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Macroporous Monolithic Layers as Efficient 3-D Microarrays for Quantitative Detection of Virus-like Particles

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The main objective of the present paper was to test the recently developed new type of 3-D protein microarray system based on glycidyl methacrylate-*co*-ethylene glycol dimethacrylate (GMA−EDMA) monolithic material for efficient and fast virus detection. The large-size synthetic particles bearing adsorption-responsible biomolecules on their surface were used as a virus model. Two affinity pairs were chosen for present study. Model virus-like particles, close to the dimensions of human viruses, were developed by means of protein (one of affinity partners) covalent binding to the outer carboxylated surface of polymer latexes (polystyrene based, 80-nm diameter). Recently, it was shown that the adsorption of similar synthetic particles was defined by a protein covering the particle surface. The corresponding complement was immobilized on the surface of prepared by photoinitiated polymerization GMA−EDMA macroporous layers. The detection of a formed biocomplementary complex between protein-bearing latex particle and immobilized affinity partner was carried out by two different methods: (1) similar to an ELISA approach using horse radish peroxidase conjugated with monoclonal antibodies and (2) direct method using two markers. In parallel, the pairing of native proteins was also evaluated. The adsorption behavior of studied particles has been additionally investigated by affinity adsorption at static and dynamic (frontal elution) conditions using the same GMA−EDMA material shaped as a short monolithic column (CIM Disk, BIA Separations, Ljubljana, Slovenia). The results obtained for these virus-mimicking supramolecular structures can be further used for the construction of a rapid, highly sensitive, and highly specific test intended for precise diagnostics of some respiratory tract infection viruses.

The detection of viral pathogens has critical importance in biology, medicine, and agriculture. Virus isolation and purification is an essential task for the provision of a full diagnostic virology service. A low concentration of viruses in biological fluids poses a permanent problem in detection and identification of viral diseases. A possible way to improve viral detection is inclusion of additional procedures in sample processing, such as chromatography. However, the production of high-quality, efficacious, and safe viruses in sufficient amounts is not a trivial task. The major requirement is to maintain biological activity during chromatographic purification and to provide a high yield. Moreover, chromatography is widely used at vaccine production where the inactivated viruses are fractionated and purified.

Obviously, in the case of conventional chromatographic particle-based supports, the interaction between a solute and an affinity ligand, or adsorption-active sites, mostly occurs inside a porous space of a sorbent where the mobile-phase flow is absent and the analyte penetrates into the pore by the force of free diffusion. Since the viruses have very low diffusion coefficients due to their large size, this process is quite limited, and accordingly, the adsorption capacity seems to be extremely low.

Chromatography on short methacrylate-based monolithic columns[1−3] appeared to be preferable to overcome many critical disadvantages of conventional separation techniques. The better mass-transfer mechanism (convection rather than diffusion) and hydrodynamic properties of monolithic sorbents,[4−7] as well as excellent biocompatibility of the initial polymer material, make the chromatographic method based on the use of such supports suitable for successful separation of different bioobjects.[3] Recently, convective interaction media (CIM) monolithic columns were applied for improved detection of two human viruses[8] and for successful concentration of two plant viruses.[9] In both cases, the ion-exchange separation mode was used, whereas our recent results demonstrated fast affinity chromatography strategy for analytical virus concentration.[10]

The development of new methods for express analysis of viruses using extremely small sample volume of human fluids remains a challenge in the diagnostic field. Microarray technology

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has great potential for clinical applications, namely, diagnostics of disease states, viral or bacterial subtyping, and even virus discovery.[11] A microarray represents an analytical device that employs the biorecognition capability of bioactive compounds (such as enzymes, antibodies, nuclear acids, microorganisms, and cells) immobilized on a solid surface. Therefore, the development of sophisticated supports as a platform for microarray preparation, as well as the choice of optimal slide chemistry, has a crucial significance. An optimal solid support for protein or virus microarrays should have the following characteristics: (1) high binding capacity, preservation of native conformation of bioligand after immobilization; (2) inert and resistant to nonspecific adsorption surface; (3) highly specific immobilization chemistry; (4) easiness of manufacture; (5) reasonable easiness of manipulations.

The great positive experience collected for glycidyl methacrylate-*co*-ethylene glycol dimethacrylate (GMA−EDMA) monolithic supports successfully used in chromatographic separations of bioobjects allows assuming the possibility to transfer their properties to another field based on affinity pair formation, e.g., microarray. In this case, a simple transition of monolithic thin layer to the inert glass surface permits us to maintain all characteristics of monolithic material.

In the present paper, we tested a new type of three-dimensional (3-D) GMA−EDMA microarrays for detection and quantitative evaluation of affinity characteristics of specially designed large-size virus-like model particles.

EXPERIMENTAL SECTION

Materials. The glass slides were obtained from BioVitrum. Monolithic disks of 12-mm diameter and 3-mm thickness (CIM epoxy disks) were provided by BIA Separations (Ljubljana, Slovenia). The mean pore size was equal to 1.5 μm whereas porosity was determined as 0.6 mL/mL sorbent. These disks were placed into specially designed cartridge also produced by BIA Separations and attached to a chromatographic system. Sephadex G-25 and G-200 used for size-exclusion chromatography (SEC) was from GE. The microarray plates (25 × 18 × 0.2 mm) based on macroporous GMA−EDMA monolithic copolymer were prepared according to the procedure described below.

