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Monolithic peptidyl sorbents for comparison of affinity properties of plasminogen activators

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Abstract

Plasminogen activators are the proteases which convert plasminogen into plasmin dissolving, in its turn, the major component of blood clots, fibrin. They are extremely useful in heart attack therapy. Modern and most appropriate way of scaled up production of these valuable proteins is gene engineering. In this case, a separation and a purification of target product become the important steps of the whole process. Recently developed *affinity chromatography on short monolithic columns* seems to be a very attractive method for these purposes. High speed of a process prevents the protein's denaturation due to temperature or/and solvents influence. The better mass transfer mechanism (convection rather than diffusion) allows considering only biospecific complexing as time limiting step. Specificity of several synthetic peptides to *plasminogen activators* have been studied by *affinity chromatography on short monolithic columns*. Peptide ligands were synthesized by conventional solid phase peptide synthesis (SPPS). The immobilization procedure was carried out as a one step process at static conditions. The results of quantitative evaluation of such affinity interactions were compared with those established for plasminogen that is the natural affinity counterpart to both proteases. Additionally, some of investigated peptides were synthesized directly on GMA–EDMA disks and their affinity properties were compared with those established for the case of immobilized ligands. The possibility of using of synthetic peptidyl ligands for plasminogen activators isolation from native cell supernatant and model protein mixtures has been demonstrated.

Keywords: Fibrinolysis; Tissue plasminogen activator; Streptokinase; Pro-urokinase; Macroporous disks; Solid phase peptide synthesis; GMA–EDMA monoliths; Ligand immobilization; Short monolithic columns

1. Introduction

Affinity chromatography is a powerful tool widely used for target protein separations. In its turn, it is known that a choice of the "right ligand" complementary to the product to be isolated represents a crucial point of this method. Despite of that the application of natural counterparts for affinity separations can be counted as a preferable way, due to high lability and high cost of natural biomolecules, like monoclonal antibodies or substrates, the synthetic ligands look more and more attractive. In particular, the peptides mimicking the active centers of proteins become the most widely used affinity sites [1,2].

Present research is devoted to the quantitative analysis of the affinity interactions between plasminogen activators (PA) and several synthetic peptide ligands by means of affinity chromatography on monolithic short columns produced from a copolymer of glycidyl methacrylate (GMA) and ethylene dimethacrylate (EDMA) [3–7]. All plasminogen activators are serine proteases activating the conversion of plasminogen into plasmin, in other words, the enzymes responsible

Abbreviations: CIM®, convective interaction media; SPPS, solid phase peptide synthesis; t-PA, tissue plasminogen activator; SK, streptokinase; pUK, pro-urokinase; K, lysine; G, glycine; R, arginine; P, proline; C, cysteine; V, valine; PBS, phosphate buffered saline; CHO, Chinese Hamster Ovary

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for the lysis of fibrin clots [8–11]. Thrombolytic agents approved for clinical use include streptokinase (SK), urokinase (UK), pro-urokinase (pUK) and tissue plasminogen activator (t-PA). SK (M_r 47,000) is a secretory protein produced by various strains of β-hemolytic *Streptococus* [12,13]. UK (M_r 54,000) is isolated from urine and conditioned media of fetal kidney cell cultures, but it is also present in conditioned media from endothelial cell cultures [14–16]. pUK is a single-chain precursor molecule, which is converted to urokinase by a cleavage of Lys^{158}–Ile^{159} peptide bond [17]. t-PA (M_r 68,000) is present in human tissues and circulates at very low concentrations in the blood [18,19]. However, nowadays t-PA as well as UK and pUK have been obtained by recombinant technology [20–22]. Despite of the fact that the mechanism of plasminogen activation by all mentioned PA is different, all of them promote proteolysis of the same Arg^{561}–Val^{562} peptide bond [23]. The main feature of plasminogen activation by t-PA is its direct affinity to fibrin [24]. The t-PA activity is based on the formation of triple complex between t-PA, plasminogen and fibrin surface. SK, UK and pUK have no affinity to fibrin.

Process of fibrinolysis represents a very complicated network of simultaneous biological events. Recently, several detailed papers devoted to step-by-step modeling of the fibrinolysis process with t-PA participation have been published [25–27]. The present one concerns the comparison of affinity properties of pUK, SK and t-PA investigated with use of affinity chromatography on short monolithic column.

The natural substrate plasminogen and several peptides were used as affinity ligands. The chosen peptides were obtained by solid phase peptide synthesis (SPPS). The first group of peptides represent the parts of one of PA binding sites on plasminogen molecule and were supposed as affinity ligands: KCPGRVVGGC (557–566), KCPGRV (557–561) and RVVGGC (561–566). Due to the direct specificity of t-PA to fibrin, the short GPRP peptide (an inhibitor of fibrin polymerization [28]) as well as different derivatives of lysine: KKKK, KKKKGPRP and dendrimer of third generation $K_{15}A$, were included in the consideration.

