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Development of a Strategy of Influenza Virus Separation Based on Pseudoaffinity Chromatography on Short Monolithic Columns

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This research is devoted to the development and optimization of fine purification processes realized on short monolithic columns (CIM disks), using influenza vaccine and viruslike synthetic particles as model objects. The pseudoaffinity mode of liquid chromatography has been used as a tool for dynamic adsorption experiments. Viruslike particles, close to the dimensions of influenza viruses, were developed by means of main antigen of influenza viruses (hemeagglutinin) covalent binding to the outer aminated surface of synthetic latex particles. The natural receptor analogues of sialic acid were used as affinity ligands immobilized on the surface of the CIM disk by different ways to achieve a high adsorption capacity. Also, some other ligands were tested as possible candidates for virus capturing. The affinity binding parameters for influenza A virus were obtained by frontal elution method at optimized chromatographic conditions and immobilization schemes. The experimental data pointed out the possibility of selective isolation of hemeagglutinin from a mixture of vaccine proteins. The results obtained by fast affinity chromatography have shown functional and sterical correspondence viruslike synthetic models to influenza viruses. Additionally, the optimization of chromatographic conditions allowed isolation of influenza virus A while maintaining its virulence. The maximum value of adsorption capacity was registered for a monolithic disk, modified subsequently by chitosan and 2,6-sialyllactose and found to be equal to 6.9×10^{12} virions/mL support.

Influenza remains, due to its annual death rate and potential to cause pandemics, a major public health concern. The efforts to control the annual spread of influenza have focused on prophylactic vaccinations. Human influenza vaccines are traditionally produced in embryonated hen's eggs and specific cell cultures. The latter crude material containing virus requires thorough purification from cell components (the components of nonviral origin) without lost of biological activity of antigenic determinates during vaccine manufacturing.

Different modes of liquid chromatography, namely, ion exchange, size exclusion, and others, as well as their combination, are widely used at influenza virus purification.[1−3] The most delicate and highly selective virus isolation can be realized using affinity chromatography. Recently obtained data illustrated that monolithic material on the base of a copolymer of glycidyl methacrylate (GMA) and ethylene glycol dimethacrylate (EDMA) seemed to be suitable for construction of biorecognizing systems in two formats,[4,5] both for isolation of viruslike particles by fast affinity chromatography and for their sensitive detection (diagnostics) using miccroarray technique. Besides that, the chromatography on short methacrylate-based monolithic columns (Convective Interaction Media, CIM, disks), demonstrating unique hydrodynamic and separation properties,[6−8] seems to be preferable to overcome many critical disadvantages of conventional separation techniques concerning large bioobjects' separation.[9−14] Because of superior mass transfer and open porous structure (totally permeable for a flowing liquid large pores), the monoliths will be able to provide very fast biospecific pair formation involving viruses that reduces the risk of product degradation.

It is known that the distinct terminal sialic acid species (*N*-acetylneuraminic acid) on the cell membrane surface serves as a hemeagglutinin-binding receptor to induce the penetration of the interior of influenza viruses by membrane fusion.[15,16] In this

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(1) Kalbfuss, B.; Wolff, M.; Morenweiser, R.; Reichl, U. *Biotechnol. Bioeng.* **2007**, *96*, 932−944.
(2) Opitz, L.; Salaklang, J.; Büttner, H.; Reichl, U.; Wolff, M. *Vaccine* **2007**, *25*, 939−947.
(3) Zhao, R.; Fang, C.; Yu, X.; Liu, Y.; Luo, J.; Shangguan, D.; Xiong, S.; Su, T.; Liu, G. *J. Chromatogr., A* **2005**, *1064*, 59−66.
(4) Kalashnikova, I.; Ivanova, N.; Evseeva, T.; Menshikova, A.; Vlakh, E.; Tennikova, T. *J. Chromatogr. A* **2007**, *1144*, 40−47.
(5) Kalashnikova, I.; Ivanova, N.; Tennikova, T. *Anal. Chem.* **2007**, *9*, 5173−5181.
(6) Josic, Dj.; Buchacher, A. *J. Biochem. Biophys. Methods* **2001**, *49*, 153−174.
(7) Branovic, K.; Lattner, G.; Barut, M.; Strancar, A.; Josic, Dj.; Buchacher, A. *J. Immunol. Methods* **2002**, *271*, 47−58.
(8) Jungbauer, A. *J. Chromatogr. A* **2005**, *1065*, 3−12.
(9) Kramberger, P.; Glover, D.; Strancar, A. *Am. Biotechnol. Lab.* **2003**, *21*, 27−38.
(10) Branovic, K.; Forcic, D.; Ivancic, J.; Strancar, A.; Barut, M.; Kosutic-Gulija, T.; Zgorelec, R.; Mazuran, R. *J. Virol. Methods* **2003**, *110*, 163−171.
(11) Kramberger, P.; Petrovic, N.; Strancar,, A.; Ravnikar, M. *J. Virol. Methods* **2004**, *120*, 51−57.
(12) Barut, M.; Podgornik, A.; Brne, P.; Strancar, A. *J. Sep. Sci.* **2005**, *28*, 1876−1892.
(13) Williams, S.; Eccleston, M.; Slater, N. *Biotechnol. Bioeng.* **2005**, *89*, 783−787.
(14) Kramberger, P.; Peterka, M.; Boben, J.; Ravnikar, M.; Strancar, A. *J. Chromatogr. A* **2007**, *1144*, 143−149.
(15) Ito, T. *Microbiol. Immunol.* **2000**, *44*, 423−430.
(16) Gambaryan, A.; Piskarev, V.; Yamskov, I.; Sakharov, A.; Tuzikov, A.; Bovin, N.; Nifant'ev, N.; Matrosovich, M. *FEBS Lett.* **1995**, *366*, 57−60.

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research, we designed a biorecognizing chromatographic system using discussed monoliths as the most efficient supports and sialic acid derivates as the basic ligands. Additionally, glucosamine-containing carbohydrates and one pharmaceutical antivirus drug as possible ligand candidates were used in our investigation. The functionality of ligands immobilized on the surface of CIM disk in different ways was studied by means of a zonal elution approach, first, using influenza vaccine and, then, intact influenza A virus. The optimized chromatographic conditions and immobilization techniques were then transferred on a determination of affinity binding parameters by a frontal elution method for virus-mimicking synthetic particles to compare those obtained for influenza A virus, grown in embryonic hen's eggs. Besides that, the affinity interaction between influenza A virus, produced in VERO cells, and natural bioactive substances as probable affinity ligands immobilized on a monolithic disk surface was also studied.

EXPERIMENTAL SECTION

Chemicals. Sialyllactose (SL), heparin (H), low weight chitosan (Ch), ammonium bicarbonate, ninhydrin, bovine serum albumin (BSA), 2-(*N*-morpholino)ethanesulfonic acid (MES), water-soluble 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (CDI), succinic anhydride, sodium periodate, Shiff's reagent, acrylamide, bisacrylamide, *N*,*N*,*N*′,*N*′-tetramethylethylendiamine, ammonium persulfate, glycine, β-mercaptoethanol, bromophenol blue, formaldehyde, sodium thiosulfate, potassium hexacyanoferrate, 1,1′-carbonyldiimidazole, bicinchoninic acid solution, snd copper(II) sulfate were from Sigma-Aldrich Chemie. Sodium dodecyl sulfate (SDS), acetic acid, methanol, and 1-hydroxybenzotriazole were from Fluka AG, $NaHB_4$ was obtained from Reanal. Dulbecco's phosphate-bufferd saline (0.0095 M was purchased from BioWhittaker. Soybean trypsin inhibitor (SBTI) was delivered by PanEco. The aqueous ammonia solution was purchased from Vecton Ltd. The mixture of 14 recombinant proteins, PageRuler Unstained Protein Ladder, for SDS-PAGE was from Fermentas GmbH. Sephadex G-25 used for size exclusion chromatography (SEC) was from Amersham Biosciences. Remantadine (Rem, the trade name of α-methyltricyclo[3.3.1.1\3,7]decan-1-methanamine hydrochloride) was purchased from Acrikhin Ltd.