Chemicals. 3-(Trimethoxysilyl)propyl methacrylate was from Fluka. 2-Hydroxy-2-methylpropiophenone (Darocur-1173, 97% pure) was purchased from Merck-Schuchard. Glycidyl methacrylate (GMA, 97% pure), ethylene glycol dimethacrylate (EDMA, 98% pure), and cyclohexanol (99% pure) were from Sigma-Aldrich. Soybean trypsin inhibitor and trypsin were purchased from PanEco (Russia). Horseradish peroxidase (HRP) and bovine serum albumin (BSA), fluorescamine (Fl), and water-soluble 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (CDI) were from Sigma-Aldrich. 1-Hydroxybenzotriazole (HOBt) was from Fluka, and 2-(*N*-morpholino)ethanesulfonic acid (MES) and Tween-20 were from Sigma-Aldrich. Dextran sulfate was from Fluka, and 3,3′,5,5′-tetramethylbenzidine (TMB) was purchased from Sigma-Aldrich.

Monoclonal antibodies against transthyretin (TTR) (mAb) and TTR were kindly donated by Prof. M. M. Shavlovsky (Institute of Experimental Medicine, Russian Academy of Medical Sciences, St. Petersburg, Russia). Suspensions of monosize latex particles were a gift from Drs. A. Yu. Menshikova and T. G. Evseeva (Institute of Macromolecular Compounds, Russian Academy of Sciences, St. Petersburg, Russia).

All buffers used were prepared by dissolving salts of analytical grade in double-distilled water and additionally purified by filtration through a 0.45-μm microfilter Milex (Millipore).

Instruments. Affinity HPMDC experiments were performed using high-performance chromatograph Gilson combining two piston pumps (303, 305) and a UV detector LKB (Unicord S 2138). The concentration of eluted protein (protein-bearing latex particles) was determined by measurement of UV absorbance at 750-nm wavelength (Lowry test) using a UV spectrophotometer (SF-26, Lomo).

The Philips 125-W mercury lamp, of wide spectrum and constant intensity of irradiation, was used for free-radical GMA−EDMA copolymerization. The spots were produced by a Biohit micropipettor (0.1−2.5 μL). The biochip washing procedure was carried out using a Maxi Mix II Thermolyne shaker. The quantitative determination of adsorbed affinity partner at a microarray format was performed using a scanning densitometer (DenScan, Lenchron) and fluorescence microscope (MLD-2, Lomo) with digital camera. The calculations were performed using the Dens computer program (Lenchrom).

Methods. (1) GMA−EDMA Microarray Preparation. *Glass Treatment.* For microarray construction, the glass surface was etched via paraffin mask with 11 M hydrofluoric acid for 30 min. The process was carried out at the intensive interfusion and resulted in formation of wells of rectangular shape and defined depth on a glass slide surface. After that, the manufactured slides were washed three times with water, boiled in 0.1 M NaOH for 40 min, washed again three times with distilled water, and, finally, dried at 100 °C.

To form the necessary further triple copolymerization double bonds, the etched glass slides were held in a 15% solution of 3-(trimethoxysilyl)propyl methacrylate in dry toluene for 17−20 h at room temperature.[12] To avoid any photochemical destruction of silane, the reactor was preliminarily wrapped up by aluminum foil. The functionalized glasses were washed three times with toluene, acetone, and ethanol and stored in ethanol in the dark. Directly before use, the glasses were dried for 1.5 h at 35 °C.

Copolymerization Process. The mixture of initiator 2-hydroxy-2-methylpropiophenone (Darocur-1173), porogenic solvent cyclohexanol, and monomers GMA and EDMA were used for preparation of polymer layers.[12] The ratio of components in reaction phase was chosen as 6:4 for GMA/EDMA and 6:4 for cyclohexanol/monomers mixture. The concentration of initiator was 1.0% from the mass of monomers. All reagents for polymerization were mixed, and the solution was purged with nitrogen for 5 min. The copolymerization process was carried out for 20 min. The plates were washed for 4 h at 70 °C consecutively by ethanol, ethanol/water (1:1), and water.

Immobilization of Affinity Ligand. The procedure of protein immobilization on a GMA−EDMA monolithic surface is well

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developed and published elsewhere.[6,13] The GMA−EDMA microarray plates were soaked with protein (soybean trypsin inhibitor (SBTI) or mAb, respectively) in 25 mM borate buffer (pH 9.3) for 16 h at 30 °C. The initial protein concentration was 5 mg/mL. Then the layers were washed using a shaker consecutively with the same reactive buffer, 2 M NaCl, and water to remove the excess of unreacted ligand from the porous volume. The amount of protein coupled was determined by Lowry test[14] monitoring the decrease of protein concentration before and after immobilization with the account of protein content in washing buffer volume. A concentration of protein was determined according to preliminary plotted calibration dependence of absorbency values on proteins concentrations obtained at maximum absorption, namely, at 750 nm.

After immobilization (in all cases, exept a few experiments for the SBTI−trypsin (TR) pair), the microarray was exposed to block unreacted epoxy groups. The blocking protocol involved a 1-h incubation at 37 °C in 25 mM borate buffer (pH 9.3), containing 5% milk.[15]

(2) Sample Labeling. *Horseradish Peroxidase Conjugates Preparation.* HRP was conjugated with mAb or TR according to a slightly modified method of Nakane and Kawada.[16] In the mAb case, 2.1 mg of HRP was dissolved in 0.525 mL of distilled water, a 0.026-mL solution of sodium periodate with a concentration of 38.5 mg/mL was added drop by drop, and the solution was incubated for 20 min at room temperature in the dark. Then SEC on Sephadex G-25 column equilibrated with 0.001 M CH_3COONa−CH_3COOH as a mobile phase was carried out to remove unbound compounds and 4 mg of mAb was added to the purified solution (pH 9.5). The obtained mixture was incubated for 2 h at room temperature with constant interfusion in the dark. For double bond reduction, 0.05 mL of a water solution of 4 mg/mL sodium borohydride was added and the reaction was allowed to proceed for 2 h at 4 °C. After overnight dialyzing against 0.015 M phosphate-buffered saline (PBS), the final concentration of dissolved protein in the obtained solution was made to 10 mg/mL by BSA. The conjugates were stored in small portions at 4 °C.