There are a few publications where GMA–EDMA monoliths are described as successful examples of solid phase for peptide synthesis followed by affinity chromatography on the obtained peptidyl sorbents [27,29–31]. According to that, some of listed peptides were synthesized directly on GMA–EDMA disks. The experimental comparison of two kinds of sorbents has been carried out.

2. Experimental part

2.1. Materials and chemicals

Macroporous monolithic disks of 12 mm × 3 mm with mean pore size of 1.5 μm and a porosity of 0.63 ml/ml sorbent (standard CIM® Epoxy Disks) as well as specially designed cartridges were from BIA Separations, d.o.o., Ljubljana, Slovenia.

Plasminogen and streptokinase were purchased from Sigma (Germany). Recombinant pro-urokinase was a commercial product of Russian Cardiological Scientific-Industrial Center (Moscow, Russia). Recombinant t-PA was kindly donated by Boehringer Ingelheim Pharma KG (Biberach, Germany). Bulk chemicals for buffer preparation were from Fluka Chemie AG (Switzerland).

Diisopropylcarbodiimide (DIC), trifluoromethanesulfonic acid (TFMSA), trifluoroacetic acid (TFA), thioanisole, ethanedithiol, hydroxybenzotriazole (HOBt), and *p*-toluenesulfonic acid (TosOH) were from Fluka Chemie AG (Switzerland). Dimethylformamid (DMF), dichloromethane (DCM), and triethylamine (TEA) were purchased from Vecton Ltd. (Russia). Boc- and Fmoc-protected amino acids were from Sigma (Germany) and Bachem AG (Switzerland).

Standard protein kit and precast gels for PAGE were purchased from Bio-Rad (Germany). All chemicals used in this method including those for gels and buffers preparation were from Fluka and Sigma.

Buffers used for affinity chromatography were prepared by dissolving analytical grade salts in doubly distilled water that had been additionally purified by filtration through a 0.2 μm pore size microfilter (Millipore Inc., USA).

2.2. Instruments

A NPS 4000 semiautomatic peptide synthesizer (Neosystem, Strasbourg, France) was used for the automated synthesis of peptides. A chromatographic instrument consisting of two piston pumps, a UV detector and a software data processing station (Golden System, Beckman, USA) fitted with Vydac C_{18} of 4.6 mm × 150 mm (particle size of 5 μm) and 22 mm × 250 mm (particle size of 10 μm) columns was used for the analysis and preparative purification of obtained peptidyl products.

For quality control and determination of yields of the synthesized product, an AAA T339 M automated amino acid analyzer (Microtechna, Prague, Czech Republic) was used. Mass spectrometric analysis was performed using ES–MS instrument produced at the Institute of Analytical Instrumentation RAS (St. Petersburg, Russia).

Affinity chromatography was carried out using a chromatographic system consisting of a peristaltic pump (2115 Multiperpex pump, LKB, Bromma, Sweden), a UV detector (2138 Uvicord S, LKB, Bromma, Sweden) and a recorder (2210 Recorder, LKB, Bromma, Sweden). To isolate t-PA from a cell supernatant and SK/pUK from a model protein mixture, a chromatographic system consisting of Waters 2690 Alliance Separations Module (USA), Waters 490 Scanning Fluorescence (USA) detector and Knauer injector (Germany) was used and a computer assisted flow control and analysis software (Andromeda 1.6 CSW) was applied for data analysis.

The concentration of proteins was determined using a UV–vis spectrophotometer SF 26 (LOMO, St. Petersburg, Russia).

SDS-PAGE characterization of isolated t-PA was carried out with use of PowerPac System (Bio-Rad, Germany).

2.3. Methods

2.3.1. Peptide synthesis

Peptides used for immobilization on GMA–EDMA disks were obtained by solid phase peptide synthesis using the Boc–Bzl strategy as described elsewhere [32]. For decapeptide, removal of acetaminomethyl (Acm) protective groups of cysteines and S–S bond formation were performed following the protocol presented in [33].

For SPPS on GMA–EDMA the native epoxy groups were preliminary converted into amino or hydroxyl groups. To introduce amino function, the disks were immersed into ethylene diamine for 4 h at 50 °C or stored overnight at RT. Hydroxyls were generated by acidic hydrolysis with 0.1 M H_2SO_4 for 6 h at 80 °C. Then, an anchoring amino acid β-Ala was introduced as described in [34]. For SPPS on monoliths the recently developed procedure based on Fmoc-chemistry was used [34]. Side-chain protected groups were *tert*-butoxycarbonyl (Boc) for lysine and arginine and acetaminomethyl (Acm) for cysteine. For hexapeptides the Acm-groups of cysteine were not removed. The solution containing 2 eq. $HgAc_2$ in 30% acetic acid per Acm group of decapeptide was used for Acm deprotection. For that 1 ml of corresponding solution was passed through the disk. After 15 min the procedure was repeated. Then, disk was washed 5 min with 50% acetic acid, 5 min with β-mercaptoethanol and 5 min with water at flow rate 4 ml/min. Oxidation of SH-groups was carried out with 10% H_2O_2 for 15 min.

