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Review

Affinity processes realized on high-flow-through methacrylate-based macroporous monoliths

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Abstract

The technology for preparation of rigid macroporous polymers suggested in the late 1980s has become a powerful instrument for the development of a novel scientific and practical field. At present, monolithic stationary phases are widely used in the processes of bioseparation (chromatography), bioconversion (enzyme reactors) as well as in other processes based on interphase mass distribution (for example, solid phase peptide and oligonucleotide synthesis). Bioaffinity modes of suggested dynamic methods are very promising for their use in different analytical processes (immunological, ecological, medical and other types of analytical monitoring), preparative isolation of blood proteins such as myoglobin, hemoglobin, immunoglobulins, etc. and also recombinant products directly from cell supernatants or lysates. For the first time, it has been shown that bioaffinity pairing with participation of immobilized on carefully designed rigid supports is very fast and the whole process of affinity separation can be realized within second time scale. The principle of bioaffinity recognition is generaly at the construction of biological reactors (for example, enzyme reactors). Improved kinetics of biocatalized reactions is explained by a minimal influence on the surface of the used sorbent. Very perspective field is the use of discussed monoliths for solid phase chemical synthesis of fragments of biological macromolecules (peptides and oligonucleotides). Several examples of these applications will be presented and discussed.
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Keywords: High-performance liquid chromatography (HPLC); Monoliths; Bioaffinity

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1. Introduction

The most popular and widely used separation procedure based on quasi-equilibrium molecular distribution between two phases is liquid chromatography (LC) or, at present, high-performance liquid chromatography (HPLC). The model of dynamic interphase mass distribution developed for a chromatography can be applied to other processes based, for example, on biological complementary interactions. Thus, there exists a definite logical tie between the aspects of effective mass transport (all adsorption types of HPLC) and biological coincidence (affinity chromatography, biosensors and bioreactors).

It is known that all biological interactions taking place in vivo are based on a formation of specific biomolecular complexes. Such pairs as enzyme–substrate, antigen–antibody and receptorial complements can be adduced as the most known examples. The similar pairing is used successfully in modern applied biology, biotechnology, immunochemistry, medicine and other practical fields [1]. In a wide range of appropriate techniques, the affinity chromatography plays a very important role. This method is based on the natural affinity of a component to be separated and being in a liquid phase to its natural biological complement, i.e. ligand, immobilized on the surface of the stationary phase. Thus, the affinity conception consists of a formation of reversibly dissociated biospecific complex.

The development of affinity ideology goes in parallel with the construction of new stationary phases and affinity ligands as well as with the new approaches to immobilization chemistry. For example, recently developed short layers of (poly)methacrylate-based monoliths (short monolithic columns) [2–9] appear to be quite useful for modern high-speed affinity chromatography. These stationary phases with well controlled and highly developed inner macroporous structure [10], provide, on one hand, significant adsorption capacity, and on the other hand, enhanced mass transition of biomolecules between two phases. As a result, the quality of separation is found to be significantly improved. Moreover, the speed of separation procedure using flat disks facilitates the recovery of the product since the exposure to putative denaturing influences such as solvents, temperature and contact time is dramatically reduced. Some of the requirements to the "ideal" affinity separation medium formulated very long time ago [11], found their successful combination in the monolithic materials presented below. These are:

- (i) insolubility;
- (ii) high permeability and high specific surface;
- (iii) absence of non-specific adsorption;
- (iv) high chemical reactivity allowing to bind ligand molecules with sufficient capacity;
- (v) high chemical stability at the conditions required for sorbent exploring and regeneration;
- (vi) high antimicrobial resistance;
- (vii) high hydrophilicity.

It is obvious that the reasonable combination of productivity and biocompatibility must be taken into account during the development of new stationary phases for affinity techniques. The final goal of such inventions should be to construct the porous space of a sorbent with the optimized morphology and topography of inner surface allowing maximum accessibility of immobilized ligands. All the factors will guarantee the high-speed processes with minimum deformation of final valuable product and high purity of isolated bioproduct.

The examples of using of very promising macroporous monoliths for modern affinity separations have been already summarized in [7,12–14]. Here, the most significant and interesting data will be presented and briefly discussed.