Ceruloplasmin (CP) was kindly donated by Prof. M. M. Shavlovsky, Institute of Experimental Medicine, Russian Academy of Medical Sciences, St. Petersburg, Russia.

The liquid inactivated purified influenza vaccine "Grippovac" was purchased from Immunotex Ltd.

Suspensions of latex particles were kindly donated by Dr. A. Yu. Menshikova and the copolymer of 2-deoxy-*N*-methacrylamido-D-glucose (MAG) with 1-vinyl-2-pyrrolidone (VP) and diethylacetal of acrolein (DAAc) [poly(MAG-*co*-VP-*co*-DAAc)' by Mr. V. A. Korzhikov, both from the Institute of Macromolecular Compounds, Russian Academy of Sciences, St. Petersburg, Russia.

The influenza A virus PR/8/34 was a kind gift of Prof. A. Egorov, Influenza Institute, Russian Academy of Medical 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. High-performance monolithic disk affinity chromatography (HPMDAC) experiments were performed using high-performance chromatograph combining two piston pumps 303 and 305 (Gilson Ltd.) and a UV detector LKB (Unicord S 2138). Also high-performance chromatograph HPLC-9 Knauer combining two HPLC pumps K-1000 and a UV detector Knauer K-260 was used. The concentration of eluted proteins was determined by BCA test using the analyzer Sunrise, Tecan Austria GmbH. Quantitative analysis of substances was realized with use of spectrophotometer SmartSpec 3000, Bio-Rad Laboratories GmbH and UV−vis spectrophotometer SF-26, LOMO for measurements of UV absorbance at different wavelengths.

Methods. (1) Amination of Sialyllactose. Amination of sialyllactose (SL) was achieved using described procedures.[17] A 25-mg sample of sialyllactose, mixture of α(2−3) and α(2−6) linked isomers, was added to 2 mL of water solution of ammonium bicarbonate with a concentration of 0.81 mg/mL. The obtained solution was stirred in an open vessel at 37 °C for 3 days. The ammonium bicarbonate (total amount, 1.63 g) was added at intervals to ensure saturation. Further, the solution was diluted with distilled water up to 20 mL and then concentrated to 2 mL in a rotary evaporator. The excess of ammonium bicarbonate was removed by repeating this procedure four times.

(2) Isolation of α-Methyltricyclo[3.3.1.1\3,7]decan-1-methanamine Hydrochloride (Remantadine) from a Pharmaceutical Form. The solution, obtained by dissolving two pills (50 mg of active substance per pill) in a mixture of ethanol, 0.01 M PBS, pH 7.3 (80:20), was filtered and then alcohol was evaporated.

(3) Preparation of Affinity Sorbents. CIM epoxy disks were used for modification by amino-bearing substances (proteins, sialyllactosylamine, chitosan, α-methyltricyclo[3.3.1.1\3,7]decan-1-methanamine). According to a previously developed approach,[18,19] the initial concentration in 0.0125 M borate buffer (pH 9. 3) of proteins and sialyllactosylamine was equal to 5 mg/mL ($V = 2$ mL). Initial quantity of α-methyltricyclo[3.3.1.1\3,7]decan-1-methanamine hydrochloride was 20 mg/mL in 0.01 M PBS (pH 7.3). The disks were exposed to modification for 16 h at 22 °C.

Chitosan's initial concentration in 0.05 M acetate buffer, pH 6.0, was equal to 5 mg/mL in the first case, and 10 and 25 mg/mL in the next two cases. The reaction occurred at stirring for 32 h at 22 °C in all cases. Then chitosan, immobilized on a disk surface, was modified by sialylactosylamine, in one case, and by 2,6-sialyllactose, in another. The reaction of activation of chitosan hydroxyl groups by 1,1-carbonyldiimidazole in 0.01 M borate buffer (pH 8.0) proceeded for 3 h at 20 °C. The small volume of a solution was loaded on a disk by syringe. The reaction of activation by 1,1-carbonyldiimidazole, taken in equimolar ratio to the amount of initial epoxy groups (4 mmol/g), in 0.01 M PBS, pH 7.0, was allowed to proceed for 3 h at 20 °C. After that the disk was washed at flow in 0.01 M borate buffer (pH 8.0) to remove unbound reagent and put into the same buffer, containing sialyllactosylamine or 2,6-sialyllactose (5 mg/mL concentration). The reaction of immobilization of affinity ligands was allowed to proceed for 24 h at 20 °C.

(17) Tsuchida, A.; Kobayashi, K.; Matsubara, N.; Muramatsu, T.; Suzuki, T.; Suzuki, Y. *Glycoconjugate J.* **1998**, *15*, 1047−1054.
(18) Ostryanina, N.; Lojkina, O.; Tennikova, T. *J. Chromatogr., B* **2002**, *770*, 35−43.
(19) Platonova, G.; Pankova, G.; Il'ina, I.; Vlasov, G.; Tennikova, T. *J. Chromatogr., A* **1999**, *852*, 129−140.

Modification of immobilized protein (SBTI, BSA) surface by sialyllactosylamine was carried out using water-soluble carbodiimide. First, a solution of water-soluble carbodiimide in 0.01 M MES buffer (pH 5.5) was added by a syringe to the cartridge with an installed disk. The reaction of activation took place over 30 min at 0 °C. Second, the disk was washed at flow with 0.01 M borate buffer (pH 8.5) to remove unbound reagent. Finally, the same buffer, containing sialyllactosylamine, taken in 4-fold excess to the carboxylic groups of protein, was loaded by syringe to the cartridge with installed disk. The reaction of immobilization was allowed to proceed for 4 h at 20 °C.

Sialyllactose (the mixture of two isomers) and heparin were immobilized on a surface of 1,1′-carbonyldiimidazole-activated methacrylate-based monolith disk (CIM CDI disk). The solution of sialyllactose (12.5 mg/mL) in 0.01 M borate buffer (pH 8.0) was loaded several times by a syringe to the cartridge with an installed disk. The reaction took place for 16 h at room temperature.

For the purpose of modification by heparin, the disk was immersed for 16 h at room temperature into 2 mL of 0.125 M borate buffer (pH 9.3) containing 10 mg of heparin.

The epoxy groups of the macroporous polymer disk were converted to primary amino groups using aqueous ammonia solution.[20] Before the immobilization, the disk was washed with 0.1 M PBS (pH 4.5). For immobilization of SBTI, succinylated according to the procedure described elsewhere[21] (SBTI-S), on an aminate surface of a sorbent, COO^- groups of SBTI-S were activated for 30 min by water-soluble carbodiimide. The disk was then immersed into this solution and after 30 min 9 mg of SLA was added. The pH of the solution was adjusted to an alkaline value by NaOH. The reaction proceeded for 4 h at 20 °C.

The disk containing the amino groups also was applied for immobilization of poly(MAG-*co*-VP-*co*-DAAc) by a direct reaction of monolith reactive groups with copolymer aldehyde groups. Before the immobilization, the aldehyde groups in a poly(MAG-*co*-VP-*co*-DAAc) chain were generated by a deprotection procedure, i.g., a removal of acetal groups, according to ref 22. The disk was immersed into a solution of 10 mg of copolymer/mL of 0.01 M borate buffer (pH 9.3), and a reaction of immobilization continued during 4 h at 20 °C. The obtained labile azomethine bonds, after consequent washing of the disk by 2 M NaCl and water, were reduced by sodium borohydride ($NaBH_4$), taken in a concentration of 10 mg/mL and a 2-mL volume. The reaction took place for 2 h at 4 °C using 0.01 M borate buffer (pH 9.3) as $NaBH_4$ solvent. Further, the sugar rings belonged to the immobilized copolymer were oxidized for 24 h by treating of $NaJO_4$, taken in 5-fold excess to the content of 2-deoxy-2-methacrylamido-D-glucose (MAG).[23] The composition of used copolymer MAG:VP:DAAc was 75:18:7 mol %. After the reaction (4 °C, in the dark, slight stirring) was finished, the disk was thoroughly washed by distilled water. Before oxidation of surface-immobilized copolymer, a blank experiment was done and the formation of aldehyde groups was verified via the reaction with Schiff's reagent. The aldehyde groups obtained were exposed to a modification by SLA for 4 h at 20 °C. For reaction, the solution with a concentration of SLA of 5 mg/mL in 0.01 M borate buffer, pH 8.5, was used.