All obtained peptides were tested by amino acid analysis after a treatment of a sample with 6 M HCl at 110 °C for 24 h. The quality of some peptides was investigated by ES–MS method. MS spectra were detected at the mode of positively-charged-ion analysis. Lyophilized samples of synthesized peptides were dissolved in 50% acetonitrile with 1% AcOH to reach a concentration of 5–10 pmol/μl. The sample volume was equal to 10 μl.

2.3.2. Immobilization procedure

Before the immobilization CIM® epoxy disk was washed by ethanol, ethanol–water (1:1) mixture and water.

In the case of plasminogen immobilization, the disk was immersed into 0.1 M sodium carbonate buffer (pH 9.3) for 2 h and, after that time, was transferred into 1 ml of 5.0 mg/ml of protein solution in the same buffer. The binding reaction was allowed to proceed over 16 h at 37 °C without any stirring. Then the disk was washed with initial pH 9.3 carbonate buffer to remove the excess of unreacted ligand from the porous volume. The amount of ligand coupled to the support was determined (i) by monitoring the decrease in absorbance at 280 nm of protein solution before and after immobilization, accounting for protein content in washing buffer volume, and (ii) by Lowry test [35].

In the case of peptides, a washed disk was immersed into 0.1 M sodium borate buffer (pH 10.0) for 2 h. The disk was then transferred into 1 ml of 5 mg/ml peptide solution in the same buffer. The binding reaction at 30 °C was carried out for 20 h without any stirring. The amount of immobilized peptide was determined according to absorbency at 230 nm of peptide solution before and after the reaction with a sorbent. The immobilization buffer was then replaced by HPMDAC mobile phase, i.e. 0.01 M phosphate buffered saline (PBS), pH 7.0. The disks were stored in PBS solution containing 0.02% sodium azide at 4 °C.

2.3.3. Determination of quantitative parameters of dynamic adsorption by affinity chromatography

The affinity characteristics of prepared affinity CIM® disks, such as maximum adsorption capacity (Q_{max}) and dynamic dissociation constants of affinity complex (K_{diss}), were evaluated on the basis of mathematical treatment of experimental adsorption isotherms resulting from frontal analysis [36]. For this purpose, model solutions of standard t-PA, SK and pUK with concentrations ranging from 0.01 to 0.5 mg/ml were passed through the corresponding disk. Unbound protein molecules were removed with 0.01 M PBS buffer and, additionally, the disk was washed with 2 M NaCl. Affinity bound proteins were eluted with 0.01 M HCl, pH 2.0. The flow rate at adsorption and desorption was 2 ml/min. Graphical treatment was performed by virtue of "Origin 6.0" computer program. The standard Lowry test was used to determine peptide concentrations.

2.3.4. Isolation of t-PA from a native CHO-cell supernatant and SK/pUK from model protein mixture by affinity chromatography

t-PA was isolated from a Chinese Hamster Ovary (CHO)-cell supernatant containing 6 μg/ml of t-PA (determined by ELISA as described in [23]). The total protein amount in the supernatant was 21 μg/ml. CHO-cell supernatant was obtained as described elsewhere [25]. SK and pUK were isolated from model protein mixture. For this purpose two kinds of mixtures were used. The first one contained 10 μg/ml of SK/pUK and total protein amount was 45 μg/ml. The other one contained 50 μg/ml of SK/pUK and total protein amount was 400 μg/ml. For separation of PA, 3 ml of the cell supernatant or 1 ml of model mixture containing SK/pUK in PBS buffer was loaded on the disks modified by different ligands and the adsorption at dynamic conditions was carried out. After a removal of ballast proteins by washing of a disk with 0.01 M sodium phosphate buffer, pH 7.0, containing 2 M NaCl, the adsorbed t-PA was eluted with 0.01 M HCl, pH 2.0. The flow rate at both adsorption and desorption was 2 ml/min.

2.3.5. SDS-PAGE

For SDS-PAGE, the lyophilized samples of affinity isolated proteins were dissolved in a solvent containing 20 mM Tris–HCl (pH 8.0), 2 mM EDTA, 5.0% SDS, and 0.02% brome phenol blue and boiled for 2 min at 100 °C. After a centrifugation, the samples were applied, in parallel with protein standard markers, onto a plate covered with precast 12.5 and 15% polyacrylamide gel. Staining of protein zones after SDS-PAGE was done using 0.1% $AgNO_3$ [37].

