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Multifunctional fractionation of polyclonal antibodies by immunoaffinity high-performance monolithic disk chromatography

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Abstract

High-performance monolithic disk chromatography (HPMDC), including its affinity mode, is a very efficient method for fast separations of biological molecules of different sizes and shapes. In this paper, protein and peptide ligands, immobilized on the inner surface of thin, monolithic supports (Convective Interaction Media or CIM® disks), have been used to develop methods for fast, quantitative affinity fractionation of pools of polyclonal antibodies from blood sera of rabbits, immunized with complex protein–peptide conjugates. The combination of several disks with different affinity functionalities in the same cartridge enables the separation of different antibodies to be achieved within a few minutes. The apparent dissociation constants of affinity complexes were determined by frontal analysis. Variation of elution flow rate over a broad range does not affect the affinity separation characteristics. Indifferent synthetic peptides used as biocompatible spacers do not change the affinity properties of the ligands. The highly reproducible results of immunoaffinity HPMDC are compared with data obtained by widely used enzyme-linked immunosorbent assay. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Monolithic disks; Immunoaffinity chromatography; Affinity sorbents; Antibodies; Proteins; Peptides; Albumin

1. Introduction

A novel separation method called high-performance monolithic disk chromatography (HPMDC) [1,2] has been pioneered more than 10 years ago. Although this method has originally been developed for the rapid separation of proteins using ion-exchange, reversed-phase, and hydrophobic interaction chromatographic modes [3–9], it was later easily extended to the separation of other classes of substances differing from proteins in both molecular shape and size [10,11].

Stationary phases for HPMDC have the shape of a flat monolithic disk with well-controlled properties [12,13]. These media are now commercially available under the name Convective Interaction Media (CIM®) disks [14]. The inner macroporous structure of these disks is similar to that of HPLC sorbents but differs significantly from classic thin flat membranes or hollow fibers used recently for "chromatography on membrane adsorbers" [2,15,16]. In contrast, the HPMDC method is truly "high-performance", analogous to modern HPLC.

Since the decrease in the number of steps in an efficient isolation of pure biological products such as proteins from complex matrixes remains a challenge, the analysis of affinity interactions may help to solve this problem. The speed of the separation procedure using disks facilitates the recovery of product since the exposure to putative denaturing influences such as solvents, temperature, and contact time is dramatically reduced. Recently, we have demonstrated affinity HPMDC in various modes including flow-injection analysis (FIA), and its use for both fast isolation/purification of recombinant proteins direct-

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ly from cellular lysates and quantitative analysis of biocomplementary pairs [17–20].

One of the significant advantages of HPMDC is the possibility of using several disks with different functionalities placed at the same time in a single cartridge. Thus, in addition to high speed of the separation and purification of biomolecules, this technology also allows combination of different chromatographic modes in a single run. A term "conjoint liquid chromatography" (CLC) [14] has recently been coined for this operational mode. This approach enables separation and purification processes that are difficult to achieve using typical current methods.

The number of reports on multifunctional chromatography using means of conventional HPLC in which several columns with different interaction functionalities are combined in series is scarce [21]. It is likely that the HPLC approach is less popular since it requires to develop a complex automated system for switching various pumps and valves. However, the rapid development of methods required for proteome analysis has revived interest in this idea [22].

In previous work, we described the use of immunoaffinity HPMDC for the fractionation of polyclonal antibodies expressed in rabbits immunized with complex protein–peptide antigenic conjugates [19]. The process is likely to find an application in both analytical and preparative processes needed by applied as well as fundamental immunology. We have shown that immobilization of different parts of a complex antigen onto the monolithic disks allowed separation of different antibodies with the affinity to the relevant antigen. The method reported here goes beyond the procedure published earlier since it allows fractionation of the same model pool of polyclonal antibodies by *a single step* using immunoaffinity conjoint HPMDC.