After the reaction was completed, in all cases, the disks were washed at flow with 2 M NaCl and, finally, with distilled water. The additional quenching of residual epoxy groups was not carried out.

(4) Quantification of Ligand Immobilization and Protein Modification. The amount of ligand, covalently bound to the sorbent surface, was calculated via the difference of ligand concentration before and after the reaction considering the ligand's content in washing fractions.

The quantitative estimation of SLA was carried out using a reaction with ninhydrin. A 0.5-mL aliquot of a 1% water solution of ninhydrine was added to 2.5 mL of the analyzing solution containing SLA. The solution obtained was intensively stirred and heated for 20 min at 120 °C in thermostated bath. The calibration curve of absorbency on SLA concentration was built at maximum absorption ($\lambda = 540$ nm). The detection limit was found as 2 μg/mL. The calibration dependence of optical density on the concentration of SLA obtained at maximum absorption, 242 nm, was also applied for determination of SLA in solutions.

Standard Lowry test[24] was used to measure a protein concentration.

Quantification of sialylactose in the solutions was carried out spectrophotometrically at maximum absorption 272 nm.

The calibration dependence of the absorbency value on heparin concentration was obtained at maximum absorption, namely at 243 nm, and used for evaluation.

The calibration curve of optical density on a concentration of chitosan obtained at $\lambda_K = 360$ nm was applied for chitosan quantification.

The 2.5 mL of 1% water solution of ninhydrin was added to 0.5 mL of analyzing solution containing Remantadine. The solution obtained was intensively stirred and heated for 20 min at 110 °C in a thermostated bath. The calibration dependence of absorbency on α-methyltricyclo[3.3.1.1\3,7]decan-1-methanamine hydrochloride concentration was established at maximum absorption ($\lambda = 540$ nm).

The iodine test was used for quantitative evaluation of poly(MAG-*co*-VP-*co*-DAAc).[23]

The concentration of ligands immobilized on CIM surface is show in Tables 1 and 2.

(5) Functionalization of Latex Particles by Hemeagglutinin (L-HA). Amino-bearing polysterene monosize nanospheres of 80-nm diameter with 1 μmol of NH_2/m^2 outer surface were applied for construction of virus-mimicking particles.

Carboxylic groups of 1 mg of hemeagglutinin (HA) were activated by water-soluble carbodiimide in 0.01 M MES buffer (pH 5.5) at 0 °C, 30-min reaction time. Water-soluble carbodiimide was taken in equimolar ratio to the carboxylic groups of HA. Unreacted carbodiimide was removed from the solution by SEC, using 0.01 M borate buffer (pH 8.5) as a mobile phase. After the activation step, the protein solution obtained was added to 4.5 mg of polymer particles (1.5 g of particles/100 mL of water) preliminarily washed

(20) Ostryanina, N.; Vlasov, G.; Tennikova, T. *J. Chromatogr., A* **2002**, *949*, 163−171.
(21) Vlasov, G.; Illarionova, N.; Izvarina, N.; Denisov, I. *Macromol. Chem.* **1985**, *9*, 239−249.
(22) Vlakh, E.; Panarin, E.; Tennikova, T.; Suck, K.; Kasper, C. *J. Biomed. Mater. Res. A* **2005**, *75*, 333−341.
(23) Lowry, O.; Posebrough, N.; Farr, A.; Randall, P. *J. Biol. Chem.* **1951**, *193*, 265−275.
(24) Korzhikov, V.; Nazarova, O.; Vlakh, E.; Panarin, E.; Diederichs, S.; Kasper, C.; Tennikova, T. *J. Appl. Polym. Sci.* In press.

Table 1. Influenza Vaccine Adsorption Capacity, Obtained for Different Affinity Sorbents[a]

disk	SLA	CP	SBTI-SLA	SBTI-S-SLA	BSA-SLA	MAG-VP-DAAc-SLA
ρ^b	15.7	0.8	0.6	1.4	3.5	5.1
Q ($\times 10^2$)[c]	0.7	7.9	26.6	16.0	1.2	1.0

[a] Conditions: The solution of influenza vaccine in the 0.05 M acetate buffer (pH 6.0) was passed through the column (disk), equilibrated with same buffer, up to totally sorbent saturation. Flow rate was 2.0 mL/min. The desorption was achieved by 12.5 mM borate buffer (pH 10.7). An intermediate washing procedure with 2 M NaCl was used. [b] ρ, SLA concentration, SLA μmol/mL sorbent. [c] Q, adsorption capacity, HA mol/SLA mol in %.

Table 2. Results Obtained by Zonal Analysis Using Different Affinity Disks[a]

ligand	ρ,[b] μmol/mL sorbent	conditions: elution, pH	test titer, HA units/mL
Rem	459.0	0.2 M NaOH, 14	7
Ch	0.3		20
SL	17.0		27
Ch-SL	2.3−13.0		27
Ch-SLA	1.0−18.5		13
H	1.6	0.4 M NaCl,7.0	13

[a] Conditions: Fixed volume (1.0 mL) of solution of influenza A virus with 1.1 mg of protein/mL concentration was loaded on the each disk at flow rate 2 mL/min. A 0.05 M acetate buffer (pH 6.2) was used as the adsorption buffer. An intermediate washing procedure with 0.4 M NaCl in 0.01 M PBS was used to release a nonspecifically bound part of protein except disk, contained heparin. [b] ρ, ligand's concentration.

Table 3. Influenza Vaccine Composition

quantity of protein, μg/mL				
		hemeagglutinin		
		A virus		B virus
total protein	ovalbumin	H1	H3	
2100	0.04	19.2	18.8	25.6

by 0.01 M borate buffer, pH 8.5. Unbound ligand was removed from the suspension by dialysis against distilled water. The amount of protein coupled to amino groups of nanospheres was determined by standard Lowry test.[24]

(6) Affinity Chromatography. (a) Isolation of Hemeagglutinin from Vaccine. The protein (HA) binding capacity for six prepared affinity disks (Table 1) was determined for the solution of vaccine with concentration of 1 mg of protein/mL of buffer solution. The vaccine composition is presented in Table 3. The titer of the stock solution of influenza vaccine on the hemeagglutination test was 1:120 for H1N1, 1:128 for H3N2, and 1:64 for B virus. Vaccine was purified by dialysis against distilled water and then concentrated up to 1 mL by rotary evaporation. The prepared solution (1 mg/mL) of influenza vaccine in 0.05 M acetate buffer (pH 6.0) was passed at 2 mL/min flow rate through each of six columns (disks) (Table 1) equilibrated with the same buffer up to total sorbent saturation. Desorption was achieved using 12.5 mM borate buffer (pH 10.7). An intermediate washing procedure with 2 M NaCl was used to release a nonspecifically bound part of proteins. Standard Lowry test[24] was used to measure a protein concentration in eluted fractions. The experiments were repeated three−five times, and experimental error was estimated as ±7%. All desorbed fractions were subjected to SDS-PAGE analysis.

(b) HPMDAC of Viruslike Particles. Chromatographic experiments were fulfilled in a mode of frontal elution with the aim to build experimental adsorption isotherms; from those, the maximum adsorption capacity (Q_{max}) was calculated. For that, the suspensions of modified nanospheres with different concentrations (0.002−0.05 mg of bound protein/mL) were passed through the disk-SBTI-SLA up to total saturation of all sorbent adsorptive sites. Flow rate was 2.0 mL/min. A 0.05 M acetate buffer (pH 6.0) was used at the adsorption step, whereas the desorption was achieved with 12.5 mM borate buffer (pH 10.7). An intermediate washing procedure with 2 M NaCl was used to release a nonspecifically bound part of protein. Standard Lowry test was used to measure a protein concentration in eluted fractions. The results of frontal analysis represent the average values calculated from linearized forms of adsorption isotherms using the Langmuir equation. At linearization, the standard deviation was found as 0.9985−0.99913 (three replicates).