3. Results and discussion

3.1. Characterization of peptide ligands

As it was mentioned above, seven peptides were chosen as affinity ligands to PA. There were five linear peptides (RVVGGC, KCPGRV, GPRP, KKKKGPRP, KKKK), one cyclic (KCPGRVVGGC) and one lysine dendrimer of third generation ($K_{15}A$). All peptidyl ligands used for immobilization on the disks were prepared by standard SPPS on polystyrene resin using Boc–Bzl chemistry. The obtained peptides were chromatographically purified and investigated by a standard method of quantitative amino acid analysis. It was found that all prepared peptides had the amino acid ratio corresponded to the expected one. Additionally, the dendrimer as well as cyclic decapeptide were analyzed by ES–MS method and very good agreement of theoretically calculated molecular masses of these compounds to those found by MS analysis was established.

Recently, the possibility of using of GMA–EDMA materials for SPPS followed by affinity chromatography on the same solid phases has been described [29–31,34]. Follow peptidilated sorbents were prepared by direct SPPS on monoliths: GMA–EDMA–β-ARVVGGC, GMA–EDMA–β-AKCPGRVVGGC and GMA–EDMA–β-AKKKKGPRP. The data found by amino acid analysis and ES–MS clearly showed that all prepared compounds had appropriate amino acid content and their molecular masses corresponded to the theoretical values. In ES–MS spectra of obtained peptides some minor peaks corresponded the inessential defects of the sequence were found. The main defects can be related to the absence of β-Ala, Val and Lys residues. According to RP-HPLC data, the yields of target peptides were found 83–86% that was sufficient for our further purpose (affinity chromatography). Ligand coupling yields were about 3 μmol/ml of sorbent.

3.2. Immobilization of affinity ligands on GMA–EDMA monoliths

Taking into account the high chemical reactivity of the original epoxy groups of GMA–EDMA macroporous polymers and high concentration of reactive amino groups in all synthetic and natural PA ligands studied, the immobilization procedure was carried out as a one step process at static conditions over 16 h [38]. Such long time of coupling procedure can be explained by the fact that, in general, the reaction between epoxy and amino groups represents very slow process [39]. Recently it was established that maximum amount of bound to epoxy monoliths ligands was achieved only after 15 h incubation [38]. Furthermore, our previous experiments have shown that the recirculation of reaction mixture through the disk did not reduce the reaction time [40]. Nevertheless, just this chemistry was used in all our investigations. The reason is that the use of other type of chemical reactions would require an intermediate derivatization of original epoxy groups that, in its turn, would need the additional reaction step, and, accordingly, additional time.

Table 1
Amount of different ligands immobilized on GMA–EDMA disks

Ligand	MM	Q_{imm} (mg/disk)	Q_{imm} (μmol/ml)
Plasminogen	90,000	1.70	0.06
KCPGRVVGGC	971	0.90	2.70
KCPGRV	728	0.70	2.90
RVVGGC	659	0.70	3.00
GPRP	424	0.45	3.10
KKKKGPRP	936	0.90	2.80
KKKK	529	0.55	2.90
Dendrimer $K_{15}A$	2,009	1.35	2.00

In our case, no intermediate spacers were inserted because the numerous papers on affinity chromatography on short monolithic GMA–EDMA columns have shown no dependence of the efficiency of biospecific separations on any inert spacers both for the cases of protein and peptide immobilized ligands [41]. The results of ligand immobilization are summarized in Table 1. As it seen from Table 1, the amount of plasminogen calculated in mg per disk is higher than the amount found for small peptides. At the same time, since the molecular mass of plasminogen exceeds significantly that of peptides, the molar amount of bound protein seems to be much lower. Obviously, that this fact can be exceptionally explained by differences in sizes of plasminogen and peptides. The surface occupied by one plasminogen molecule is accessible for more than one peptide molecule, but the total occupied surface for both cases should be the same. Additionally, the obtained ligand density was in a good agreement with declared optimal ligand's capacity (not more than 20 μmol/ml sorbent) necessary to realize the mechanism of affinity adsorption [7,42]. With GMA–EDMA monoliths it was shown that the use of exceeding this recommended ligand density led to non-specific adsorption of back-side proteins [29].

3.3. Affinity chromatography

3.3.1. Dissociation constants

The quantitative parameters of affinity paring between PAs and plasminogen/peptide ligands were found from frontal

analysis data. It is well known that this experimental approach is sectioned into a few steps: (1) saturation of available adsorption centers on a surface by molecules of biocomplement dissolved in mobile phase; (2) if necessary, elution of nonspecifically adsorbed (hydrophobic or ionic interactions) part of the protein of interest, and, finally, (3) desorption of a product bound specifically to the ligand. A linearization of experimentally obtained adsorption isotherms based on frontal elution curves allows calculation of the affinity parameters of pairing, e.g. dissociation constants and maximum adsorption capacity.