2. Experimental

2.1. Materials and chemicals

Standard monolithic poly(glycidyl methacrylate–co-ethylene dimethacrylate) (GMA–EDMA) disks 12 mm in diameter and 3 mm thick (CIM® disks) with epoxy functional groups were provided by BIA Separations, Slovenia. The mean pore size measured by mercury intrusion porosimetry was equal to 1 μm whereas a porosity was determined as 0.7 ml/ml sorbent. A specifically designed cartridge (BIA Separations) was used to install the disks into the chromatographic system.

Amino acid derivatives were purchased from Fisher Biotech, USA. Diiso-propylcarbodiimide (DIC), trifluoromethanesulfonic acid (TFMSA), trifluoroacetic acid (TFA), thioanisole, ethanedithiol, hydroxybenzotriazole (HOBt), and *p*-toluenesulfonic acid (TosOH) were purchased from Fluka (Buchs, Switzerland). 4-Dimethyl-aminopyridine (DMAP) and 1-1′-carbonyldiimidazole (CDI) were obtained from Merck–Schuchard (Germany). Dimethylformamide (DMF), dichloromethane (DCM), and triethylamine (TEA) were purchased from Vecton (Russia), and purified according to procedures described elsewhere [23].

Mixtures of polyclonal antibodies against the synthetic ligand bradykinin (BK) were produced by immunization of rabbits with BK covalently conjugated to bovine serum albumin (BSA). BSA, succinylic anhydride, and water soluble 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide (CDII) were from Sigma Bioscience (USA), Reachim (Armenia) and Sigma–Aldrich (Germany), respectively.

The sera and their reprecipitated (three times, 32–35% aqueous ammonium sulfate) immunoglobulin fractions were prepared using standard procedures.

Protein A was a kind gift of Pasteur Scientific Research Institute of Epidemiology and Microbiology (St Petersburg, Russia). Bovine serum albumin and highly purified gelatin were purchased from Sigma–Aldrich. Horseradish peroxidase used for preparation of conjugates for enzyme-linked immunosorbent assay (ELISA) was delivered by Sigma Bioscience (USA).

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

2.2. Instruments

The NPS 4000 synthesizer (Neosystem, France) was used for the automated synthesis of peptides.

Synthetic nonapeptide hormone bradykinin (RPPGFSPFR), and two peptidyl ligands nonapeptide (GVVKNNFVP) and tripeptide (GGG) were synthesized on benzhydrylamino polystyrene support beads (*p*-methylbenzhydrylamine·HCl resin, Neosystem Labs., France) using *tert*.-butoxycarbonyl –benzyl (Boc/Bzl) strategy and purified by RP-HPLC. The detailed synthetic approach was published elsewhere [24]. A chromatographic system consisting of two HPLC pumps, UV detector, plotter (all from Waters, USA) and semipreparative 16×300 mm column packed with 20 μm Nucleosil C_{18} was used for the isolation of synthesized peptides from the reaction mixture.

Amino acid analysis of peptides was carried out using a T 339 automatic amino acid analyzer (Mikrotechna, Czech Republic).

The concentrations of peptides were determined from the absorbance at maximum wavelength using a UV–Vis SF-26 spectrophotometer (LOMO, Russia). The concentration of proteins (immunoglobulins) in eluates was determined using a standard Lowry method [25] and UV–Vis spectrometry. Immunoaffinity HPMDC separations were carried out with a Gilson chromatographic system (France) combining two piston pumps (model 303 and 305) and a 118 UV–Vis detector.

The titers of antibodies were measured using ELISA in the 96-well microplate format (Medpolymer, Russia). The optical absorption of the solutions in the wells was determined at 492 nm using an Immunoenzyme Colorimetric Analyzer KAI-TS-01 (Scientific Research Center of Biological Instrumentation, Russia).