(c) Zonal Analysis of Influenza A Virus. The method of zonal adsorption−desorption runs was used to optimize the chromatographic conditions for six prepared affinity disks (Table 2). For that, a fixed volume (1.0 mL) of influenza A virus solution with 1.1 mg of protein/mL concentration was loaded on each disk at a flow rate 2 mL/min. A 0.05 M acetate buffer (pH 6.2) was used for adsorption, whereas the desorption in all cases except for disk, modified by heparin, was achieved by 0.2 M NaOH, 14. A intermediate washing procedure with 0.4 M NaCl in 0.01 M PBS (pH 6.5) was used to release a nonspecifically bound part of protein, except the disk containing heparin as a ligand. All collected fractions were analyzed by the hemeaglutination test and BCA assay to measure the protein concentration. The experiments were repeated three times, and experimental error was established as ±7−10%.

(d) HPMDAC of Influenza Virus (Frontal Analysis). Influenza A virus (A/PR/8/34) was grown in the allantoic sacs of 10-day-old embryonic eggs and then clarified by 0.2-μm filtering. The hemeagglutination titer of the stock solution of influenza virus was more than 32 HA units. These samples were used for testing using the frontal elution method on the disk with immobilized SBTI, modified by sialyllactosylamine (disk-SBTI-SLA) and the disk with immobilized SBTI-S modified by sialyllactosylamine (disk-SBTI-S-SLA).

The influenza A virus PR/8/34 (H1N1) grown on VERO cells was also used in chromatographic experiments without additional clarification. Harvested virus had a titer of ~200 HA units/mL according to the hemeagglutination test. Affinity binding parameters for these samples of influenza A virus were obtained by a frontal analysis method on two affinity disks: the disk modified by sialyllactose (disk-SL) and the disk with immobilized chitosan further modified by sialyllactose (disk-Ch-SL).

Table 4. Data of Frontal Analysis of Influenza A Virus and Hemeagglutinin-Bearing Latex Particle[a]

	virus				L-HA
disk[b]	SBTI-S-SLA	SBTI-SLA	SL	Ch-SL	SBTI-SLA
ρ	1.4	0.6	17.0	13.0	0.6
Q_{max}	0.14	0.11	0.19	0.47	0.03
$Q \times 10^{-12}$	2.0	1.6	2.8	6.9	1.9*
titer	20	20	27	67	not data

[a] Conditions: The suspensions of modified nanospheres with different concentrations (0.002−0.05 mg of protein/mL) were passed through the disk with immobilized ligand up to total saturation of all adsorptive sites of sorbent. The solution of influena A virus with different concentrations (0.03−3.0 mg of protein/mL) were passed through the disk with immobilized ligand up to total saturation of all adsorptive sites of sorbent. Flow rate was 2.0 mL/min. A 0.05 M acetate buffer (pH 6.2) was used as the adsorption buffer. Desorption was achieved high-alkaline buffer. An intermediate washing procedure with 0.4 M NaCl in 0.01 M PBS (for virus) and 2 M NaCl (for viruslike particles) was used to release a nonspecifically bound part of protein. [b] (1) $Q = ((Q_{max}k)/M)/\upsilon$. (2) Q (*) $= (Q_{max}/Q')$, where $Q' = q/N.Q$, maximum adsorption capacity (particles/mL sorbent) calculated using eqs 1 and 2; Q_{max}, maximum adsorption capacity,protein amount (mg) calculated from frontal data per milliliter of sorbent; k, value of ratio of hemeagglutinin molecule amount in virion to total molecules proteins amount in virion, equaled 0.74; M, molecular mass of hemeagglutinin; υ, mol quantity(number) of hemeagglutinin per virion; Q', protein amount (mg) bound to one latex nanosphere; q, protein amount bound to total number of nanospheres in a solution; N, number of nanospheres in a solution; ρ, affinity ligand's concentration (SLA, SL, or Rem μmol/mL sorbent); titer, units of hemeagglutination (HA units/mL), determined by hemeagglutination test.

The general procedure was as follows: the solution of influenza A virus of different concentrations (0.03−3.0 mg of protein/mL) was passed through the disk with immobilized ligand up to total saturation of all sorbent adsorptive sites. Flow rate was 2.0 mL/min. A 0.05 M acetate buffer (pH 6.2) was used at the adsorption step. Desorption was achieved with 0.0125 M borate buffer (pH 10.7) for disk-SBTI-SLA and disk-SBTI−S-SLA cases, whereas in other cases, the elution was performed using 0.2 M NaOH. An intermediate washing procedure with 0.4 M NaCl in 0.01 M PBS (pH 6.5) was used. All collected fractions were analyzed by the hemeaglutination test; standard Lowry test[24] or BCA assay[25] were used to measure the protein concentration. The desorbed fractions from disk-SBTI-SLA were subjected by culturing on Madin−Darby Canine Kidney (MDCK) cells. The results of frontal analysis (Table 4) represent the average values calculated from linearized forms of adsorption isotherms built with use of the Langmuir equation. At linearization, the standard deviation was equal to 0.9980−0.9985 (three replicates).

RESULTS AND DISCUSSION

The first task solved in this research was to isolate hemeagglutinin from a crude vaccine to use it later as a specific protein for virus modeling. The second task was to optimize the immobilization step of the chosen affinity ligand, namely, sialyllactose, to distance this small molecule from a rigid surface and, correspondingly, to provide it additional flexibility. For that, the proteins of various molecular size, synthetic copolymer, and natural polysaccharide were used as intermediate spacers. And, finally, the last task was to study comparatively the adsorption behavior of virus-mimicking particles and real influenza virus using fast affinity chromatography on the most effective solid matrix (monolith).

Affinity Ligands and Their Immobilization on Monolith Surface. As mentioned earlier, *N*-acetylneuraminic acid derivative as pseudoaffinity ligand for virus separation was chosen due to its complementation to hemeagglutinin located at the virus membrane. To distance the active center of immobilized affinity ligands from a sorbent surface, the sialyllactose (Figure 1a), with its second sugar ring as a short intermediate spacer, was chosen as a ligand to provide the better access of binding sites for isolated bioobjects.

The foresight of some peculiarities of carbohydrate derivates' functionality such as conformational changes, sterical accessibility, and binding to bioobjects ability induced us to studying and optimization of the immobilization step. First, sialyllactose, after its amination, was bound to the surface of monolithic epoxy GMA−EDMA sorbent (Figure 2a). Amination of sialyllactose was carried out practically with 100% conversion.[17] It should be noticed that the amount of a ligand immobilized directly (~16 μmol/g support) was significantly less than the conversion of epoxy groups (4 mmol/g support) achieved by, for example, sorbent treatment with ammonium hydroxide. In this case, the quantity of introduced amino groups was found to be 1−2 mmol/g support. In contrast, the conversion of epoxy groups of a sorbent at SLA binding was only 0.4%. Therefore, the initial sialyllactose was immobilized on the CDI-activated surface of a disk (Figure 2b) to compare the influence of two schemes of affinity ligand immobilization on the final ligand concentration. It was established that the amount of immobilized sialyllactose was practically identical for two used chemical methods (Tables 1 and 2).

In the third case, the protein ceruloplasmin (CP), which contains sialic acid on its surface, was used as a probable ligand. It is known that every molecule of ceruloplasmin has 32 *N*-acetylneuraminic acid residues.[26] So far, CP contains a sufficient number of amino groups, which can be used for its covalent binding to epoxy GMA−EDMA matrix (Figure 2c). The final amount of immobilized ceruloplasmin (0.05 μmol/g support) corresponded to 0.8 μmol of sialo-sugar residues/mL of sorbent.

The next approach was introduction of additional spacer between a matrix and sialyllactosylamine to facilitate access of the large bioobject to the active site of the immobilized small ligand. For that, SBTI was used as an affinity inert intermediate. The immobilization of SBTI was performed by ordinary means via the amino group of the protein, whereas the following binding of SLA to the protein surface was carried out using carbodiimide chemistry (Figure 2d). Most likely that parallel direct binding of amino derivates of sialyllactose to a sorbent surface did not take place because the reactive epoxy groups were hydrolyzed at the activation step, and thus, they lost the ability to bind primary amino groups. The quantitative yield at a second step can be characterized by a number of SLA molecules conjugated to one protein molecule. It was found to be equal to 4.6 molecules, which corresponded to 0.13 μmol of SBTI and 0.6 μmol of SLA (Table 1).