2. Common principles of affinity pairing

The major term for successful realizing of any two-phase dynamic process based on affinity pairing (in particular, affinity chromatography) is maximally unlimited formation of specific complex between a soluble substance P and a ligand L bound to the insoluble support [15]. It is known that the formation of biological complexes involves the participation of ionic, hydrogen and hydrophobic bonds, London's dispersion forces, dipole–dipole and charge–transfer interactions, etc.

According to the assumption that only one substance from the mixture has the affinity to be functionalized by complementary ligands sorbent, such interactions can be mathematically described by the following equations:

$$P + L \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} PL \quad (1)$$

$$\frac{\mathrm{d}[PL]}{\mathrm{d}t} = K_1[P][L] - K_{-1}[PL] \quad (2)$$

$$K_{\text{diss.eff.}} = \frac{K_{-1}}{K_1} = \frac{[P][L]}{[PL]} = \frac{(Q_{\max} - Q^*)C^*}{Q^*} \quad (3)$$

where K_1 is the rate constant of complex formation, K_{-1} the rate constant of complex dissociation, $[L]$ the concentration of ligand's binding sites, $[P]$ the concentration of soluble protein, $[PL]$ the concentration of formed complex, $K_{\text{diss,eff.}}$ the effective constant of dissociation of the affinity complex, $Q_{\max}$ the maximum capacity of binding of soluble complement by a unit of affinity sorbent (usually determined at static conditions), and Q^* the amount of soluble product bound to the volume unit of sorbent at equilibrium concentration C^*.

In affinity chromatography, so-called frontal elution (or frontal analysis) is generally used to obtain the isotherms and thereby, the affinity constants of investigated interactions. Apparent dissociation constants K_{diss} and theoretical adsorption capacities $Q_{\max}$ of the affinity pairs can be easily calculated from the adsorption isotherms [16,17]. The experimentally determined curves fit most often the Langmuire

equation:

$$Q = \frac{Q_{\max} C}{K_{\text{diss}} + C} \tag{4}$$

where Q is the amount of protein adsorbed on the affinity sorbent from a protein solution of concentration C. Eq. (4) can be rewritten to linearized forms as:

$$\frac{C}{Q} = \frac{C}{Q_{\max}} + \frac{K_{\text{diss}}}{Q_{\max}} \tag{5}$$

$$\frac{1}{Q} = \frac{K_{\text{diss}}}{Q_{\max}} + \frac{1}{Q_{\max}} \tag{6}$$

and both K_{diss} and $Q_{\max}$ can be obtained from the respective plots. These two constants quantitatively reflect the quality of the conjugate formed by interaction protein–ligand. Moreover, they allow optimization of the entire process and quantification of the external effects influencing the affinity pairing [18].

The dissociation constant, K_{diss}, accounts only for the part of active ligands depending on the conditions of their immobilization on the surface. As a rule, these characteristics differ significantly from the ones measured in a solution. The decrease of absolute value of this parameter confirms the change of the ligand's space structure which resulted in its binding with functionalized sorbent's surface. In contrast, the increase in the discussed characteristics may be related to non-specific adsorption of soluble complement both on the support and molecular surface of formed affinity complex [19].

The main request in successfully carrying out affinity chromatography (dynamic and reversible affinity pairing) is that the formation of the complex of macromolecular solute with bound covalently to the stationary phase ligand has to be maximum adequate to the pairing in a solution. Obviously, the complements should have maximum steric freedom within the porous space of a sorbent. It means that the porosity, or designed morphology, of the used stationary phase is the most important criterion.

3. Immobilization of biospecific ligands on monoliths

The substances used as affinity ligands can be divided into two categories [20]:

(i) general ligands, such as dyes, amino acids, Proteins A and G, lectin, coenzymes, metal chelates, etc.;
(ii) specific ligands, such as enzymes and substrates; antibodies and antigens; hormone and receptors; and others.

The wide range of various ligands and corresponding biocomplementary partners were summarized in the recently published brilliant review by Labrou and Clonis [21].

3.1. Chemistry

The chemistries of covalent binding of bioligands to solid supports are dependent on the reactive groups available at the surface of used sorbent. One common approach represents the direct attachment of biomolecules (natural or synthesized) to a solid phase. Extensive descriptions of various methods used for immobilization of bioligands can be found in the literature [22,23].