2.3. Methods

2.3.1. Synthesis and immobilization procedures

Succinylation of BSA (BSA-S) and the following preparation of BSA-S–BK conjugate were achieved using described procedures [26]. Protein A–peroxidase conjugate for the ELISA test was prepared using the periodate method [27]. A procedure developed previously was used for direct immobilization of BSA and BK ligands [17,19]. To avoid non-specific Coulombic interactions of antibodies with affinity support, the quenching of unreacted epoxy groups with 2-aminoethanol was omitted. Since a flow applied has lead to decreasing of immobilization yield, the static conditions were used in all cases.

The epoxide functionality of disks was converted to primary amine groups enabling covalent immobilization of both succinylated BSA-S and BSA-S–BK conjugates. The disks were immersed in 1 mol/l aqueous ammonia solution and heated to 40 °C for 3 h. After the reaction was completed and the mixture cooled, the disk was washed with water, 1 mol/l NaCl, and water again. The presence of amino groups was qualitatively tested using standard ninhydrin reaction. Before the immobilization, the disk was washed with 0.1 mol/l sodium phosphate buffer (pH 4.5). Succinylated protein (BSA-S) or BSA-S–BK conjugate were dissolved in 2 ml of the same buffer to obtain a 4–5 mg/ml solution. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (160-fold molar excess) was added to the protein solution. The disk was immersed in this solution and allowed to react with stirring at room temperature for 4 h. The disk was then washed with phosphate-buffered saline (PBS), water, 1 mol/l NaCl, water, and, finally, with the chromatographic buffer.

The amount of immobilized ligands was estimated from the difference in peak areas before and after the reaction using ion-exchange HPMDC on a DEAE CIM® disk [20].

Spacer peptides GVVKNNFVP and GGG were immobilized using the procedure for direct immobilization of BSA and BK. After checking the non-specific adsorption of antibodies of interest on the disks bearing only the spacer, specific ligands (BSA and BK) were attached to the spacers using carbodiimide chemistry.

2.3.2. Immunoaffinity chromatography

Four disks with immobilized ligands corresponding to all three parts constituting the conjugate used for the immunization (BSA, BK, and BSA-S) and the conjugate itself (BSA-S–BK) were placed in a single cartridge; 500 μl of mixed antibodies with a protein concentration of 2 mg/ml were loaded at the adsorption step using 0.01 mol/l sodium phosphate buffer pH 7.0 containing 0.15 mol/l NaCl as the mobile phase. After the adsorption step had been completed, the cartridge was dismantled and mono-

specific antibodies adsorbed on each disk were eluted separately using 0.01 mol/l HCl (pH 2.0) as a desorbing solution. Different flow rates in a range 1–10 ml/min were used in both adsorption and desorption steps. The order of disks within the cartridge was changed to elucidate the amount of crossreactive antibodies.

2.3.3. Dynamic affinity adsorption

Frontal analysis was used to determine the apparent affinity constants. Solutions of antibodies in 0.01 mol/l sodium phosphate buffer, pH 7.0, containing 0.15 mol/l NaCl with a concentrations range of 0.05–1 mg/ml were flushed through the disk. The strongly adsorbed specific antibodies were eluted using 10 mmol/l HCl, pH 2.0. The apparent dissociation constants of the affinity complexes were calculated according to Ref. [28].

2.3.4. ELISA test

Determination of titers of the antibodies (Abs) was carried out using a solid-phase immunoenzyme analysis in 96-well microtiter plates. The diluted solutions of Abs with a total protein concentration of 10 mg/ml were used as initial samples. A 0.01 mol/l sodium phosphate buffer solution pH 7.4, containing 1% NaCl and 0.05% Twin-20 (PBST buffer) was used to wash the wells and dilute the Protein A–horseradish peroxidase conjugate. PBST with 0.1 mol/l sodium phosphate was used to dilute the antibodies solutions.

