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Solid Phase Peptide Synthesis on Epoxy-Bearing Methacrylate Monoliths

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Abstract: Monoliths based on a copolymer of glycidyl methacrylate (GMA) and ethylene dimethacrylate (EDMA) can be used directly as sorbents for affinity chromatography after solid phase peptide synthesis. The quality of the synthesized products, the amount of grown peptides on a support and the reproducibility of the process must be considered. A determination of the quantity of the introducing β-Ala (and, consequently, the total amount of synthesized peptide) was carried out. Three peptides complementary to recombinant tissue plasminogen activator (t-PA) have been synthesized using Fmoc-chemistry on GMA-EDMA disks. The peptidyl ligands were analysed by amino acid analysis, ES-MS and HPLC methods.

The affinity binding parameters were obtained from frontal elution data. The results were compared with those established for GMA-EDMA affinity sorbents formed by the immobilization of the same but separately synthesized and purified ligands. The immobilization on GMA-EDMA disks was realized using a one-step reaction between the amino groups of the synthetic ligand and the original epoxy groups of monolithic material. The affinity constants found for two kinds of sorbent did not vary significantly. Finally, the directly obtained affinity sorbents were tested for t-PA separation from a cellular supernatant.

Keywords: macroporous disks; solid phase peptide synthesis; ligand immobilization; affinity HPMDC; t-PA; binding parameters

INTRODUCTION

The Merrifield cross-linked polystyrene support [1] is widely used for peptide synthesis, but it has several definite disadvantages (for example, high hydrophobicity) limiting its application. It should be noted that until the 1980s, the supports represented mainly dispersed beads. Later, attempts to find new types and shapes of alternative solid phases were undertaken and so-called monoliths [2] appeared. The chemical base and geometric shape of such supports vary widely. They include planar surfaces of glass [3], cellulose [4,5] and cotton [6] in the shape of slides or sheets, polymeric (polyethylene and polypropylene [7,8], polystyrene [9,10], polyacrylate [11,12] and polyethylene terephthalate [13]) films, disks, membranes and polymeric pins [14].

In this case, the synthesized target peptides are not removed from the support and the functionalized carriers are used for further investigations based on affinity interactions [15]. Various

Abbreviations: HPMDAC, high performance monolithic disk affinity chromatography; SPPS, solid phase peptide synthesis; GMA-EDMA, glycidyl methacrylate-co-ethylene dimethacrylate; CIM®, Convective Interaction Media; t-PA, tissue plasminogen activator; HPLC, high performance liquid chromatography; ES-MS, electrospray mass-spectrometry; PBS, phosphate buffered saline; CHO, Chinese hamster ovary; K, lysine; G, glycine; R, arginine; P, proline; C, cysteine; V, valine; t_R, retention time.

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combinatorial approaches for chemosensor (and biosensor) surfaces have been summarized recently [16,17]. The application of polymer-based supports for the synthesis of peptide libraries allows very rapid testing of large numbers of compounds [18].

Solid phase bound peptidyl ligands are also widely used in affinity chromatography [19–22]. The standard procedure of preparation of peptidyl sorbents for affinity chromatography includes: (1) peptide synthesis; (2) purification and lyophilization of the product and (3) covalent immobilization of the ligand on a solid matrix. Obviously, a better way seems to be *in situ* preparation of such affinity sorbents using solid phase peptide synthesis (SPPS) followed directly by biospecific separation. In this case, a single support has to satisfy the demands for both procedures. Thus, the solid phase has to provide high permeability, its functional groups should be easily accessible to biological molecules and it should ensure high chemical and mechanical stability in organic media. Furthermore, sufficient hydrophilicity is required to avoid a denaturation of the separated biological product [23,24].

Over the past decade, so called ultra-short monolithic columns of optimized macroporous structure based on a copolymer of glycidyl methacrylate (GMA) and ethylene dimethacrylate (EDMA) have become available for the separation and purification of biological molecules [25–29]. The main advantage of these monolithic sorbents is their extremely high hydraulic permeability and the dominance of convection over diffusion mechanism of mass-exchange under dynamic conditions. These circumstances allow very high flow rates [30,31]. After chemical conversions of the original epoxy groups, GMA-EDMA monolithic disks are used as efficient stationary phases for high-speed separations of biological molecules in ion-exchange, reversed phase, hydrophobic interaction and affinity modes [31,32]. Moreover, the same sorbents are stable in organic solvents that allow their use for solid phase peptide synthesis. Recently, GMA-EDMA monoliths were used for both peptide synthesis and affinity chromatography [11,12,33].