There is only one phase in the mold at polymer monoliths preparation. Therefore, almost any monomer which is not suitable for standard polymerization in aqueous suspensions may be used to form such kinds of supports. This greatly increases the variety of surface chemistries that can be obtained [24]. The list of monomers includes chemistries varying from very hydrophilic [acrylamide and 2-(acrylamido)-2-methyl-l-propanesulfonic acid], through the reactive [glycidyl methacrylate, (chloromethyl)styrene, 2-vinyl-4,4-dimethylazlactone] and protected [4-acetoxystyrene] to hydrophobic monomers [styrene and butyl methacrylate] [2,9,25–28]. Another route that increases the number of functionalities for monoliths is chemical modification and grafting [3–6,24].

Up to now, the most frequently used affinity chromatography monoliths are those based on the macroporous copolymer of glycidyl methacrylate and ethylene dimethacrylate (GMA–EDMA). In contrast to the sorbents to be activated, GMA–EDMA polymer contains original epoxy groups. Among the different designs of monolithic supports offered at present in the world market, the short GMA–EDMA beds seem to be more convenient for affinity separations. In fact, the most consequent and significant results on affinity separations are still obtained only by using these kind of monolithic supports. The high speed of the separation across the flat monolithic disks facilitates the product recovery since the exposure to punitive denaturing influences such as solvents, temperature and contact time is dramatically reduced. Together with the open channel-like morphology of disk-shape solid phases (CIM® disks, BIA Separations, Ljubljana, Slovenia), this allows carrying out the immobilization in a single step reaction between these groups of a sorbent and amino groups of immobilized ligand under very gentle "biocompatible" conditions [18,29–38].

Hahn et al. [39] have developed a novel immobilization strategy on GMA–EDMA monoliths leading to a tailored functional surface structure. The model ligand, namely a peptide directed against lysozyme, has been conjugated with glycidyl methacrylate before the polymerization. Further, the monolithic terpolymer was performed using the mixture of this peptidyl conjugate, glycidyl methacrylate and ethylene dimethacrylate and tested for affinity isolation of lysozyme. The authors noticed that, in contrast to the immobilization of the same peptide, a better ligand position was achieved as indicated by smaller affinity constant value.

3.2. Spacers

In a recently published review [20], the problem of necessary spacer introduced between a ligand and sorbent surface has been discussed. In particular, it has been declared that the "ideal" spacer should have:

(i) proper length (at least three atoms);
(ii) no active center which could cause non-specific adsorption between a support and a sample;
(iii) bifunctional ends of the molecule to react with both a substrate and a ligand.

Many investigations in the field of conventional affinity chromatography strongly recommended the use of such spacers to enhance ligand's accessibility and binding. In this case, a ligand is separated from the matrix for the distance of a spacer length. Additionally, spacer molecule also provides an increase in protein flexibility compared to the direct coupling with the matrix. Finally, this fact might even improve the biological activity of immobilized ligand (for example, enzyme) [40].

In the experiments with the model system including glucose oxidase (GOX) [41], the influence of intermediate spacer used at enzyme immobilization on GMA–EDMA short monoliths on enzymatic activity was studied. Thus, it was shown that the substrate's conversion was relatively low for the case of direct immobilization of enzyme on epoxy sorbent. The result was significantly improved when ethylendiamine or glutaraldehyde were introduced as the spacers separated GOX from the support surface.

On the other hand, Luo et al. [42] immobilized macromolecular Protein A and small L-histidine on macroporous GMA–EDMA monoliths both directly and via the spacer. The obtained affinity columns (in this case, there were not short beds of a few mm length but much longer rods) were used for the isolation of IgG from human serum. The authors have shown that the columns which contained the spacer indicated some extent of non-specific adsorption controlled by passing bovine serum albumin (BSA).

From the extensive experience of our group, either macromolecular or low molecular mass bioligands can be directly immobilized on poly(glycidyl methacrylate-co-ethylene dimethacrylate) monoliths using nucleophilic reaction of their amino groups with epoxy functionalities of a matrix without significant influence ligand's activity (for example [30]).