Protein Labeling with Fluorescamine. For conjugate preparation, a 0.5-mL solution of fluorescamine in acetone with a concentration of 2 mg/mL was added to 2 mL of TR solution in 0.1 M sodium borate buffer (pH 8.3) with a concentration of 2.5 mg/mL. The reaction mixture was held for 20 min in the dark; after that, the conjugate obtained was passed through the column with Sephadex G-25 to remove free label. The fraction containing Fl conjugate was collected and lyophilized.

(3) Latex Particle Modification. Carboxyl-bearing polysterene monosize microspheres of 80-nm diameter with 0.775 μg·equiv COO^-/m^2 were applied for construction of virus-mimicking particles. According to the method described elsewhere,[17,18] 3.6 mg of particles (0.1 mL of latex suspension containing 3.6 g of particles in 100 mL of water) was activated with CDI and HOBt using 10 mM MES buffer (pH 5.5), at 0 °C and 15-min reaction time. CDI and HOBt were taken in equimolar ratio to the surface carboxylic groups. After the activation step, SEC on Sephadex G-25 column with 10 mM borate buffer, (pH 8.5−8.7) as a mobile phase was performed to remove soluble admixtures. Then 3.0 mg of individual protein (TTR) was added to the activated particles. In TR−Fl or TR−HRP conjugation cases, 1.5 mg of protein was also taken for the above-described reaction. The chemical reaction of protein chemisorption onto particle surface was carried out at 20 °C for 4 h. Unbound protein was removed by SEC on Sephadex G-200 column, and 10 mM sodium phosphate buffer (pH 7.0) was used as a mobile phase. The amount of immobilized protein on latex surface, and, consequently, the part of the occupied surface, was determined by standard Lowry test.[14] The particles coated with TR−Fl conjugate were stored in phosphate buffer in the dark (pH 7.0) at 4 °C.

(4) Quantitative Analysis on GMA−EDMA Microarrays. To evaluate the parameters of affinity binding (K_{diss}, Q_{max}), two model pairs SBTI−TR and TTR−mAb to TTR have been used. The detection of biocomplementary complexes obtained was carried out by fluorescence and immunoenzyme assays. In the SBTI−TR pair case, the direct assay (Figure 1a) was used, while the TTR−mAb complex was tested by indirect "sandwich" mode (Figure 1b).

The quantitative determination of affinity-adsorbed protein was performed by either fluorescence microscope (fluorescent marker) or optical densitometry at a visual spectrum field for the HRP−marker case. To build adsorption isotherms, the equal volumes of protein solutions of different concentrations were spotted and, after formation ligand−affinant pair, carefully washed to extract the unbound part of protein to be analyzed. The adsorbed protein amount was calculated by comparison of analyzed zones with a parallel line of differing concentration control spots. The use of a relative method to measure the amount of analyte, namely, the calibration curves obtained at identical analysis conditions, allowed unification of probable incorrectness related to nonhomogeneous distribution of signal intensities over the spot. The examples of calibration curves obtained for SBTI−TR pair are presented in Figure 2. All manipulations with microarrays containing spotted fluoro-labeled protein were carried out in the dark to prevent extinguishment of fluorescence. The removal of unbound protein in all experiments was made according to the following washing procedure: the microarray was washed with shaking for 30 min at 0.01 M PBS buffer containing 0.05% Tween-20, 2 M NaCl for 30 min, and, finally, distilled water for 10 min. Before measurements, the layer was dried for 40 min at 22 °C.

Indirect Analysis. Immunoenzyme Assay. In the TTR case, the samples of individual protein and TTR−latex particles with concentrations ranging from 0.002 to 0.2 mg of protein/mL in PBS buffer were spotted (1 μL probe) onto a microarray surface previously modified by mAb. The reaction of affinity binding was proceeded for 15 min. In all experiments, the washing procedures described in earlier followed sample spotting. Further, the microarray surface was saturated with the solution of enzyme-labeled antibody to TTR with a concentration of 10 mg of protein/mL and the reaction proceeded for 15 min. After washing, a microarray was incubated consecutively with EDTA−citrate buffer (pH 5.0)