In all recent publications on affinity chromatography using ultra-short monolithic macroporous layers as efficient stationary phases [38,40,41], the values of constants of dissociation of biospecific complexes were found to be very close to those obtained in the solutions. This means that the macroporous optimized design of these sorbents (additionally to extremely high speed of this type of affinity processes and, accordingly, very short time for each experiment) can provide a unique opportunity to construct, investigate and quantitatively compare different biocomplementary pairs under conditions close to physiological. The same approach has been used in this research.

Despite of the fact that the short monolithic columns allow using of very high flow rates, all experiments of this research were carried out at 2 ml/min. Recently, the influence of flow rate (up to 10 ml/min) on recovery of biological product has been demonstrated by our group [40]. It was established that both K_{diss} and maximum adsorption capacity did not depend on flow rate. However, the higher flow rate, the higher the sample volume has to be loaded to achieve the saturation. Thus, flow rates of 7–10 ml/min that would be very attractive from the point of time benefit is offset by lost of valuable biological material because of the increasing through passing. Therefore, flow rates 2–5 ml/min are a good compromise allowing the completion the process within a few minutes.

Obviously that in the range of investigated ligands, plasminogen have been accepted as "zero-point" on a scale of measured interactions. As expected, the strongest specific interactions were found for substrate–enzyme pair, e.g. plasminogen–t-PA, plasminogen–SK and plasminogen–pUK (Table 2). The dissociation constant of these complexes appeared at least one order of magnitude lower than those established for other partners. It should also be stated that found affinity parameters are also very close to the values measured in the solution. Thus, the Michaelis–Menten constants for complexes plasminogen–SK and plasminogen–pUK were found as 7.7×10^{-7} [43,44] and 4.0×10^{-7} M [45,46], respectively. In the case of t-PA, the Michaelis–Menten constants for complex plasminogen–t-PA varied from 6.5×10^{-5} up to 1.4×10^{-7} [9]. These data corresponds also to that established for the case of plasminogen immobilized on Sepharose. There, the dissociation constant of plasminogen–SK complex was equal to 6.3×10^{-8} M [47]. The obtained in present work data confirms a high degree of protection of natural structure (and, accordingly, biological function of proteins immobilized on the surface of GMA–EDMA monoliths), as well as the advantages of chosen monolithic stationary phases: well controlled structure of the operating surface of the flow-through channels (pores), rapid intrapore mass transport not dependent on molecular diffusion, high concentration of reactive epoxy groups ensuring high surface density of functional ligands covalently bound, etc.

Table 2 demonstrates also the values of dissociation constant of investigated complexes between immobilized syn-

Table 2
Affinity parameters of the PA interactions with immobilized and directly synthesized on GMA–EDMA disks peptides

Ligand[a]		t-PA		SK		pUK	
		K_{diss} (M)	Q_{max} (μmol $\times 10^2$ ml^{-1} of sorbent)	K_{diss} (M)	Q_{max} (μmol $\times 10^2$ ml^{-1} of sorbent)	K_{diss} (M)	Q_{max} (μmol $\times 10^2$ ml^{-1} of sorbent)
	Plasminogen	9.3×10^{-7}	0.6	7.7×10^{-7}	0.3	5.0×10^{-7}	0.6
	KCPGRVVGGC	3.3×10^{-6}	1.8	1.8×10^{-6}	0.9	3.5×10^{-6}	2.5
←	KCPGRV	2.1×10^{-6}	1.5	1.3×10^{-6}	0.8	5.0×10^{-6}	2.9
	RVVGGC	3.6×10^{-6}	2.5	3.2×10^{-6}	1.5	6.7×10^{-6}	3.6
	GPRP	2.2×10^{-6}	3.2	1.1×10^{-5}	0.9	1.2×10^{-5}	3.8
	KKKKGPRP	1.7×10^{-6}	3.9	2.5×10^{-5}	2.5	2.0×10^{-5}	4.4
	KKKK	1.5×10^{-6}	2.1	1.5×10^{-4}	2.8	2.2×10^{-4}	5.3
	Dendrimer $K_{15}A$	5.0×10^{-5}	4.0	–	–	4.4×10^{-4}	5.5
	β-AKCPGRVVGGC	3.3×10^{-6}	1.8	2.7×10^{-6}	0.9	2.3×10^{-6}	1.9
→	β-ARVVGGC	3.6×10^{-6}	3.4	4.0×10^{-6}	1.8	7.5×10^{-6}	4.2
	β-AKKKKGPRP	2.1×10^{-6}	2.8	1.2×10^{-4}	2.5	1.3×10^{-4}	5.6

Conditions: For estimation of affinity constants different sample volumes with different concentrations of PA (0.01–0.50 mg/ml) were pumped through the disks with immobilized ligands to produce the breakthrough curve (PBS, pH 7.0); intermediate rinsing procedure with 2 M NaCl was carried out and specifically bound t-PA was eluted using 0.01 M HCl (pH 2.0); desorbed fraction of t-PA was collected and analyzed by Lowry test; K_{diss} and Q_{max} presented in the table are average values of those calculated from linearized forms of the Langmuir equation (Origin 6.0) with the r^2 equal to 0.9756–0.9998.