Korol'kov et al. [33] immobilized synthesized peptide bradykinin (BK) and used it as the selective ligand to isolate anti-BK antibodies. The results of these experiments showed that the immobilization of small peptide ligands could be also carried out without any spacer generally recommended in conventional (e.g. on packed columns) affinity chromatography and, in particular, in some cases of affinity processes realized on monoliths [41,43]. It is likely, that the $-COO-CH_2-CH(OH)-CH_2-$ segment that is formed by the opening of the epoxide ring of 2,3-epoxypropyl (glycidyl) functionality plays the role of a short but effective spacer.

The effect of introduced spacer on immunoaffinity binding has been studied more in details by Ostryanina et al. [35]. The authors compared the isolation of antibodies against BSA and bradykinin using CIM® disks with protein and peptide ligands immobilized without any spacer as well as using a specially synthesized "neutral" nonapeptide and terpeptide as intermediate linkers. As a result, very similar affinity constants for all cases of biointeractions were calculated from linearized adsorption isotherms. The adsorption of both kinds of antibodies and model protein (BSA) did not occur on the disks modified with only nonapeptide and terpeptide spacers with no affinity ligands attached.

Further, Vlakh et al. [37] have shown that, despite the absence of intermediate spacers between a ligand and a surface of monolithic medium, the short biocomplementary peptides demonstrated very high affinity to tissue plasminogen activator (t-PA).

3.3. Direct solid-phase peptide synthesis

The peptides synthesized by conventional solid phase procedure [44] are usually cleaved from a resin and can be used for the preparation of affinity stationary phases. In this case, the synthesized and purified peptides are immobilized on the chosen support using different kinds of chemical reactions. Obviously, a better way seems to be in situ preparation of such affinity sorbents using solid-phase peptide synthesis (SPPS) followed directly by the bio-specific separation on the same solid phase. In this case, the used single support has to satisfy the demands of efficient matrix for both the procedures. Thus, this matrix has to provide high permeability, its functionalities should be well accessible to biological molecules and, additionally, it should ensure high chemical and mechanical stability in organic media. Besides, the affinity sorbents intended for the isolation of biological substances should also be sufficiently hydrophilic to avoid the denaturation of a separated biological product.

The discovery of new affinity ligands has been recently substantially accelerated using methods of modern combinatorial chemistry and affinity screening. In this case, the support with "grown" peptide is used directly for the identification of optimal variants of a desired biocomplement. The application of carbohydrate polymer-based supports for synthesis of peptide libraries allows very rapid testing of very large numbers of investigated compounds [45]. In this range, the pioneer work of R. Frank based on the use of cellulose membrane as a solid phase has to be mentioned [46]. This approach known as spot-synthesis becomes more and more popular in regards to searching for new drugs and studying of complex mixtures of natural metabolites. In parallel with synthetic and analytical methodologies, the new and new supports for these purposes are developed. For example, chemically modified polypropylene and polyethylene [47,48], polyethyleneterephthalate [49], glass [50] can be mentioned here.

Ultra-short GMA–EDMA monolithic beds have been also described as the successful example of solid support for peptide synthesis [33,51–57].

It has been shown that GMA–EDMA monoliths with directly produced peptidyl ligands can be easily and effectively used for following high-speed affinity chromatography. Such "2-in-1" combination of the processes of chemical conversion and biospecific separation represents an appropriate way to create highly productive and time stable media for analysis and isolation of biologically valuable products. In particular in the papers [51–54], a series of peptides complementary to human blood coagulation factor VIII (FVIII) were synthesized and the properties of these peptides were tested in affinity separation of FVIII. Vlakh et al. presented a few practical examples of application of affinity sorbents obtained via direct SPPS on GMA–EDMA monoliths [55–57]. There, several peptidyl ligands of different length complementary to plasminogen activators have been synthesized using Fmoc-chemistry. This approach allowed direct obtaining of sorbents suitable for affinity chromatography avoiding a cleavage of synthesized peptides from a carrier followed by their isolation, purification and analysis. The affinity binding parameters were found from experimental frontal analysis data. The results have been compared with those established for CIM® affinity sorbents prepared by immobilization of the same but preliminarily synthesized on conventional resin peptides on the disks using one step reaction with epoxy groups of monolithic material. It has been shown that the affinity constants of these two kinds of sorbent did not vary significantly. Directly obtained affinity sorbents have been used for fast and efficient on-line analysis as well as semi-preparative isolation of recombinant t-PA from crude cellular supernatant.