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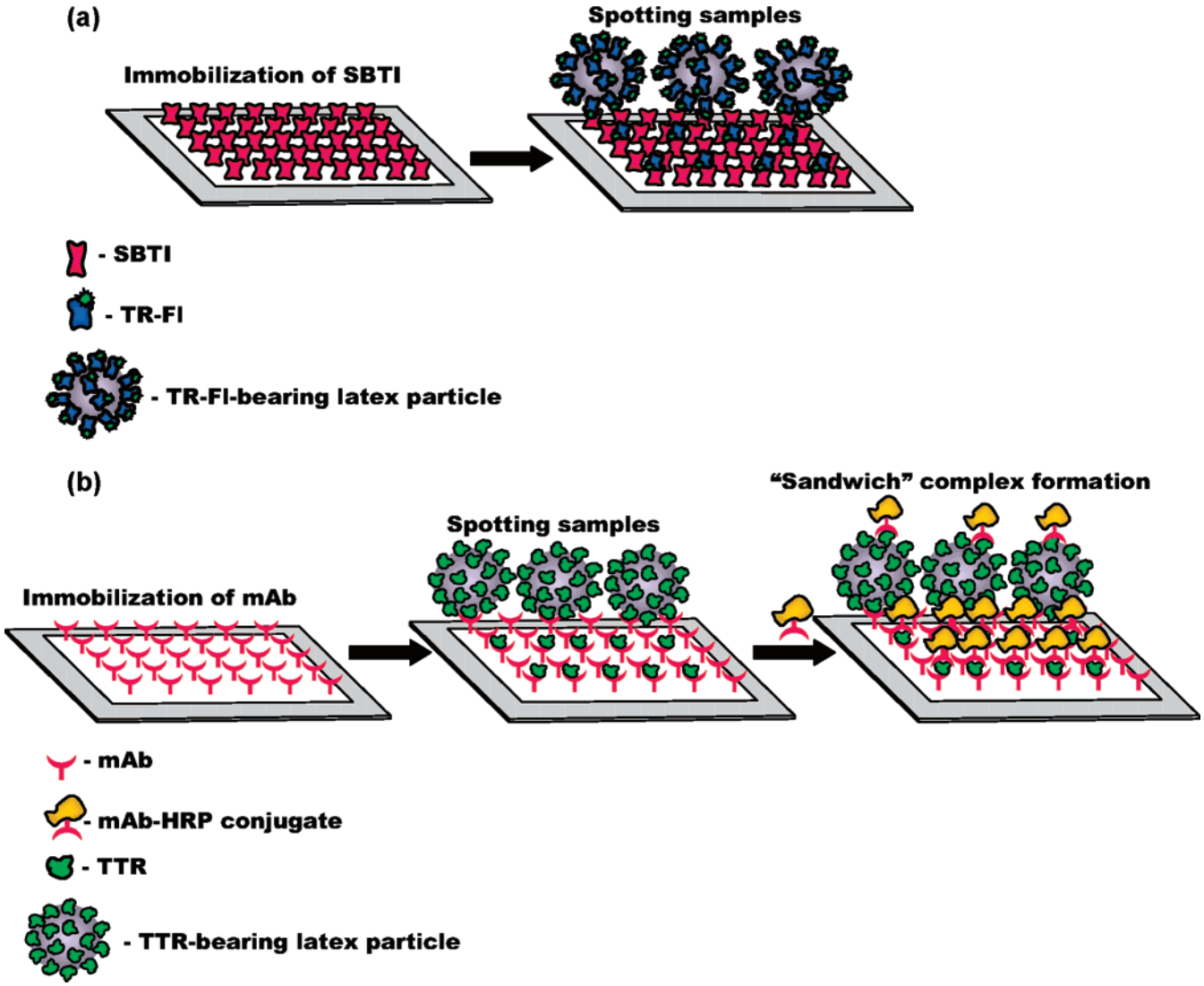

Figure 1. The graphic image of two types of assay: (a) direct and (b) indirect or "sandwich" modes.

for 15 min and 10% solution of dextran sulfate in the same buffer for 10 min and then transferred again into EDTA−citrate buffer (pH 5.0). A substrate solution (0.25 mg/mL TMB in 0.01 M EDTA−sodium citrate buffer, pH 5.0, containing 0.125% H_2O_2) was added, and the enzyme reaction proceeded at room temperature for 1 min.

Direct Analysis. The samples of conjugates of native TR (TR−Fl or TR−HRP) and TR−Fl−latex or TR−HRPn-latex particles with concentrations of trypsin varied in the range from 0.001 to 0.2 mg of protein/mL were spotted onto the biochip surface previously modified by SBTI. The spotted volume was 1.0 μL. The reaction of affinity binding proceeded for 15 min, and then the microarray was carefully washed using a shaker. To obtain colorized complexes (SBTI−TR labeled by HRP), the microarray was incubated for 1 min in substrate solution (0.25 mg/mL TMB in 0.01M EDTA−sodium citrate buffer, pH 5.0, containing 0.125% H_2O_2).

(5) Quantitative Analysis by High-Performance Monolithic Disk Affinity Chromatography (HPMDAC). *Affinity Ligands Immobilization Procedure.* The standard procedure[6] was used for the immobilization of SBTI on CIM epoxy disk. In the case of mAb, the protein solution with a concentration of 5 mg/mL in 25 mM borate buffer (pH 9.3) was loaded hydraulically by a syringe to the cartridge with installed disk. The binding reaction was allowed to proceed over 16 h at 30 °C without any stirring. Then the disk was washed with initial carbonate buffer, pH 9.3, 2 M NaCl and, finally, with operative buffer (PBS, pH 7.0) to remove the excess of unreacted ligand (protein) from sorbent porous volume. The amount of ligand coupled to the support was determined by the Lowry test[14] monitoring the decrease of protein concentration before and after immobilization with the account of protein content in washing buffer volume. The affinity sorbents were stored in PBS at 4 °C.