One hundred microliters of 100 μg/ml solution of each part of complex BSA-S–BK immunogen in 0.1 mol/l sodium carbonate buffer, pH 9.6, as well as the complete immunogen (BSA, BSA-S, BK, and BSA-S–BK) were pipetted in separate rows of wells to obtain the first adsorbed layer. Control row with no antigen was kept next to the each analyzed one. A row with no reagents was left empty to measure the non-specific adsorption of the plate. The binding time was 20 h at 4 °C.

The wells were then washed with PBS and 150 μl of 1% gelatin solution in 0.1 mol/l PBS was added immediately into each well. This mixture was incubated for 1 h at 37 °C. After washing with PBST repeated six times at 1-min intervals, solutions of antibodies from crude serum fraction dissolved in PBST as well as a solution of monospecific immunoglobulins isolated using HPMDC and adjusted to pH 7.0 were added into each well in the following mode: 100 μl of analyzed solution at the initial concentration was introduced in the first well of the row with the corresponding antigen. The concentration of the solutions pipetted into each next well of the same row was decreased to one half. The same procedure was used to fill the wells of the control rows covered with plain gelatin that did not contain any antigen. The incubation proceeded for 1 h at 37 °C. After removing the Ab solutions and six repeated washings with PBST, 100 μl of Protein A–peroxidase conjugate solution in PBST (1:1500 dilution) was added into each well and kept for 30 min at 37 °C. After another six repeated washings with PBST, 100 μl of a 0.5 mg/ml solution of *o*-phenylenediamine in 0.1 mol/l sodium citrate–phosphate buffer, pH 5.0, containing 0.03% H_2O_2 were added. The mixture was kept in the dark at room temperature for 30 min. The reaction was terminated by an addition of 100 μl of 2 mol/l H_2SO_4. Absorbance measured at 492 nm was compared with that of control wells covered with neutral gelatin. The results obtained were interpreted by the immunotitration method [19].

3. Results and discussion

Recently, we have demonstrated that HPMDC is suitable for fast affinity separations based on strong interactions between natural biological complements [17–20]. These results confirmed the rapid formation of biocomplementary pairs, which generally does not affect the high speed of affinity separation. This observation was explained by significantly improved mass transport resulting from carefully designed inner porous space of the porous polymer separation media helping the adsorption–desorption process to occur without steric limitations imposed by both pore size and shape [2]. Our procedure for fast isolation of specific antibodies from polyclonal pools allows isolation of significant quantities of antibodies from both preliminary precipitated blood serum fraction and crude blood serum within a few minutes [19]. This study is to confirm that the isolation can be

carried out efficiently in a short period of time. To achieve this, we have used the polyclonal pool of antibodies described recently to be able to compare the results obtained by the present method with the previous ones. It is worth noting that the main purpose of these experiments is to demonstrate a new method using well-known objects. We used a similar approach in our previous studies concerned with affinity HPMDC to quantitatively evaluate the binding of human serum albumin (HSA) and immunoglobulin G (IgG) to immobilized recombinant Protein G with genetically separated IgG and HSA binding sites [29].

3.1. Multifunctional fractionation of polyclonal pools of antibodies

We used the pool of polyclonal antibodies obtained by immunization of rabbits with the covalent conjugate (BK–BSA-S) as a model complex natural mixture [19]. Such a pool typically contains both monospecific antibodies against each part of the conjugate used for immunization and some so-called "crossreactive" antibodies [30,31] that have epitopes for complementary binding to all parts of the complex antigen.

To achieve a well-controlled fractionation of the polyclonal pool of antibodies, each structural part of the antigen, i.e. BK, BSA, BSA-S, and the complete conjugate (BSA-S–BK) were immobilized on individual disks. Quantitative results of this covalent binding are shown in Table 1.