Furthermore, Vlakh et al. demonstrated the results of specially developed methods of quality control of peptides synthesized on monoliths. Using different modes of chemical procedure and ESI–MS as analytical tool, it was shown that the obtained peptidyl products had a very satisfactory level of purity which could not afford any changes of K_{diss} compared with the same characteristics established for the case of immobilized ligand [56].

4. Examples of bioaffinity pairs investigated by the use of monolithic stationary phases

At present, the novel separation techniques based on the use of short monolithic beds and called recently as high-performance monolithic disk chromatography (HPMDC) [7] is successfully used for analysis and separation of biological molecules including proteins, nucleic acids, peptides, etc. Theoretical concept of this type of monolithic stationary phases was recently developed and discussed in [5,7,8,12].

Practically, in all recent publications on affinity chromatography on ultra-short methacrylate-based monolithic beds the various factors, such as surface density of immobilized ligands, flow rate, concentration of complementary soluble partner, temperature, which affect binding parameters in affinity mode of HPMDC have been analyzed (for example [18]). As it was already discussed above, to compare the data obtained, the affinity interactions were evaluated by a treatment of linearized adsorption isotherms and expressed in the terms of dynamic dissociation constants of formed complexes K_{diss} as well as theoretical adsorption capacities Q_{max}. The found values of dissociation constants of biospecific pairs reflected their high thermodynamic strength. This means that the macroporous design of such sorbents, additionally to extremely high speed of HPMDAC experiments and, accordingly, in a very short operative time, provide a unique opportunity to construct, investigate and quantitatively compare different biocomplementary pairs under the close to physiological conditions.

4.1. Antigen–antibody

The antigen–antibody complex is one of the most often used biocomplementary pairs in different biotechnological processes [58]. Similarly, an increasing number of modern diagnostic and therapeutic technologies are based on the interaction between antibodies with high affinity and their specific antigens [59]. The very popular separation technique immunoaffinity-chromatography (IAC) is based on these biocomplementary interactions, including those using not antigens but the ligands of general specificity (Proteins A, G, L) [60–62].

IAC represents the dynamic approach in which the stationary phase contains an antibody or an antigen and the separated substance is distributed between stationary and mobile phases. The first preparative using of IAC was reported by Campbell in 1951 when the antigens immobilized on *p*-aminobenzyl cellulose was used for purification of corresponding antibodies [63]. At present, this chromatographic method is widely used for the isolation of different biological materials [64], as well as for bioanalytical procedures which, for example, help to quantify the accumulation of antibodies in stressed human or animal organisms (immunomonitoring) [65].

In a greater part of the experiments on affinity chromatography, the macroporous ultra-short methacrylate-based monoliths with corresponding immobilized ligands have been used for the separation of antibodies [18,31–37,66–69] (Table 1, Part a).

More information about using of IA HPMDC can be found in a recently published review [14].

4.2. Enzyme–substrate

Enzymes are able to form highly specific complexes with a variety of substances including their respective substrates. The combination of several binding sites on the surface of enzyme molecule permits the formation of a relatively large number of biospecific complexes with its participation. For example, the antigenic sites of the enzyme can be determined

Table 1
Applications of macroporous methacylate-based monoliths in affinity processing