Affinity Chromatography. Chromatographic experiments were carried out by a frontal elution method to evaluate the parameters of affinity binding of two chosen model pairs.[6,13]

The solutions of individual proteins (TR or TTR) and latexes modified by corresponding proteins with different concentrations ranging from 0.01 to 1.3 and 0.003 to 0.1 mg of protein/mL, respectively, were passed at flow rate of 3 mL/min through complementary functionalized corresponding disks. PBS buffer, pH 7.0, was used at the adsorption whereas the desorption was achieved with 0.01 M HCl, pH 2.0. An intermediate washing procedure with 2 M NaCl was used to release a nonspecifically

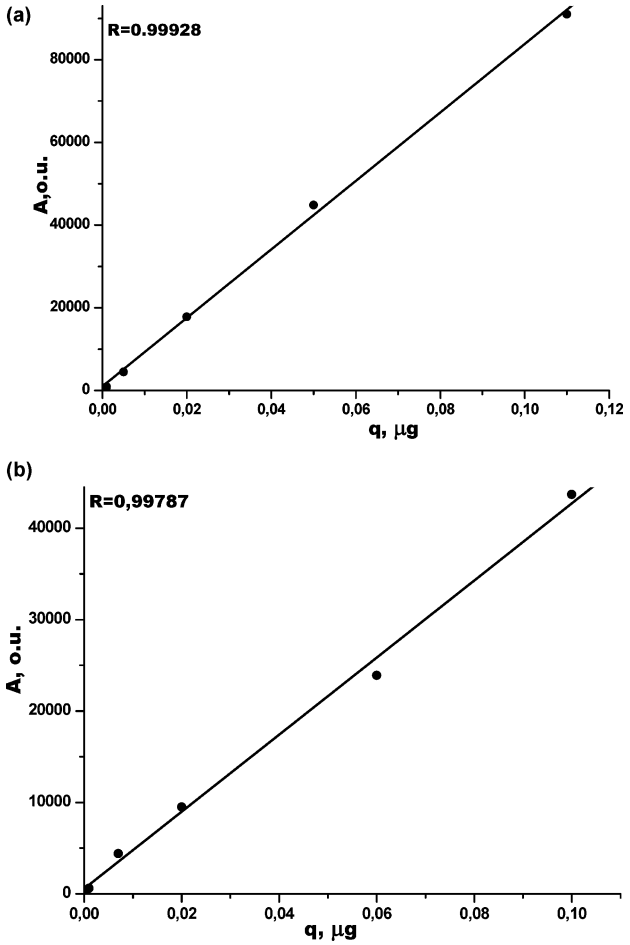

Figure 2. Calibration curves used for quantitative determination of protein (SBTI-TR affinity pair) on microarrays. (a) fluorescent marker; (b) immuno-enzyme.

bound part of protein. The standard Lowry test was used to measure the protein concentration in the desorbed fractions.

The static adsorption experiments were performed immersing mAb−CIM disk into 8.5−17 μg of protein/mL of TTR-l latex or TTR solutions with different volumes. The incubation was allowed to proceed for 15 and 90 min at room temperature. Then the disks were washed by pumping through monolithic column PBS buffer, and desorption was achieved using 0.01 M HCl.

The quantitative affinity characteristics of studied pairs have been calculated on the basis of mathematical treatment of experimental adsorption isotherms resulting from frontal analysis. The results represent the average values calculated from linearized forms of adsorption isotherms by the Langmuir equation.

RESULTS AND DISCUSSION

GMA−EDMA Microanalytical System Preparation. The priority at the development of microarray systems for virus detection is manufacturing of an appropriate design of porous support that could provide both nonrestricted penetration of virus particles and large proteins into 3-D porous space and sufficient adsorption capacity. The advantage in application of GMA−EDMA monolithic material is the possibility of direct formation of a porous structure in a wide range of pore sizes (0.1−10 μm). The experimental work on optimization of polymerization parameters, such as a choice of initiator and its concentration, reaction time, and variation of porogenic composition, has been recently carried out and described elsewhere.[19]

Table 1. Characteristics of GMA−EDMA Monolithic Sorbents Used in Two Formates

format	sorbent unit length, cm	porosity, %	pore diam, μm	sorbent unit vol, mL	specific surface, m^2/g
disk	0.30	60	1.5	0.34	15.0
layer	0.02	60	1.1	0.09	28.0

According to the procedure developed, the manufacture of a microarray based on a GMA−EDMA monolithic layer includes three steps: (1) glass etching process for well formation, (2) glass surface modification (functionalization) step using 3-(trimethoxysilyl)propyl methacrylate to introduce double methacrylic bonds capable of copolymerizing with GMA and EDMA monomers, and (3) GMA−EDMA layer formation in prepared wells by photoinitiated polymerization.

In contrast to that described elsewhere[3] and widely used for analytical separations of biological molecules,[20] GMA−EDMA monolithic columns, which are generally produced using thermoinitiated polymerization,[21] the ultrathin GMA−EDMA slices are obtained by photopolymerization and represent a nonflowing system with quite similar porous design. As seen from the comparative Table 1, the thin layers obtained have more advanced specific surface in comparison with CIM materials due to the increasing part of smaller pores. However, as has been shown in our experiments, this fact does not influence essentially the adsorption capacity of large-size particles because of steric inaccessibility of this part of porous surface.