These affinity disks were installed in the single housing and a single adsorption run was carried out. The simplicity of the commercially produced cartridge allows easy rearrangement of the sequence of the disks in a stack, as well as reinsertion of a single disk for subsequent desorption. We observed that no elution occurs using typical buffers such as 200 mmol/l KSCN, pH 7.4, or 100 mmol/l glycine, pH 2.0–3.5. Quantitative desorption of bound proteins was only achieved under strongly acidic conditions with a 10 mmol/l HCl solution (pH 2.0) [17,20,32]. To prevent denaturation of isolated antibodies, the pH values of eluted fractions were immediately adjusted to neutral. Since the exchange of the mobile phase inside our short monolithic layer proceeds very quickly and complete desorption is achieved in a few seconds, inactivation of antibodies is negligible as confirmed by very high binding to antigen detected by ELISA.

Table 2 shows data for fractionation of serum using individual disks with immobilized different parts of complex immunogen as well as the results obtained using the stacking procedure. The adsorption capacity of the single disks appears to be different from that of stacked set. The only reason for this difference is the adsorption of crossreactive antibodies with close affinity to the specific antigen that together with monospecific antibodies contribute

Table 1
Quantitative data on the immobilization of different affinity ligands

Ligand (M_r)	Immobilized ligand	
	mg/ml disk	μmol/ml disk
Bradykinin, BK (1060)	1.5	1.40
Bovine serum albumin, BSA (66 000)	3.0	0.05
Succinylated bovine serum albumin, BSA-S (72 000)	3.0	0.04
Conjugate of bradykinin with bovine serum albumin, BSA-S–BK (92 000)	7.4	0.08

Immobilization procedure: see Experimental. Five immobilization procedures were carried out for each ligand and standard deviation was found to be ±10%.

Table 2
The data on serum fractionation using the disks with different immobilized antigens

Antibodies	Quantity of antibodies (Abs) recovered from serum immunoglobulin fraction	
	Monospecific Abs, μg/mg serum	Monospecific+ crossreactive Abs, μg/mg serum
Anti-BK	21	61
Anti-BSA	13	53
Anti-BSA-S	14	55
Anti-BSA-S–BK	58	100

Chromatographic conditions: flow rate 2 ml/min; detection 278 nm; loading: 500 μl serum precipitated fraction with protein concentration of 2 mg/ml. Ten subsequent runs with single disks as well as with each combination of four disks were carried out and the standard deviation of presented values was to be ±5%.

to the overall binding. Changing the sequence of the disks with different immobilized antigens can be used to detect the crossreactive antibodies. In this case, the disk installed at the top of the stack adsorbs both its ''own'' and crossreactive immunoglobulins. Changing the order of disks in the cartridge makes it possible to separate quantitatively all types of antibodies from the whole serum fraction. Ten subsequent runs with single disks as well as with each combination of four disks were carried out. The results confirm the high reproducibility of the method. In addition, it is also possible to quantitate antibodies in a blood serum.

Fig. 1 compares two affinity chromatograms obtained at different flow rates in order to demonstrate the time scale of the process. As an example, the single disk with bound BSA was used to isolate the fraction of anti-BSA and crossreactive immunoglobulins from blood serum.

3.2. Effect of spacer on immunoaffinity binding

It should be emphasized that the immobilization of ligands is an important issue in our process. While a well-controlled procedure was recently developed for protein immobilization directly on GMA–EDMA disks using nucleophilic addition of amino groups of the ligand to epoxide functionalities of the matrix [17–20,27,32], the chemistry of immobilization of both succinylated BSA and BSA-S–BK conjugate is different. Specifics of the immobilization of short peptides on the pore surface of the support has been described elsewhere [19].