Target molecule	Affinity ligand	Reference
(a) Antigen–antibody interaction		
Polyclonal IgGs from rat liver	Annexin	[66]
Polyclonal IgGs from rabbit antiserum	Protein A	[66]
Recombinant Protein G from *E. coli* cell lysate	Human IgG	[29,30,34]
Antibradykinin polyclonal IgGs from rabbit bloom serum	Bradykinin	[31,33]
Flag-HAS	Anti-Flag monoclonal IgG	[67,68]
Human IgGs	Protein A	[69]
Polyclonal bovine IgGs	BSA	[18,35]
Human IgGs	Recombinant Protein G	[35,36]
Monoclonal antibodies against clotting factor VIII	Protein A	[41]
Tissue plasminogen activator	Monoclonal antibodies	[37]
(b) Enzyme–substrate interaction (bioreactors)		
4-Nitrophenyl acetate	Carbonic anhydrase	[71]
Cytochrome C	Trypsin	[43,74]
L-Benzoyl arginine ethyl ester	Trypsin	[27,43]
Casein	Trypsin	[27,43]
Transferrin	Trypsin	[41]
BSA, OVA	Trypsin	[41]
Saccharose	Invertase	[41]
Glucose oxidase	Concanavalin A	[41]
Glycoproteins from plasma membranes of rat liver	Concanavalin A	[41]
β-D-Glucose	Glucose oxidase	[41,76]
Oxaloacetic acid	Malate dehydrogenase	[76]
Citric acid	Citrate lyase	[75,76]
Isocitric acid	Isocitrate dehydrogenase	[76]
Purivic acid	Lactate dehydrogenase	[76]
Adenosin 5′-diphosphate (synthesis)	Polynucleotide phosphorylase	[77]
Polyriboadenylate(phosphorolysis)	Polynucleotide phosphorylase	[77]
Plasminogen and its fragments	Tissue plasminogen activator	[37,55–57]
Plasminogen and its fragments	Streptokinase	[37,55–57]
Plasminogen and its fragments	Pro-urokinase	[37,55–57]
(c) Enzyme–inhibitor interaction		
Carbonic anhydrase from human erythrocytes	*p*-(Amino methyl) benzoyl sulphonamide	[71]
Soluble trypsin	Soybean trypsin inhibitor	[18]
Different inhibitors	Human recombinant acetyl-cholinesterase	[78]
(d) Receptor–ligand interaction		
Polyclonal bovine IgGs	Microbial receptors Protein A	[34]
Recombinant human IgGs	Protein G	[32]
Recombinant human IgGs	Protein L	[32]
Ceruloplasmin	Synthetic peptide corresponding to fragment of Menkes ATPase	[79,80]
(e) Complementary interaction		
Poly(U)	Poly(A)	[38]

by investigation of the antigenic structure of the peptide chain in experiments with specific antibodies to this enzyme. Enzymes form complexes with substrates and their analogues, allosteric effectors, metal ions, etc. The type of complex formed determines the mode of the action in chemical processes that take place in the living cell. Excellent review by Freitag [70] summarizes such data using mentioned interactions in analytical chemistry and biochemistry for the elucidation of binding mechanism, the determination of binding strength and screening of substrate/inhibitor molecules.

The monolithic high-throughput stationary phases discussed in this review were used in heterogeneous biocatalysis, where immobilized enzymes were bound covalently to the surface of inert support (Table 1, Part b). The first step of any enzymatic reaction is the formation of specific (affinity) complex between the immobilized enzyme and soluble substrate. The characteristics of such pairing between immobilized enzyme and soluble substrate should be close to those of the same pair formed in a free solution.

The first attempt to construct a flow-through enzyme reactor on the base of GMA–EDMA monoliths was described in [71]. The authors immobilized carbonic anhydrase and used obtained affinity unit for the conversion of two low molecular mass substrates: 2-4-nitrophenyl acetate and chloro-4-nitrophenyl acetate. Different experimental conditions (recirculation flow rate) were investigated. In these experiments, it was shown that monolithic support provide enhanced mass transport. It meant that the substrate was converted much

faster and the diffusion was no longer a limiting factor for enzyme–substrate interaction.

Subsequent experiments carried out on differently designed methacrylate-based monoliths (disks and rods) have shown that proteases can be successfully immobilized and used for enzymatic hydrolysis of their substrates. The most frequently used enzyme was trypsin [27,40,43,72–75].

Other types of enzymes which have been investigated using the same monoliths were invertase, glucose oxidase [73,75], isocitrate dehydrogenase, L-lactate dehydrogenase, citrate lyase, malate dehydrogenase [76], polynucleotide phosphorylase (PNPase) [77] and serine proteases: tissue plasminogen activator, streptokinase and pro-urokinase [37,55–57].

The applications of bioreactors described above were of analytical scale. However, there are many options and good prospectives for the large-scale use of enzymes immobilized to monolithic supports [40].

A very simple and practically suitable construction of bioreactor were suggested by Platonova et al. [77]. In this work, the immobilization of PNPase from *Thermus thermophilus* on GMA–EDMA disk was studied and the parameters of polyriboadenylic acid synthesis as well as its phosphorolysis were established. The authors presumed that the used mode of immobilization of PNPase on macroporous support practically had no disadvantages and could be recommended as a base for designing of simple semi-scale bioreactors.