[a] (→) Directly synthesized peptide; (←) immobilized peptide.

thetic peptides and PAs. Here, the complexes of PAs with the group of peptides mimicking the sequence of plasminogen binding site can be characterized as the strong ones. The values of K_{diss} are of the same order, 10^{-6} M, and very close to K_{diss} determined for natural substrate. In the case of t-PA and SK, neither extension of peptide length nor cyclization of peptide chain did influence K_{diss} values. In contrast, for the case of pUK, some differences depending on peptide length and its sequence have been observed.

It is known that t-PA has affinity to fibrin and its degradation products. The adsorption of t-PA on fibrin surface is passed including so called "lysine site". It had been demonstrated the possibility of the separation of t-PA on Lysine–Sepharose column [48] with K_{diss} found as 8.0×10^{-5} M. In our experiments, most probably due to the small size of amino acid ligand, we did not observe any interactions between lysine immobilized on rigid macroporous material of monolithic GMA–EDMA disk and t-PA. At the same time, the hetero- and homolysine peptides revealed very strong pairing with t-PA. The most significant difference between t-PA and SK/pUK was the high t-PA affinity to KKKK and KKKKGPRP as well as to GPRP peptide that played an inhibiting role in t-PA–fibrin interaction. The use of branched lysine dendrimer molecule led to the decreasing of K_{diss} up to 5.1×10^{-5} M.

Contrary to t-PA, K_{diss} of complexes between SK/pUK and KKKK peptide were found around 10^{-4} M. Indeed, these proteases have no affinity to fibrin, its derivatives and other agents of fibrinolysis process; so this fact can be explained by manifestation of rather ionic or different weak interactions than real affinity. In the case of dendrimer $K_{15}A$, no specific interaction was observed during saturation with SK-containing solution. For pUK, the values of K_{diss} were comparable with those found for KKKK peptide.

As to GPRP and KKKKGPRP peptides, the analysis of data presented in Table 2 enables to follow the impairment for both SK and pUK up to 10^{-5} M. It is clear that the value of K_{diss} obtained for KKKKGPRP peptide, in general, depends on GPRP fragment. The values of K_{diss} for GPRP–SK/pUK pairs confirm the fact of affinity interaction that may occur because of the presence in the sequence of such amino acids like Gly, Pro and Arg which are also included in the PA binding site on plasminogen molecule.

The investigation of interactions of SK/pUK with peptides obtained by direct SPPS on GMA–EDMA had also been done. It was established that both types of peptidyl supports exhibited the similar constants of affinity complex dissociation (Table 2). This fact indirectly testified the high quality of peptides obtained by direct SPPS and made this approach very attractive for affinity matrix preparation. Especially, it can be very important for the cases of searching or screening of appropriate ligands among the wide range peptides. The main advantages of this approach comparatively to traditional way are shorter reactions times, the use of less amount of chemicals that, respectively, makes the procedure cheaper. Furthermore, the most important difference between discussed cases is that direct synthesis of a ligand on the polymer ensures single-point binding to a surface whereas the covalent immobilization can involve more than one reactive groups of a peptide. This risk is increased with increasing of peptide length. In contrast to "random" conformation of immobilized peptidyl sequences, the direct synthesis on monolithic sorbent allows an obtainment of the surface conformation similar to a "brush".

The differences between immobilized and directly synthesized peptides may probably arise in two cases. At first, if the quality (chemical structure) of synthesized ligands is not comparable to that of immobilized analogues. The way of preparation of affinity sorbent by immobilization of preliminary synthesized and purified peptides ensures the high quality of bound ligands of any reasonable length. From our experience, the direct solid phase synthesis of peptides containing more than 10–12 amino acids meets some problems that lead to the appearance of failure sequences [34]. This, in its turn, may worsen the affinity pairing process and, consequently, affect the affinity parameters. Secondly, the more peptide length, the more difference between spatial structures of immobilized and directly synthesized peptides. As it was mentioned above, the immobilization process of ready peptide can involve more than one reactive groups of a peptide whereas the direct synthesis of a ligand on the polymer ensures single-point binding to a surface. This fact may also alter the affinity pairing.