Functionalization of GMA−EDMA Polymer by Affinity Ligands. Antibodies are still in fashion and extensively used in microarrays and diagnostic kits.[15,22−24] Therefore, the affinity pair transthyretin−mAb to TTR has been chosen for the present research. The choice of the second investigated pair, namely, SBTI−TR, was made since its affinity binding parameters have been recently studied on GMA−EDMA monolithic stationary phases.[6,10]

For arrays, it is important that the method of ligand immobilization does not require tedious protocols of support modification to introduce a reactive group.[25] In the GMA−EDMA case, the availability of original epoxy groups allows carrying out a one-step reaction with amino-bearing compounds. Besides, the suitable immobilization strategy that has already been developed for CIM monolithic columns was easily transferred for the same thin layer in microarray format. The ligands' density obtained on CIM disks and layers are presented in Tables 2 and 3. The layers prepared in that way keep the specific activity for a long time on

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Table 2. Experimental Data Obtained from TTR-mAb Pair on CIM Disk and a Microarray[a]

established affinity characteristics	CIM disks: dynamic method	CIM disks: statis method	microarray: HPR label
$\rho \times 10^{-16}$, ligand molecules/mL of sorbent	3.3		0.9
k_{diss} (TTR−MAb) $\times 10^7$, M	2.9	2.5	1.0
Q'_{max} (TTR) $\times 10^2$, particles/ligand molecules[b]	3.8	1.0 (15 min)	1.6
Q''_{max} (TTR−latex) $\times 10^7$, particles/ligand molecules[b]	7.0	2.0 (15 min) 6.0 (90 min)	3.0

[a] Experimental conditions. (a) Chromatography: The solutions of sample (TTR or TTR−latex particles) with differenct concentrations of TTR were pumped through a CIM−mAb disk at 3 mL/min flow rate; the CIM−mAb disk was immersed into the same solutions. A PBS buffer, pH 7.0, was applied for adsorption whereas the desorption step was achieved with 0.01 M HCl, pH 2.0; to eliminate any nonspecific adsorption of protein, a washing procedure with 2 M NaCl was used. A standard Lowry test was used to measure protein concentration. (b) Microarry sandwich method: The solutions of samples (TTR or TTR−latex particles) with differenct concentrations of TTR in PBS buffer were spotted onto the microarray surface modified by different ratios of mAb to TTR. See details in the Experimental Section. The results of fraontal analysis in both formats represent the average values calculated from linearized forms of adsorption isotherms by the Langmuir equation. [b] $Q'_{max} = (Q_{mas}/M)(N_A/\rho)$ and $Q''_{max} = (Q_{mas}/Q^*)/\rho$, where $Q^* = q/N$, Q_{max} is maximum absorption capacity, protein amount (mg) calculated from frontal data, $Q'(Q'')_{max}$ is maximum adsorption capacity using eqs 1 and 2, M is molecular mass, N_A is the Avogadro constant, Q^* is protein amount (mg) bound to one latex microsphere, q is protein amount bound to total munber of microspheres in a solution, N is number of microspheres in a solution, and ρ is number of immobilized ligand molecules calculated from the Lowry text.

Table 3. Experimental Data for TR−SBTI Pair Obtained by the Direct Method on Microarray and Affinity HPMDC and CIM Disk[a]

	affinity chromatog	microarray: fluorescent label	microarray: HPR label
$\rho \times 10^{-17}$ ligand molecules/mL of sorbent	0.7	0.7	0.6
$k_{diss} \times 10^5$, M	1.1	1.0	0.6
Q'_{max} (TR) $\times 10^1$, particles/ligand molecules[b]	1.5	1.4	9.5
Q''_{max} (L−TR) $\times 10^5$, particles/ligand molecules[b]	7.2	7.4	4.5

[b] $Q'_{max} = (Q_{mas}/M)(N_A/\rho)$ and $Q''_{max} = (Q_{mas}/Q^*)/\rho$. For definition of terms, see footnote *b* in Table 2.

Table 4. Characteristics of Protein-Bearing Latex Particles

samples	max no. of protein molecules coupled to one latex microsphere: theoretical[a]	max no. of protein molecules coupled to one latex microsphere: experimental[b]	covering of latex surface with protein, %
TR−latex	1600	1100	68.8
TR−FI−latex	1600	830	51.9
TR−FIRP−latex	400	390	97.5
TTR−latex	585	270	46.2

[a] $S_{sphere}/S_{protein\ cross\ section}$. [b] $N_{molecules\ of\ protein}/N'_{microspheres}$.

storage in a refrigerator (PBS buffer, 4 °C). It should be noted that in several experiments the milk blocking procedure has been excluded from a protocol and the data obtained in this case appeared practically identical to those established with a blocking step. This fact allows simplification and speeding up the analysis.

Virus-Mimicking Particles Construction. The beads used for development of model virus-like particles must meet requirements such as availability of surface adsorptive ligands and particle sizes close to virus dimension. The latex particles (80 nm) chosen for development of virus models were close to the dimensions of human viruses (80−120 nm). The covalent binding of different ligands (TR and TTR, as well as TR−Fl and TR−HRP) to the surface of carboxylated latex can occur only after activation of carboxylic groups with water-soluble carbodiimide.[17,18] The characteristics of macroparticles obtained are presented in Table 4. Filling of the latex surface with protein was estimated as a ratio of the calculated and experimentally found numbers of protein globules coupled to one latex particle (%). Theoretically possible value means a number of protein molecules capable to be placed on a surface of one spherical particle and was defined by a ratio of outer surface area of spherical particle to protein cross-sectional area. The latter was calculated from the protein radius: about 2 nm for SBTI, TR, and TR−Fl, 4.0 nm for TR−HRP, and 3.3 nm for TTR. Data from the Lowry test allow determination of the experimental amount of protein molecules bound to one latex microsphere. Recently it was established that the properties of a formed surface could be related to ones of bound ligands.[10,26]

Quantitative Analysis. To evaluate the efficiency of the developed test system, the affinity binding parameters of model biospecific pairs, namely, maximum adsorption capacity (Q_{max}) and thermodynamic constant of affinity pair formation (K_{diss}), were determinated by a frontal analysis procedure on monolithic ultrashort columns, as well as on a microarray plate. The adsorption isotherm obtained from frontal analysis data is demonstrated in Figure 3a for the example of the TR−Fl−SBTI pair, whereas its linearized form is given in Figure 3b. Latex particles bearing TR or TTR, as well as corresponding native proteins, have been chosen as a soluble part of the studied pairs, and respectively, SBTI and mAb played a role of counterparts immobilized on a CIM epoxy disk or GMA−EDMA layer.