To increase the amount of SLA bound to the protein spacer, all active carboxylic groups located on the protein surface were

(25) Smith, P.; Krohn, R.; Hermanson, G.; Mallia, A.; Gartner, F.; Provenzano, M.; Fujimoto, E.; Goeke, N.; Olson, B.; Klenk, D. *Anal. Biochem.* **1985**, *150*, 76−85.

(26) Yamashita, K.; Liang, C.-J.; Funakoshi, S.; Kobata, A. *J. Biol. Chem.* **1981**, *256*, 1283−1289.

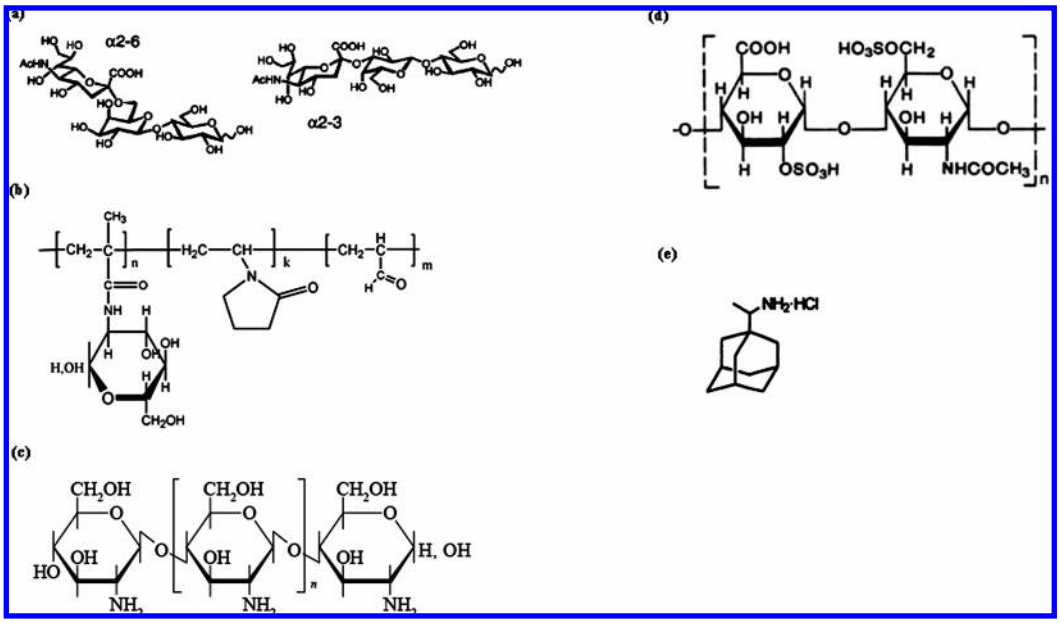


Figure 1. Chemical structure of ligands. (a) Sialyllactose, mixture of α(2−3)- and α(2−6)-linked isomers, (b) polyMAG-VP-DAAc, (c) chitosan, (d) heparin, and (e) α-methyltricyclo[3.3.1.1\3,7]decan-1-methanamine (Remantadine).

used as the next immobilization approach. For that, the same inert "spacer" (SBTI) was succinylated, and this step provided ∼100% yield of amino group substitution. Such a procedure (Figure 2e) has permitted us to obtain 1.37 μmol of SLA attached to 0.176 μmol of SBTI (per mL of sorbent); namely, one molecule of SBTI contained 7.8 SLA molecules (Table 1). The procedure of immobilization allowed excluding direct SLA binding to a sorbent surface.

It is known that spacer size can perform the fundamental function in sterical orientation of a ligand at its binding to the affinity partner.[20] That is why a larger protein molecule as an inert spacer, namely, BSA instead of SBTI, was also tested. The enlargement of protein surface used for SLA immobilization resulted in sufficiently high surface density of sialic ligand (3.5 μmol of SLA /mL of sorbent) (Table 1). In this case (Figure 2f), 25 SLA molecules appeared to be bound to 1 molecule of BSA. This result allowed expection of higher affinity adsorption capacity regarding hemeagglutinin purification.

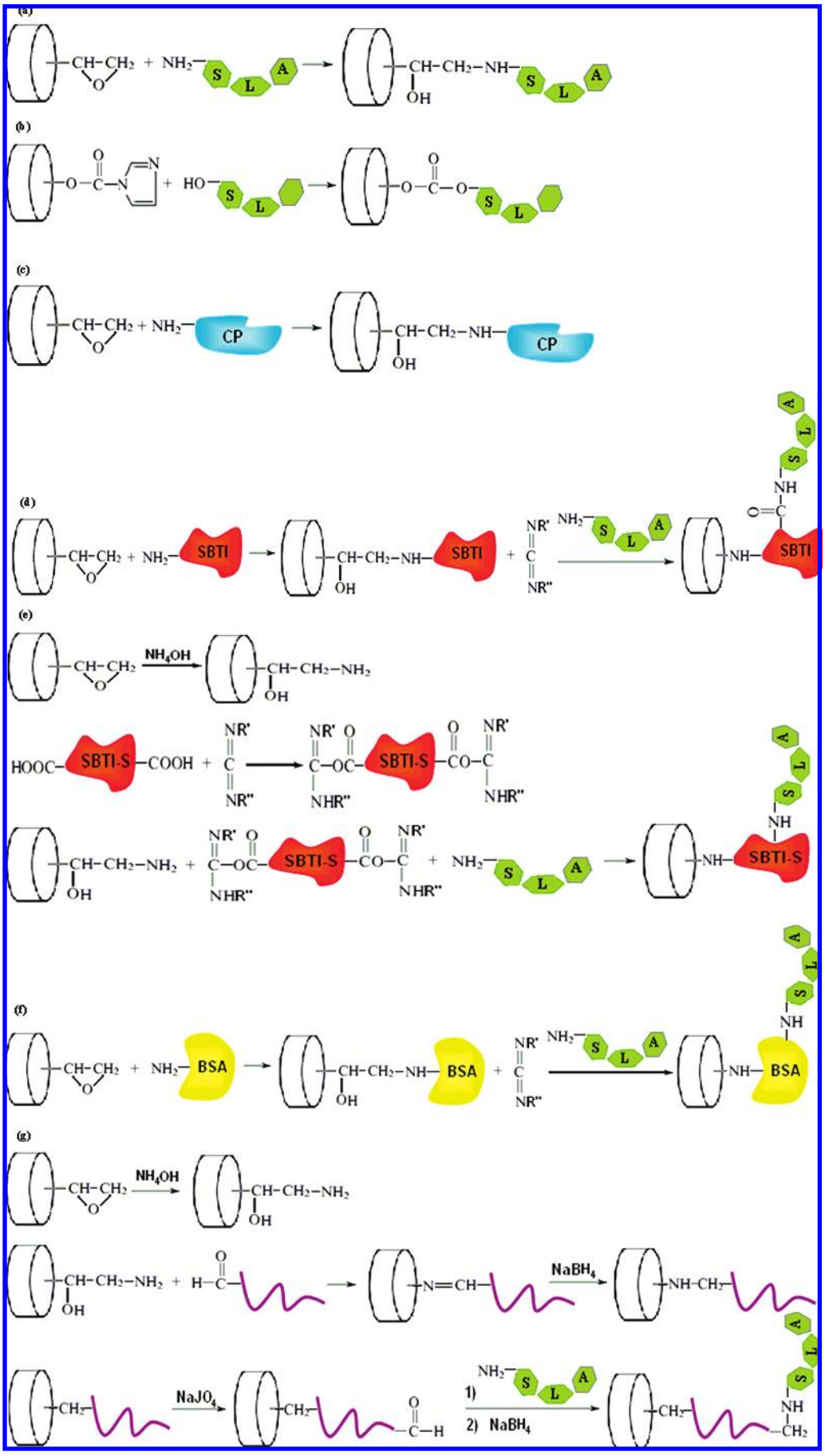
It is known that polymers and, in particular, copolymers of VP are widely used in the medico-biological field.[23,27,28] It was assumed that water-soluble and biocompatible copolymer poly-(MAG-*co*-VP-*co*-Ac) (Figure 1b) being used as a macromolecular spacer could provide a significant flexibility to a small specific ligand in comparison with its direct coupling or via protein coupling to the solid support. In this case, the formation of an affinity matrix consisted of three steps (Figure 2g): (1) covalent coupling of preliminary activated (aldehyde-bearing) copolymer, (2) formation the secondary aldehyde groups by periodate oxidation of vinylsaccharide residues, and (3) immobilization of SLA on a polymer backbone using a direct reaction between amino and aldehyde groups. The developed procedure was yielded in 0.8 μmol of copolymer that corresponded to 5.1 of μmol SLA (per mL of sorbent) (Table 1).