The present literature suggests a number of linkers

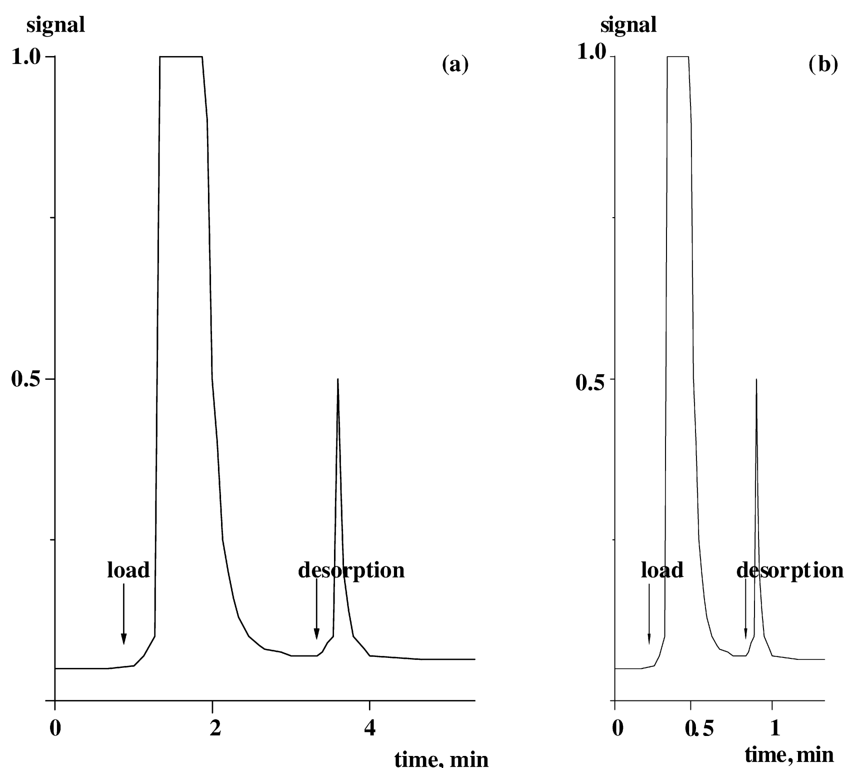

Fig. 1. Examples of immunoaffinity HPMDC: isolation of monospecific *anti*-BSA antibodies at different flow rates. Conditions: stationary phase: CIM BSA disk: BSA was immobilized with use of intermediate nonapeptide GVVKNNFVP spacer; mobile phase: buffer A: 0.01 mol/l sodium phosphate buffer, pH 7.0, containing 0.15 mol/l NaCl; buffer B: 0.01 mol/l HCl, pH 2.0; loading: 500 µl serum solution with protein concentration of 2 mg/ml; detection 278 nm; (a): flow rate 1 ml/min, and (b): flow rate 6 ml/min.

to insert between the solid surface of the stationary phase and the immobilized biocomplement (ligand) [33–35]. These spacers improve the mobility of the ligand during its interaction with affinity partner residing in the mobile phase. A careful choice of spacer may even help to prevent non-specific adsorption. Therefore, peptide linkers that are compatible with the isolated antibodies but do not interact with them can be successfully used.

In our experiments, we compared the isolation of antibodies against BSA and bradykinin using CIM® disks with protein and peptide ligands immobilized without any spacer and with specially synthesized nonapeptide and tripeptide as linkers. Frontal analysis was used to obtain the adsorption isotherms and thereby the affinity constants of all involved interactions. The experimental isotherm fits the Langmuir type well [19]. The linearization of this equation allows calculation of K_{diss} with standard deviation r^2 0.9985–0.9995 (10 replicates). Although these dissociation constants are apparent since their magnitude is affected by the adsorption of different antibodies (monospecific+crossreactive), this quantitative approach is useful for comparison of results. Table 3 shows that dissociation constants of affinity complexes do not reveal any negative or positive effect of either nonapeptide or tripeptide spacer.

Table 4 demonstrates that the experimental K_{diss} values, which characterize thermodynamic stability of affinity complexes, are not affected by the flow rate used at the elution step. In addition, no adsorption of both antibodies and model protein BSA occurs on the disks modified with only nonapeptide and tripeptide spacers with any attached affinity ligands. This result indicates that the modified porous polymer is well suited for affinity separations.