3.3.2. Isolation of t-PA from a CHO-cell supernatant and SK/pUK from model protein mixtures

The GMA–EDMA sorbents functionalized with plasminogen and peptide ligands were used for the isolation of t-PA from a CHO-cell supernatant and SK/pUK from model protein mixtures. The protein amounts desorbed from the disks were measured by Lowry test. For t-PA isolation a supernatant containing 6 μg t-PA/ml was used. At the same time, the total protein amount in the supernatant was 21 μg/ml. The possibility of t-PA separation with GPRP and lysine-derived peptides from a cell supernatant was preliminary described and discussed [41,44]. Data presented in Table 3 concern the application as affinity ligands of the plasminogen and peptides mimicking its PA binding site. The total amounts of t-PA isolated from the supernatant using peptidylated sorbents were found about 89–100% of loaded t-PA that is higher than in the case of plasminogen disk (78%). The purity of isolated products were analyzed by SDS-PAGE (Fig. 1). Except t-PA no other proteins were found.

Table 4 demonstrates the results of SK/pUK separation from a model protein mixture. Protein mixture consisted of phosphorylase B, BSA, ovalbumin, ribonuclease A, lysozyme, carbonic anhydrase, trypsin inhibitor and SK/pUK. Presented results clearly show that only plasminogen and peptides mimicking its PA binding site can be used for SK/pUK isolation. These results are in a very good agreement with K_{diss} of investigated pairs and, moreover, with the mechanism of fibrinolysis process catalyzed by

Table 3
Amounts of t-PA isolated from a crude cell supernatant by affinity HPMDC

Ligand[a]		t-PA amount (μg)	Yield (%)
	Plasminogen	14	78
←	KCPGRVVGGC	17	94
	KCPGRV	16	89
	RVVGGC	16	89
→	β-AKCPGRVVGGC	18	100
	β-ARVVGGC	16	89

Conditions: adsorption buffer PBS, pH 7.0, desorption buffer 0.01 N HCl; intermediate washing buffer 2 M NaCl; flow rate 2 ml/min; loading: 3 ml of supernatant; total amount of t-PA in 1 ml of supernatant was 6 μg; desorbed fractions of t-PA were collected and analyzed by Lowry test.

[a] (→) Directly synthesized peptide; (←) immobilized peptide.

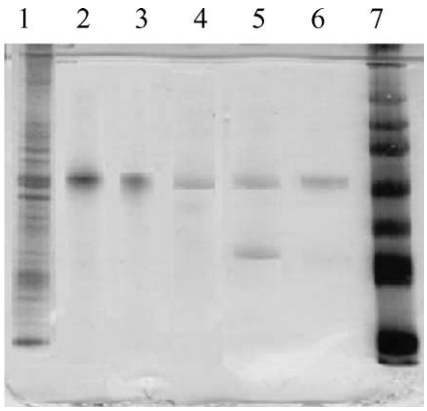

Fig. 1. 12.5% SDS-PAGE quality control of t-PA isolated from CHO-cell supernatant. Lane 1: crude CHO-cell supernatant; lanes 2–4: t-PA isolated with directly synthesized KCPGRVVGGC peptide, immobilized KCPGRVVGGC peptide and plasminogen, respectively; lane 5: t-PA standard; lane 6: t-PA isolated with immobilized KCPGRV ligand; lane 7: standard protein markers.

these enzymes. The use of GPRP and linear/branched lysine derivatives as affinity ligands demonstrated non-selective PAs' adsorption. It was exhibited in much higher amounts of adsorbed proteins than real SK/pUK content in loaded mixtures. The presence of foreign proteins in the eluat was also confirmed by SDS-PAGE. The total amounts of SK isolated from a protein mixture using peptides mimicking plasminogen's PA binding site were found to be from 34 to 68% of loaded SK that is higher than in the case of plasminogen disk (24%). Fig. 2 illustrates a purity of SK isolated using corresponding ligands. Only SK was found in the eluates from plasminogen-, KCPGRVVGGC - and KCPGRV-disks, whereas RVVGGC-disk isolated also minor amounts of trypsin inhibitor. The analogous situation was observed for pUK separations, but total amounts of isolated enzyme were approximately two times higher, e.g. 72–84%. This fact is in a good agreement with Q_{max} data. Q_{max} for pUK isolation was also two–three times higher than for SK. Presented on Fig. 2 gel testifies the separation process. As well as for SK, only three ligands, e.g. plasminogen, cyclic decapeptide and KCPGRV, were found to be suitable for pUK isolation. Both kinds of sorbents revealed an equal results.