Chitosan (Figure 1c) represents a natural linear polysaccharide composed of randomly distributed β-(1−4)-linked D-glucosamine (deacetylated unit) and *N*-acetyl-D-glucosamine (acetylated unit). Its biocompatibility, bioactivity, and antiviral properties make it a very attractive substance for applications as a biomaterial in the pharmaceutical and medical fields.[29,30] The used immobilization procedure (Figure 2h) allowed obtaining 0.3 μmol of macroligand/mL of sorbent. Two- and fivefold increases of initial concentration of chitosan for immobilization led to increases of ligand's amount up to 1.0 and 2.3 μmol of substance/mL of sorbent, respectively, and, correspondingly, to 18.5 μmol of SLA and 13.0 μmol of SL (per mL of support) (Table 2).

Heparin is a member of the glycosaminoglycan (GAG) family of carbohydrates consisting of variably sulfated disaccharide units (Figure 1d). GAGs are negatively charged molecules, which interact with positively charged amino acids by electrostatic forces. GAGs can also interact specifically with proteins and a wide variety of pathogens including enveloped and nonenveloped viruses.[31] In our research, heparin was immobilized on a monolithic surface (Figure 2i) in the amount of 1.6 μmol/mL CDI-activated matrix (Table 2).

Remantadine (Figure 1e), or α-methyltricyclo[3.3.1.1\3,7]decan-1-methanamine hydrochloride, has anti-influenza viral properties. This antiviral drug inhibits the integral membrane protein M2 activity.[32] The immobilization scheme is presented in Figure 2j. The high immobilization capacity of α-methyltricyclo[3.3.1.1\3,7]-decan-1-methanamine hydrochloride can be related to easy penetration of this compact ligand to the small pores of a sorbent, where however, the ligand practically is not accessible for large objects.

(27) Couvreur, P.; Vauthier, C. *Pharm. Res.* **2006**, *23*, 1417−1450.
(28) Rybalko, S.; Nesterova, N.; Diadiun, S.; Danylenko, G.; Danylenko, V.; Guzhova, S.; Maksimov, Y.; Arkadiev, V.; Ivans'ka, N.; Maksymenok, O.; Vrnycianu, N.; Zhtrebtsova, E.; Grygoreva, T. *Acta Biochim. Pol.* **2001**, *48*, 241−246.
(29) Dhawan, S.; Singla, A.; Sinha, V. *AAPS PharmSciTech*. **2004**, *5*, 1−7.
(30) Chirkov, S. *Appl. Biochem. Microbiol.* **2002**, *38*, 5−13.
(31) Zahn, A.; Allain, J.-P. *J. Gen. Virol.* **2005**, *86*, 677−685.
(32) McCown, M.; Pekosz, A. *J. Virol.* **2005**, *79*, 3595−3605.


(a)
(b)
(c)
(d)
(e)
(f)
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SLA
CP
SBTI
BSA
SBTI-S
NH4OH
NaBH4
NaJO4
1)
2) NaBH4

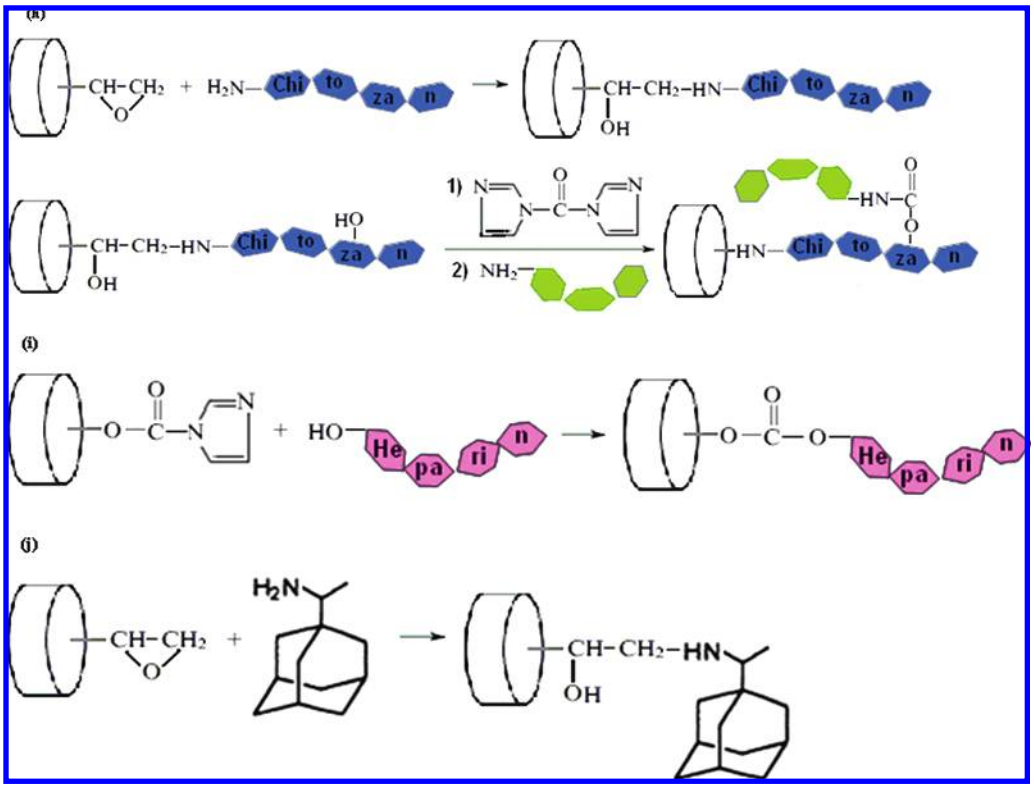

Figure 2. Immobilization schemes. (a) Sialyllactosylamine (SLA) immolilization on epoxy surface of monolithic disk, (b) α(2−6)-sialyllactose (SL) immolilization on CDI-activated surface of monolithic disk, (c) ceruloplasmin (CP) immolilization on epoxy surface of monolithic disk, (d) soybean trypsin inhibitor (SBTI) immolilization on epoxy surface of monolithic disk and its modification by SLA, (e) succinylated soybean trypsin inhibitor (SBTI-S) immolilization on aminated surface of monolith disk and its modification by SLA, (f) bovine serum albumin (BSA) immolilization on epoxy surface of monolithic disk and its modification by SLA, (g) poly(MAG-*co*-VP-*co*-DAAc) immolilization on aminated surface of monolithic disk and its oxidation and modification by SLA, (h) chitosan immolilization on epoxy surface of monolithic disk and its modification by SLA or SL, (i) heparin immolilization on CDI-activated surface of monolithic disk, and (j) α-methyltricyclo[3.3.1.1\3,7]decan-1-methanamine hydrochloride (Remantadine) immolilization on epoxy surface of monolith disk.

For all cases of ligand immobilization, it was shown that the treatment of residual epoxy groups by 0.5 M H_2SO_4 (1 h at 55 °C) with the aim of their inactivation did not influence the final adsorption capacity. That is why no quenching of unbound epoxy groups of a sorbent was carried out.

HPMDAC Analysis. Fast affinity chromatography was used as the most appropriate tool to check and compare the efficiency of obtained supports.