In the paper [37], the results on quantitative analysis of a range of modeled pairs between tissue plasminogen activator and its probable natural (monoclonal antibodies, plasminogen, fibrinogen) and synthetic complements (linear peptides, poly-L-lysine) were compared to make the right choice of the possible ligand suitable for t-PA isolation. There, the frontal elution curves have been used to evaluate quantitatively the affinity interactions between t-PA and its immobilized counterparts.

Established in the next series of experiments, the values of dissociation constants of affinity complex PA-synthetic ligands, e.g. the pairs of different plasminogen activators and the peptides imitating the parts of binding center on substrate (plasminogen) molecule, clearly demonstrated high affinity of all of them to mentioned above plasminogen activators [55–57].

4.3. Enzyme–inhibitor

The first affinity process bound on monolithic GMA–EDMA disks with immobilized inhibitor of carbonic anhydrase-*p*-(aminomethyl) benzoyl sulphonamide was developed for the isolation of mentioned enzyme from human erythrocytes (Table 1, Part c) [71]. The authors studied the interactions between the enzyme and the inhibitor at different pH. They noticed that at pH > 6.0, the protein was not denatured retaining totally its enzymatic activity.

Ostryanina et al. [18] used affinity pair soluble trypsin-immobilized soybean trypsin inhibitor (TR-SBTI) to study the effects of experimental conditions (flow rate, surface density of immobilized ligands, temperature) on biocomplementary pairing in HPMDAC.

In [78], the monolith with immobilized human recombinant acetylcholinesterase was used for on-line inhibition studies. The authors noticed the increased enzyme stability and system automation which allows a large number of compounds to be analyzed in continuous.

4.4. Reception–ligand

So-called receptor affinity chromatography based on the specificity and reversibility of receptor–ligand interactions has been described in papers (Table 1, Part d) [30,32,34,36,79,80].

In particular, Berruex et al. [32] studied the specific interactions between antibodies and immobilized group specific ligands, such as microbial receptors Proteins A, G and L. All of these ligands were known to bind IgG in a highly specific manner. The supports with immobilized receptors were used for fast biospecific separation of different types of IgGs including recombinantly produced humanized antibodies.

Other type of the receptor–ligand interactions was described in the papers [79,80]. Ceruloplasmin (Cp) (Ferroxidase, EC 1.16.3.1, a copper-containing plasma glycoprotein) is a major transporter of copper ions to non-hepatocyte cells through the blood stream of mammalians [81]. Highly specific Cp binding to the receptor localized on cellular surface is indispensable for the copper ion transfer through the cell membrane. The authors [80] have used monolithic disk affinity chromatography to identify the site on Cp molecule that was responsible for interacting with copper transferring Menkes ATPase. They synthesized and immobilized on macroporous GMA–EDMA monolithic disk the peptide identical to the fragment of Menkes ATPase involved in copper ion transfer and studied pairing of ceruloplasmin with this peptidyl ligand. The chromatographically obtained dissociation constant K_{diss} (1.5×10^{-6} mol/L) was found to be close to the value calculated for the interaction of the same peptide with antibodies against the peptide–hemocyanin conjugate (1.1×10^{-6} mol/L) [82]. This fact indirectly testified to a specific interaction between ceruloplasmin and investigated fragment of Menkes ATPase.

4.5. Complementary polynucleotides

The use of hybridization of complementary strands of DNA, RNA and its analogues, one of which is immobilized on solid supports, has become very popular technique in molecular biology and is applied for detection, isolation and genetic analysis of specific sequences [83,84]. When defined polynucleotide sequence is immobilized on solid support, the specific pairing of complementary bases of partner molecule being in a solution takes place. Thus, a specific adsorption of hybridized polynucleotide and its separation

from any non-complementary partners present in complex mixture occur [85].

The authors of this paper have realized the first experiments using affinity monolithic disk chromatography to study the hybridization between complementary polyribonucleotides polyuridylic acid [poly(U)] and polyadenylic acid [poly(A)] (Table 1, Part e) [38]. This paper reports the data on immobilization of poly(A) on CIM® disk using covalent binding to the surface of polyribonucleotide via its adenine base. Similar to the proteins and peptides, the determination of dissociation constant of poly(A)–poly(U) duplex was carried out. To evaluate both parameters of affinity binding, Q_{max} and K_{diss}, usually applied for proteins frontal analysis procedure was also used.

