel. Studies of gel drops used in the first-generation biochips with confocal microscope showed that immobilized probes were concentrated mainly on the surface of gel elements [7]. Current technology for the manufacturing of hydrogel microchips is based on copolymerization of gel components with chemically modified probes. Gel polymerization and immobilization of probes occur simultaneously, and immobilized probes are distributed uniformly within the gel drop volume. Uniform distribution of probes was confirmed using confocal microscopy of DNA and protein microchips [7,13]. It must be stressed that the amount of probe per square unit immobilized on three-dimensional chips is two to three orders of magnitude higher than that on two-dimensional chips, resulting in higher sensitivity of assays.

In this work, hydrogel glycan microchips were used for quantitative assay of antibodies and lectins with fluorescent detection. In all cases, only specific interaction with chip-immobilized saccharides was observed, whereas the level of background signals was very low. The detection limit of on-chip assays (i.e., the minimum concentration of the analyzed protein that gave a reliable positive signal) was comparable to the standard plate assay methods. However, much smaller amounts of saccharides are necessary for the preparation of microchips as compared with assays on 96-well plates. The working amount of chip-immobilized compound is several picomoles per gel element—three orders of magnitude less than the amount of PAA conjugates used for 96-well plate coating (~1 nm/well). At the same time, the potential capacity of the chip is 100 times higher than the capacity of the microplate.

Another main advantage of microchips over standard assays is the possibility of simultaneous analysis of samples for many parameters, for example, affinity to several ligands immobilized on the same microchip. Such an experiment is illustrated in Fig. 6, where the affinity of ricin to different saccharides was tested on a single chip.

Mixing of reaction solution allowed us to decrease significantly the duration of the assays: 2–3 h for incubation and development steps and 10 min for washing. This effect was demonstrated previously for protein chips by us [27] and for DNA chips by others [35,36]. A method for determination of association constants for binding of compounds with chip-immobilized ligands is proposed, and the calculated association constant values correlate well with the literature data.

The technology of hydrogel microchip manufacturing allows one to combine different compounds (DNA, proteins, and saccharides) on one microchip. Previously, we developed the method of quantitative immunoassay of biological toxins using microchips with immobilized antibodies [13]. Here, we have described microchips with antibodies and saccharides immobilized in gel elements on the same biochip. Combined microchips containing different types of probes can be designed for assaying various samples or sera when simultaneous detection of different biomolecules is necessary.

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