It is worth noticing that the use of GMA–EDMA–plasminogen and GMA–EDMA–peptidylated sorbents over 1 year (about 50 cycles) and their storage at 4 °C was not accompanied by any significant loss of ligands and, accordingly, in a decrease in adsorptive capacity. Control experiments with

Table 4
Amounts of SK/pUK isolated from a model protein mixture by affinity HPMDC

Ligand[a]		SK		pUK			
		Mixture 1		Mixture 1		Mixture 2	
		Total protein amount (μg)	Yield[b] (%)	Total protein amount (μg)	Yield[b](%)	Total protein amount (μg)	Yield[b] (%)
	Plasminogen	12	24	36	72	8	80
	KCPGRVVGGC	22	44	39	78	10	100
←	KCPGRV	17	34	37	74	–	–
	RVVGGC	39	68	42	84	–	–
	GPRP	45	90	56	112	–	–
	KKKKGPRP	52	104	61	122	15	150
	KKKK	54	108	69	138	–	–
	Dendrimer $K_{15}A$	58	116	74	148	–	–
→	β-AKCPGRVVGGC	25	50	38	76	10	100
	β-ARVVGGC	49	98	51	102	–	–
	β-AKKKKGPRP	55	110	65	130	16	160

Conditions: adsorption buffer PBS, pH 7.0, desorption buffer 0.01 N HCl; intermediate washing buffer 2 M NaCl; flow rate 2 ml/min; loading: 1 ml of protein mixture; mixture 1 contained 50 μg/ml SK/pUK and total protein concentration was 400 μg/ml; mixture 2 contained 10 μg/ml pUK and total protein concentration was 45 μg/ml; desorbed fractions of SK/pUK were collected and analyzed by Lowry test.

[a] (→) Directly synthesized peptide; (←) immobilized peptide.

[b] Yields of isolated protein product(s) were calculated concerning of loaded PA amounts.

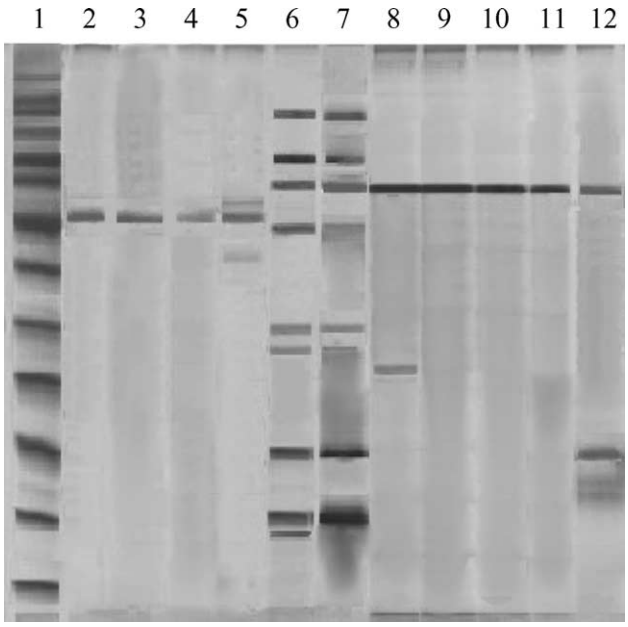

Fig. 2. Fifteen percent SDS-PAGE quality control of SK and pUK isolated from model protein mixture. Lane 1: standard protein markers; lanes 2–4: SK isolated with directly synthesized KCPGRVVGGC peptide, immobilized KCPGRV peptide and plasminogen, respectively; lane 5: SK standard; lane 6: model protein mixture consisting of phosphorylase b, BSA, pUK, ovalbumin, carbonic anhydrase, trypsin inhibitor, lyzozyme and ribonuclease A; lane 7: pUK isolated with immobilized KKKKGPRP ligand; lane 8: pUK standard; lane 9–12: pUK isolated with disks containing directly synthesized KCPGRVVGGC, immobilized KCPGRVVGGC, plasminogen and immobilized RVVGGC ligands, respectively.

t-PA separations a year later showed that the amounts of t-PA isolated by GMA–EDMA–peptide monoliths was the same, but, these for GMA–EDMA–plasminogen disk was at 10% off the initial values.

4. Conclusions

The results of this work have clearly shown that ultra-short monolithic stationary phases (CIM® disks) with well controlled porous design providing enhanced mass transfer represent the appropriate solid phases for in vitro investigations of complex biological processes. Using the discussed technique and the approach of construction of model affinity pairs, it was possible to discern the difference of affinity properties of investigated plasminogen activators. Moreover, it was shown that the biospecific properties of immobilized after preliminary synthesis and directly synthesized on GMA–EDMA monoliths peptide ligands did not vary significantly. This result can be taken into account for the future development of related methodologies, for example, spot synthesis on similar materials followed by fast screening analysis. The quantitative data on affinity adsorption together with the evaluation of strength of affinity pairs allows making a balanced choice of stable and efficient peptide ligands to isolate valuable biological product. It was shown, that peptides mimicking plasminogens' PA binding site can be successfully used for affinity isolations of tested bioproducts. Thus, they definitely may be considered as an alternative to large and labile protein molecule of plasminogen.

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