The adsorption isotherms resulting from frontal experiments were used for calculation of these values. The effect of surface occupation by poly(A) and the data on comparing of static and dynamic experiments were demonstrated. It seemed that the increase of ligand concentration did not strongly affect K_{diss} of studied duplexes. In all cases, the experimentally determined dissociation constants were found close to 10^{-4} mol/L. The shown data indicates that only 1.5–3.5% of immobilized poly(A) are accessible for a soluble poly(U) that can be explained by steric interference of reacted macromolecules.

5. Conjoint affinity processes

One of significant advantages of affinity chromatography on short monolithic beds is the unique opportunity of simultaneous use of several separation units (disks) with different functionalities placed in a single cartridge. This approach enables separation and purification processes that are difficult to achieve using standard methods. In the first report on this approach, Josic et al. [66] have isolated monospecific polyclonal antibodies obtained against calcium-binding protein annexin. The cross-reacted antibodies complexing with similar proteins from the annexin family were removed from the antiserum by passing it through the disk with immobilized corresponding antigens. Later, the name “conjoint liquid chromatography” has been coined for this operational mode [73]. In the paper [41] concerning the isolation of monoclonal antibodies against clotting factor VIII, the process was carried out using the combination of two disks, e.g. strong anion-exchanger and Protein A affinity unit. Both IgG (bound to the Protein A disk) and accompanying proteins (bound to QAE disk) from mouse ascites were retarded and eluted separately.

Ostryanina et al. [35] reported on the method of fractionation of pools of polyclonal antibodies against bradykinin in a single step using conjoint immunoaffinity HPMDC. Such pools typically contain both monospecific antibodies against each part of a conjugate used for immunization and some cross-reactive antibodies that have epitopes for complementary binding to all parts of the complex antigen [86,87].

To achieve a well-controlled fractionation of described polyclonal pool of antibodies, each structural part of complex antigen, e.g. BK, BSA, succinylized bovine serum albumin (BSA-S), and the complete conjugate (BSA-S-BK) were immobilized on individual disks. Four affinity disks were installed in a single housing and adsorption step was carried out. The simplicity of the commercially produced cartridge allows easy rearrangement of the sequence of the disks within the stack, as well as reinsertion of only individual disks for subsequent desorption. The data on specific fractionation of rabbit blood serum using individual disks with immobilized different parts of complex antigen, as well as the results obtained using the procedure that includes the stack of disks were obtained. The adsorption capacity of single disks appears to be rather different from that of the stacked set. The reason for this was explained by the adsorption of cross-reactive antibodies with affinity close to the specific antigen that together with monospecific antibodies contributes to the overall binding. Changing the sequence of the disks with different immobilized antigens can be used to detect these cross-reactive antibodies. This method demonstrated very high reproducibility and enabled quantification of antibodies in the blood serum. The authors also compared results obtained by such multifunctional fractionation of pools of polyclonal antibodies with those afforded by widely accepted enzyme-linked immunosorbent assay (ELISA). In contrast to the indirect enzyme-linked immunosorbent assay, the HPMDC separations enables direct determination of antibodies or antigens even in complex biological matrixes such as crude blood serum. In addition, high speed of the HPMDC approach allows carrying out many separations in series within a short period of time.

6. Conclusions

Recently developed monolithic stationary phases have revolutionized protein and polynucleotide chromatography combining speed, capacity, and resolution in a unique manner. Since such stationary phases contain no particles but only flow-through pores, the usual mass transfer restrictions based on interphase mass distribution (including chromatographic separations) are not observed and extremely fast separations become possible. Especially significant advantages are observed for the cases of bioprocesses based on strong affinity interactions between the biological complements located in mobile (liquid) and solid (stationary) phases. This indicates that enzyme–substrate, antigen–antibody, and other biological pairs in conjunction with high performance affinity chromatography can be used for the on-line process monitoring of high value biotech products.

The design of disk-shaped methacrylate supports allows the combination of different ligands within one chromatographic cartridge. It is then possible to apply a unique multidimensional chromatography or affinity multifunctional approach to complex biofractionations. As well, these

supports can be effective sorbents for solid phase peptide synthesis or enzymatic bioconversion.

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