(1) Isolation of Hemeagglutinin from Vaccine. To compare the affinity sorbents, the method of loading of influenza vaccine of a defined concentration on a column (disk) up to total sorbent saturation was applied. It is known, that the fusion peptide in a hemeagglutinin molecule changes its conformation at acidic conditions, and at pH 5.5, it is in an active state.[33−35] The influence of pH of elution buffer on affinity binding was studied. It was found that considerable differences in bound hemeagglutinin amount were not observed at pH 5.0−6.5, and practically no binding was established at pH 8. The elution by salt buffer led to release of nonspecifically adsorbed proteins, whereas the sharp change of pH of eluting buffer resulted in affinity complex destruction and desorption of hemeagglutinin. This fact was confirmed by the result of electrophoresis in 15% polyacrylamide gel (Figure 3).

Abnormal low binding efficiency of support modified directly by silalyllactosylamine (Table 1) most likely resulted from sterical limitations at close contact of a large protein molecule and macroporous support surface with immobilized small ligands. Obviously, a 1-nm-length spacer (lactose ring) is not sufficient for unhampered interaction.

Extension of the distance between affinity ligand and polymer surface by insertion of protein spacers positively influenced adsorption capacity. Both disks, modified by sialyllactose via SBTI, have shown a good binding capacity of hemeagglutinin. The disk-SBTI-SLA demonstrated maximum effectiveness (Table 1). These two affinity supports were chosen for the next experimental run.

The low adsorption capacity of disk-BSA-SLA and disk-CP (Table 1) can be explain by effect of screening of a big part of bound ligand active sites, which can arise as a result of the big size of intermediate proteins (BSA and CP) covalently bound to the rigid sorbent surface. In contrast to BSA, CP has its own saccharide chains containing the residues of sialic acid, but the mechanism of their binding HA activity is not clear.[26] That is why this aspect can be a reason for low affinity adsorption capacity of discussed support.

Insertion of poly(MAG-*co*-VP-*co*-Ac) as a macromolecular spacer into the separation system and its subsequent modification

(33) Carr, C.; Chaudhry, C.; Kim, P. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 14306−14313.
(34) Cross, K.; Burleigh, L.; Steinhauer, D. *Expert Rev. Mol. Med.* **2001**, *6*, 1−18.
(35) Ramalho-Santos, J.; Pedroso de Lima, M. *Biochim. Biophys. Acta* **1998**, *1376*, 147−154.

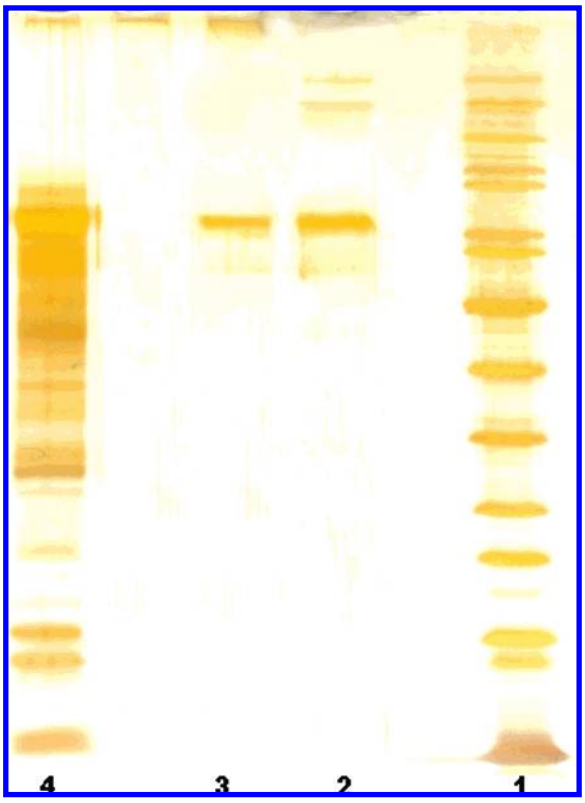

Figure 3. SDS-PAGE profile of desorbed fractions by HPMDAC of influenza vaccine. (1) markers, (2) fraction desorbed by 2 M NaCl, (3) fraction desorbed by 12.5 mM borate buffer (pH 10.7), and (4) influenza vaccine.

by affinity ligands was assumed the acquisition of multipoint binding at the interaction of the virus particle with such a conjugate. However, in contrast to the protein spacer, the used copolymer was not inert to all isolated protein components of the vaccine. Obviously, the total adsorption capacity in this case was a sum of adsorbed HA by both copolymer itself and SLA-ligands (Table 1).

Zonal Elution of Influenza A Virus. This chromatographic approach was used with the aim to optimize the conditions of virion adsorption from a crude sample and its effective elution. The maximum stability of the virus was established at pH 6.0−7.0 according to hemeagglutination test data. Any alteration in virus activity was not detected under the conditions of changes in concentration buffer within 0.05−1.0 M. The total desorption of virus was achieved using alkali solution, and immediately, the alkaline pH was adjusted to a neutral value in collected fractions.

The disk modified by heparin, operates most probably as an ion exchanger.[31] In our case, the virus was eluted by 0.4 M NaCl (Table 2). The follow two steps of washing were carried out consequently by 2 M NaCl and an alkaline solution that permitted desorbtion nonspecifically bound proteins. The hemagglutination test has shown the absence of specific virus activity at these fractions. For other affinity disks, the virus was discovered only in fractions eluted by alkaline solution (Table 2).

Two next studied chitosan-bound supports were modified by different derivatives of sialyllactose. It was found that amination of sialyllactose led to decrease of affinity binding capacity (Table 2). The disk bearing on this surface a big amount of α-methyl-tricyclo[3.3.1.1\3,7]decan-1-methanamine hydrochloride displayed rather low capacity (Table 2). This fact can be explained by (1) a short length of used ligand and, consequently, its low accessibility, and (2) lower than hemeagglutinin concentration of M2 protein responsible for specific virus attachment to this drug.[36,37] Thus, disk-Ch-SL and disk-SL, were chosen for further chromatographic experiments (Table 2).

HPMDAC of Viruslike Particles and Influenza A Virus. The SBTI-SLA disk was used to study the adsorption behavior of virus-mimicking synthetic particles. Recently, it was shown that the adsorption of similar synthetic units was defined by a protein covering the particle surface.[4,5] This part of the research was concentrated on the comparison of chromatographic behavior of real virus and the corresponding model. The affinity binding parameters were obtained from frontal analysis data for viruslike particles and compared to those obtained for influenza A virus. Adsorption isotherms of viruslike particles and influenza A virus, obtained on disk-SBTI-SLA and disk-SBTI-S-SLA, are presented in Figure 4a and b.

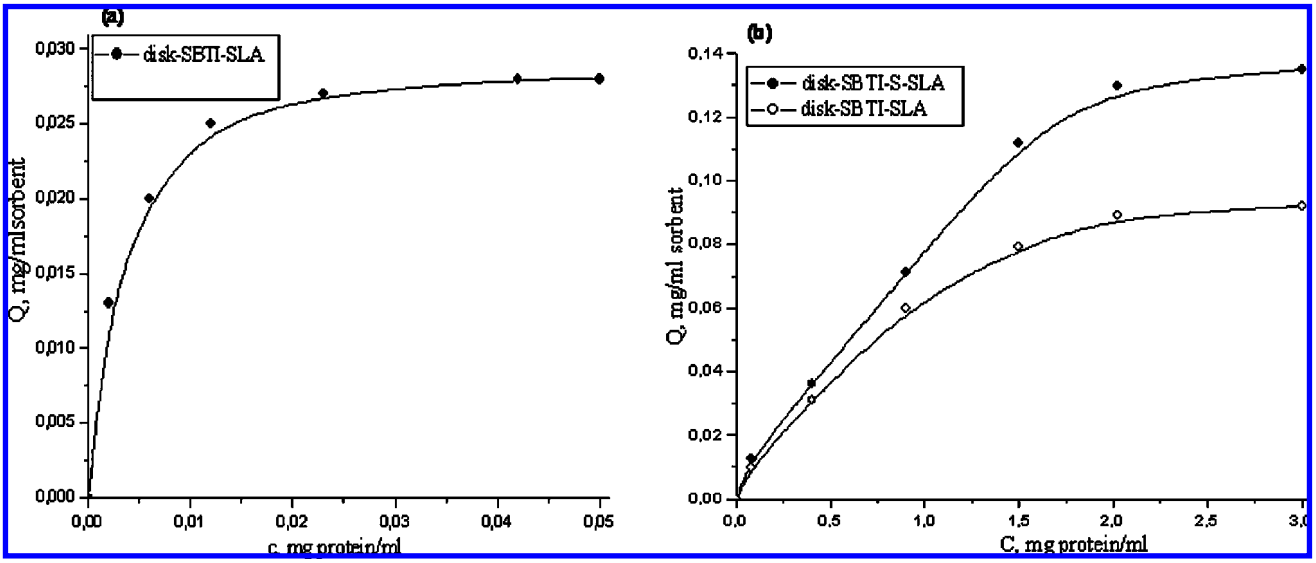

Figure 4. Adsorption isotherms of viruslike particles (a) and influenza A virus (b) on disk-SBTI-SLA and disk-SBTI-S-SLA.