The method of solid-phase densitometry based on a comparison of intensities of standard and analyzed spots was used for detection and quantitative estimations in microarray format.

HPMDAC Analysis. Fast affinity chromatography was used as the most appropriate tool to check and compare some factors important for microarray operations. These are ligand immobilization capacity with preservation of 3-D protein structure and affinity binding capacity of target objects that, in its turn, can be counted as a criterion of affinity ligand access. As the maximum adsorption capacity does not change at different flow rates for the case of monoliths, 8.8 column volume/min (3 mL/min) flow rate has been chosen as a reasonable optimum in all chromatographic experiments.

Similar to common rules of chromatography on monolithic stationary phases, the openess of the porous surface allows easy

(26) Menshikova, A.; Dmitrieva, I.; Kuchuk, V.; Skurkis, Yu.; Evseeva, T.; Shabsel's. B. *Russ. Colloid J* **1999**, *61*, 740.

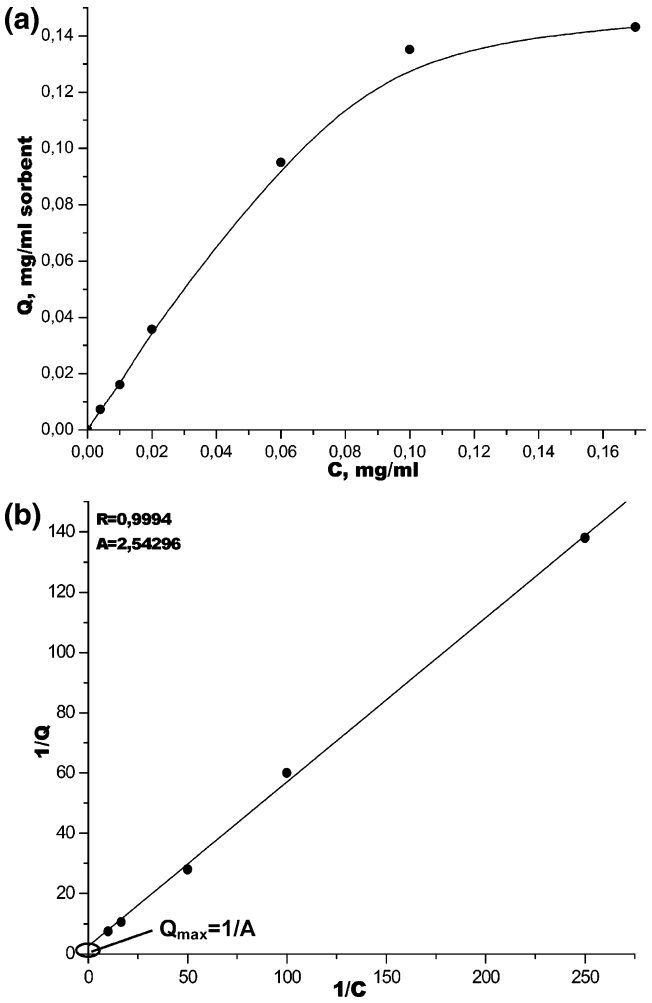

Figure 3. The adsorption isotherm (a) and its linearized form (b) built on the base of microarray data. SBTI-TR is the examined pair with use of fluorescent marker; C is a concentration of TR in spotted solutions; Q is the amount of adsorbed protein determined by fluorescence microscope from preliminary built calibration curve.

access of affinity ligands regarding their binding to the large supramolecules (particles). This rule is fulfilled even at static conditions; however, in this case, to reach the value of dynamic binding capacity, much more time is required (Table 2). This result can be explained by the very low diffusivity of protein-bearing latex particles within a rigid macroporous space in the absence of convective flow. Indeed, the calculated value of their diffusivity at convection (dynamics) by almost 3 orders exceeds the value of the free diffusion coefficient.[10] Evidently, K_{diss} remains practically constant for various methods of affinity interaction (Table 2). The significant decrease of maximum adsorption capacity for protein-bearing latex particles in comparison with native proteins agrees with earlier published results.[10]

Quantitative Analysis on Microarrays. Typically, the analysis in clinical diagnostics is usually based on a "yes" or "no" principlel e.g., it is necessary only to confirm or to reject the presence of target marker. However, to know the quantitative content of virus in a sample of biological material can be quite useful, for example, at vaccine production. Moreover, quantitative evaluation on an opaque 3-D layer usually meets a particular problem. We suggest a simple and effective method of quantitative estimation of affinity complexation, realized on thin opaque layers based on the GMA−EDMA copolymer. Besides that, in contrast to the most known arrays, the constructed devices can be used repeatedly because a destruction of affinity complex can be achieved by short-time boiling of a device in 0.01 M HCl (pH 2.0). Then a biochip is carefully washed by water/buffer and can be used again without any risk of loss of ligand activity.

In this study, we have tested the capability of transfer of the widely used ELISA technique to our 3-D microarray format. In this case, mAbs were covalently bound to the polymer solid-#phase and captured TTR−latex particles were quantified using the next adsorption layer of mAbs labeled with horseradish peroxidase. The quantification is based on a registration of light-absorbing product of the enzyme−substrate (TMB) reaction. The limit of sensitivity for the sandwich immunoenzyme assay was found as 7.1×10^7 TTR-bearing latex particles/spot (12 fmol/spot) and 1.8×10^{10} TTR molecules/spot (30 fmol/spot).