Table 4
Influence of flow rate used in immunoaffinity HPMDC on K_{diss} values measured for BSA–*anti*-BSA Abs complex

Flow rate (ml/min)	0.5	2	6	10
K_{diss} (μmol/l)	3.0	2.4	3.1	3.1

Conditions: the same as for Table 3.

3.3. Comparison of data obtained by immunoaffinity HPMDC and ELISA

Obviously, the results of HPMDC fractionations of pools of polyclonal antibodies should be compared with other methods. We used the popular and widely used ELISA [36,37]. Table 5 summarizes the results of recovery of different monospecific antibodies obtained by both methods. It shows a clear quantitative relation between both methods we used. This suggests that the multifunctional approach to immunoaffinity HPMDC is suitable for the fractionation of polyclonal pools. ELISA tests also shown in Table 5 demonstrate that antibodies isolated by HPMDC keep their biological activity while their purity is more than one order of magnitude higher.

Table 3
Affinity characteristics (K_{diss} and q_{max}) found by frontal analysis approach for ligand–antibody pairs using the disks with or without peptide intermediate spacers

Ligand	Spacer	Amount of immobilized ligand (average values)		q_{max}, mg/ml disk	K_{diss}, μmol/l
		mg/ml disk	μmol/ml disk		
BSA	None	5.9	0.09	4.2	2.4
	GGG	2.9	0.04	1.4	3.8
	GVVKNNFVP	1.5	0.02	0.6	3.0
BK	None	5.0	4.7	1.4	2.6
	GGG	1.5	1.4	0.4	1.5
	GVVKNNFVP	1.2	1.1	0.2	1.2

Conditions: flow rate 2 ml/min; detection 278 nm, concentration range of 0.05–1 mg/ml, standard deviation (r^2) at linearization of isotherms 0.9985–0.9995 (5–10 replicates, Excel).

Table 5
Comparison of serum fractionation data obtained by immunoaffinity HPMDC and enzyme-linked immunosorbent assay (ELISA)

Antibodies	Immunoaffinity HPMDC quantitative part of monospecific antibodies in serum, μg/mg serum Ig-fraction	ELISA, titer of monospecific antibodies in	
		Serum	Immunoaffinity HPMDC eluates
Anti-BK	21	1300–2600	–
Anti-BSA	13	1100–2200	46 400–92 800
Anti-BSA-S	14	1050–2100	–
Anti-BSA-S–BK	58	4400–8800	–

Chromatographic conditions: flow rate 2 ml/min; detection 278 nm; loading: 500 μl serum precipitated fraction with protein concentration of 2 mg/ml. ELISA titers were determined according to the procedure described elsewhere [36]. Ten subsequent HPMDC runs and 10 corresponding ELISA tests were carried out and standard deviation of presented values was found to be ±5 and ±15%, respectively.

It may not be absolutely correct to compare these two methods directly. The ELISA method is more sensitive and allows running a large number of parallel assays simultaneously. However, in contrast to the indirect ELISA, the HPMDC separations enable direct determination of antibodies (or antigens) even in complex biological matrixes such as crude blood serum. In addition, the high speed of the HPMDC approach allows carrying out many separations in series within a short period of time. Thus, HPMDC not only allows estimation of concentrations of the individual antibodies but also enables the ''semipreparative'' separation of significant amounts of biomolecules.

4. Conclusions

HPMDC can be successfully used for the fast and quantitative fractionations of complex biological mixtures such as pools of polyclonal antibodies. Immunoaffinity HPMDC using macroporous methacrylate stationary phase (CIM® disks) is not affected by biocompatible spacers. The apparent dissociation constants of affinity complexes, K_{diss}, were also determined and their independence on flow rate was demonstrated. The results of HPMDC serum fractionation were verified by ELISA test and a good correlation between the methods was observed. The results presented here support the use of fast multiaffinity HPMDC for effective semi-preparative separations of complex biological mixtures.

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