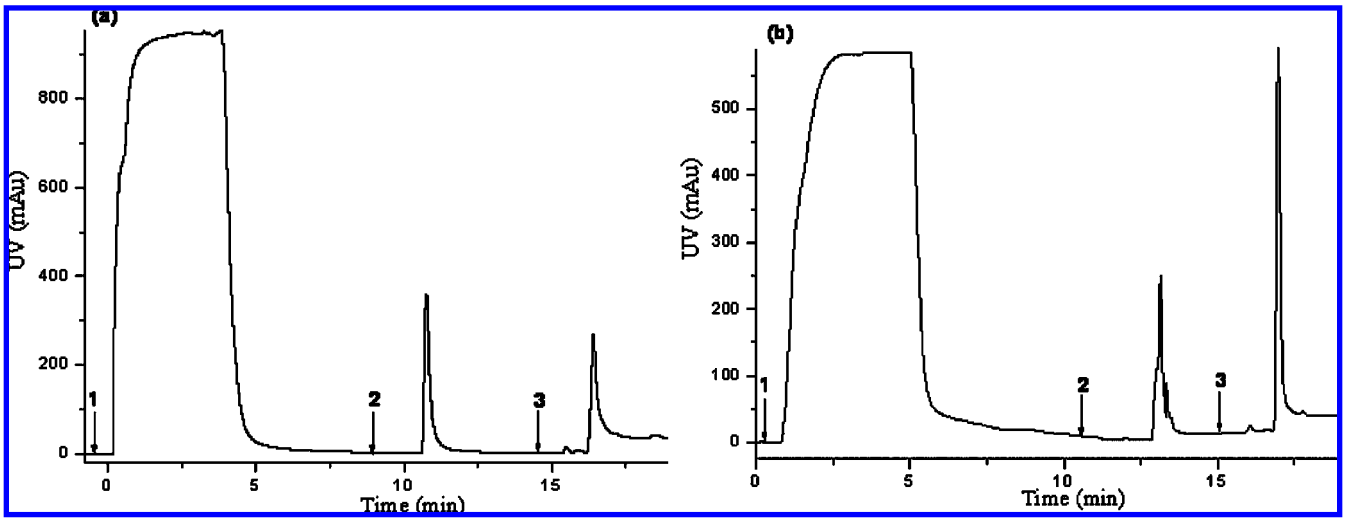

Figure 5. Chromatograms of influenza A virus (frontal analysis) on the following: (a) disk, sialyllactose, (b) disk-chitosan-sialyllactose; (1) sample loading (0.05 M acetate buffer, pH 6.2), (2) washing by 0.4 M NaCl in 0.01 M PBS (pH 6.5), and (3) virus elution with 0.2 M NaOH (pH 14). Flow rate was 2.0 mL/min.

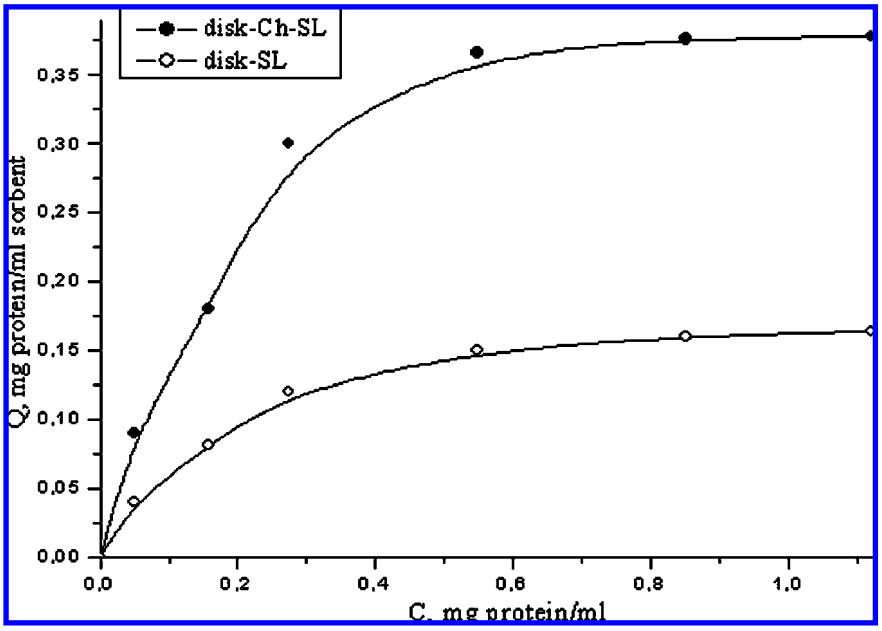

Figure 6. Adsorption isotherms of influenza A virus on disk-sialyllactose and disk-chitosan-sialyllactose.

Similar values of adsorption capacity were discovered for influenza A virus with the use of disk-SBTI-SLA and disk-SBTI-S-SLA (Table 4). The absence of difference in maximum adsorption capacity, expressed in particle amounts per milliliter of sorbent for hemeagglutinin-bearing latexes and real virus was observed (Table 4). The identity of Q_{max} values proves the close similarity of the virus and its synthetic model with regard to their geometrical and functional characteristics. It was found that the use of strong alkaline elution buffers did not influence virus structure that was approved by their cultivation (eluted fractions) in MDCK cells.

The disks modified by 2,6-sialylactose, directly and via chitosan, allowed isolation of ~1.5 and ~4 times more virus particles per milliliter of sorbent due to the high affinity ligand's concentration in comparison with other investigated matrixes (Table 4). Nevertheless, the best adsorption properties were demonstrated by disk-Ch-SL that can be related to cooperative adsorption effect including the interactions between virus and both chitosan and sialylic parts. The chromatograms for influenza A virus using disk-SL and disk-Ch-SL (frontal analysis) are shown in Figure 5a and b. Adsorption isotherms of influenza A virus, obtained on disk-Ch-SL and disk-SL, are presented on Figure 6a and b.

CONCLUSIONS

The different ways of modification of a monolith surface by various ligands were considered. The manner of representation (immobilization, introduction) of sialyl derivates on the surface of macroporous monolithic support was optimized.

The selectivity of sialyllactose and its derivate as affinity ligands for hemeagglutinin isolation from inactivated influenza vaccine was demonstrated.

(36) Zebedee, S.; Lamb, R. *J. Virol.* **1988**, *62*, 2762−2772.
(37) Masaki, I.; Mizuno, T.; Kawasaki, K. *J. Biol. Chem.* **2006**, *281*, 12729−12735.

It is shown that the sorbents, bearing sialyllactose, immobilized on a surface via low weigh protein as spacer and polysaccharide has the incontrovertible efficiency for virus isolation.

Also, optimization of chromatographic conditions was allowed isolating influenza virus A while maintaining virulence. The best candidates as affinity ligands were revealed from screening chromatographic experiments, namely, zonal analysis.

The results obtained by fast affinity chromatography showed functional and sterical correspondence viruslike synthetic models with influenza virus.

Taking into consideration such parameters as amount of hemeagglutination units and adsorption capacity, it is possible to tell that sialyllactose and chitosan can be used as ligands for concentration of influenza virus.

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