According to preliminary static experiments carried out with GMA−EDMA disks, 90 min was needed to achieve the maximum affinity adsorption capacity obtained at dynamic conditions. As the average thickness of the microarray's layer is 7.5 times less than a half-length of CIM column, 15-min affinity binding appears to be enough to reach the same Q_{max}.

The value of maximum adsorption capacity obtained by such a method was found slightly less than that established at both dynamic and static HPMDAC modes (native protein and TTR−latex cases) (Table 2). However, this difference can not be explained by the correct comparison of data obtained using direct and sandwich technologies. It should be noted that calculated K_{diss} obtained for the chosen affinity pair remains practically constant for various formats (disk and layer).

As an alternative to the sandwich assay, protein-containing samples (native TR or TR-bearing latex particle) were labeled with either fluorescent or horseradish peroxidase tags followed by direct detection after affinity binding (first case) and enzyme reaction with TMB (second case). The images of microarrays with colored and fluorescent zones of TR and TR-bearing particles are presented in Figure 4. Data obtained by the direct method (Table 3) indicate that affinity binding parameters of the SBTI−TR pair, both in affinity HPMDC and microarray formats, for the case of fluorescent marker are rather close. Slightly lower values, obtained

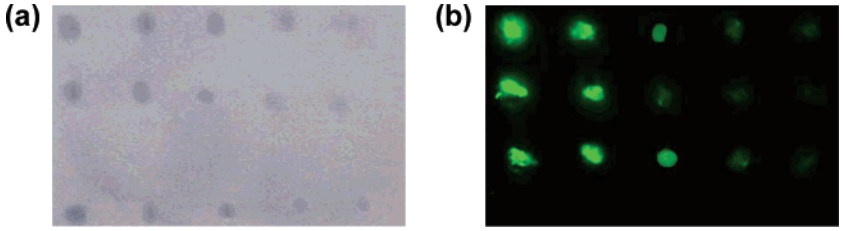

Figure 4. The images of colored (a) and fluorescent (b) zones of TR and TR-bearing particles. *Conditions:* (a) Line 1: TR-HRP-bearing latex particles; Line 2: TR-HRP; Line 3: calibration (b) Line 1: TR-Fl-bearing latex particles Line 2: TR-Fl; Line 3: calibration See details in Experimental.

with the HRP label, can be explained by the essentially larger size of the HRP molecule in comparison with TR that definitely causes the limitations at affinity binding to immobilized partner. However, as it follows from Table 3, the value of K_{diss} in the case of HRP labeling is almost half in comparison to that obtained at fluorescent analysis.

The limit of sensitivity of the discussed model test system was found as 2.1×10^7 TR-bearing latex particles/spot (3.5 fmol/spot) and 2.5×10^{10} TR molecules/spot (42.0 fmol/spot) for an immunoenzyme assay. The use of a fluorescent label permits us to detect 9.8×10^7 TR-bearing particles/spot (16.3 fmol/spot) and 20×10^{10} TR molecules/spot (0.3 pmol/spot). Most probably, increase of the sensitivity limit for the case of the fluorescent marker was caused by decaying of the fluorescent signal during conjugate preparation.

The data obtained confirm the assumption of similar adsorption behavior of macroparticles on two types of supports based on the GMA−EDMA polymer. Optimal porous structure provides the opportunity of efficient affinity interactions, including those with participation of very large objects. Moreover, maximum adsorption capacities, as well as thermodynamic constants of affinity interaction for studied affinity pairs, seemed to be close, and insignificant differences can be reasonably explained. The detecting system based on use of a fluorescent label was optimized and can be recommended for application in test systems for virus infection discovery.

CONCLUSIONS

Recently it has been shown that the permeability of porous space of GMA−EDMA monolithic sorbents appeared to be sufficient for efficient separation of large particles and quite similar to the well-studied process developed for individual proteins. In this paper, the analogous separation process was transferred to the nonflowing separation system, namely, specially designed GMA−EDMA macroporous monolithic layes (microarrays).

Similar to data obtained in high-throughput chromatographic format, for the case of GMA−EDMA microarrays, the adsorption capacity decreased with increase of size of analyte from native protein to protein-bearing latex particles.

For the first time, the quantitative characteristics of affinity binding were measured for microarray systems. The detection limit for the sandwich immunoenzyme assay was found as 7.1×10^7 TTR-bearing particles/spot, whereas for thr direct method it was $(2.1-9.8) \times 10^7$ TR-bearing particles/spot depending on marker used. The affinity binding parameters obtained for the microarray were rather close to those established at affinity chromatographic conditions with use of the same GMA−EDMA monolith as a stationary phase."

ABBREVIATIONS

CDI	1-ethyl-3(3-dimethylaminopropyl)-carbodiimide
CIM	Convective Interaction Media
Fl	fluorescamine
GMA−EDMA	glycidyl methacrylate-*co*-ethylene glycol dimethacrylate
HOBt	1-hydroxybenzotriazole
HPMDC	high-performance monolithic disk affinity chromatography
HRP	horseradish peroxidase
mAb	monoclonal antibody
MES	2-(*N*-morpholino)ethanesulfonic acid
PBS	phosphate buffered saline
SEC	size-exclusion chromatograph
SBTI	soybean trypsin inhibitor
TMB	3,3′,5,′-tetramethylbenzidine
TR	trypsin
TTR	transthyretin

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