Three peptides representing the parts of the t-PA binding site on the plasminogen molecule were chosen to be synthesized on a monolithic GMA-EDMA matrix followed by use of the obtained sorbents directly in affinity separations. They were KCPGRV, RVVGGC and KCPGRVVGGC (557–562, 561–566 and 557–566 of the plasminogen sequence, respectively). These peptides were also synthesized by conventional SPPS and then immobilized on GMA-EDMA disks; the high specificity of the chosen ligands to t-PA was confirmed by high performance monolithic disk affinity chromatography (HPMDAC) [34].

The quality of the peptides directly synthesized on monolithic disks was evaluated by amino acid analysis, RP-HPLC and ES-MS methods. The affinity properties of these monolithic sorbents were compared with those obtained by the same but separately synthesized and immobilized peptides. The frontal elution approach was used to build experimental adsorption isotherms and to calculate the quantitative affinity parameters of the analysed biocomplementary pairs.

EXPERIMENTAL

Materials and Chemicals

Macroporous monolithic disks with a mean pore size of 1.5 μm and a porosity of 0.63 ml/ml sorbent (CIM® Epoxy Disks, BIA Separations, d. o. o., Ljubljana, Slovenia) were used as a solid phase. The disks were installed into specially designed cartridges from the same manufacturer.

Ammonia solution, dimethylformamide (DMF), dichloromethane (DCM), phenol and methanol were from Vecton Ltd (Russia). Diisopropylcarbodiimide (DIC), trifluoromethanesulfonic acid (TFMSA), trifluoroacetic acid (TFA), thioanisole, ethanedithiol and hydroxybenzotriazole (HOBt) were purchased from Fluka Chemie AG (Switzerland). Diisopropylamine (DIPA), *N*, *N*-diisopropylethylamine (DIPEA), piperidine, acetic anhydride, *N*-methylimidazole (NMI), 4-dimethylaminopyridine (DMAP) were obtained from Merck-Schuchard (Germany).

Boc-protected amino acids for solid phase synthesis on Merrifield resin — benzhydrylamino polystyrene support beads (p-methylbenzhydrylamine resin HCl, 1% DVB, 200–400 mesh, Neosystem Laboratories, France) were from Fisher Biotech (LJ, USA). Fmoc-protected amino acids were from Sigma (Germany) and Bachem AG (Switzerland).

The buffers used for affinity chromatography were prepared by dissolving the analytical grade salts in doubly distilled water that had been additionally purified by filtration through a 0.45 μm Milex microfilter (Millipore, Austria).

t-PA standard and CHO-cell supernatant were kindly donated by Boehringer Ingelheim Pharma KG (Biberach, Germany) and Mr A. Tappe, Institute

of Technical Chemistry, University of Hannover (Germany). Standard protein kits for PAGE were purchased from Sigma (Germany). The chemicals for the gel and buffer preparations were from Fluka and Sigma.

Instruments

The peptidyl ligands obtained by SPPS on Merrifield resin were analysed and purified by RP HPLC. The chromatographic instrument consisted of two piston pumps, a UV detector and a software data processing station (Golden System, Beckman, USA). Vydac C_{18} columns (pore diameter 300 Å) 4.6 × 150 mm (particle size 5 μm) and 22 × 250 mm (particle size 10 μm) were used for analytical and preparative purposes, respectively.

For analytical control of the synthesized peptides, an AAA T339 M automated amino acid analyser (Microtechna, Prague, Czech Republic) was used. MS analysis was performed using an ES-MS instrument manufactured at the Institute of Analytical Instrumentation RAS, St Petersburg, Russia. IR spectra were obtained with a Specord M80 (Karl Zeiss, Jena, Germany).

High-performance monolithic disk affinity chromatography (HPMDAC) was carried out using a chromatographic system consisting of a peristaltic pump (2115 Multiperpex pump), a UV detector (2138 Uvicord S) and a recorder (2210 Recorder), all from LKB, Bromma, Sweden. The concentration of proteins was measured using a UV-VIS spectrophotometer SF 26 (LOMO, St Petersburg, Russia). SDS-PAGE characterization of isolated t-PA was carried out with the use of a Mini Protean II System (Bio-Rad, Hercules, CA, USA).

Methods

Samples investigated by IR spectroscopy consisted of 5 mg finely dispersed peptidyl polymer and 600 mg KBr. The spectra were detected from 200 to 4000 cm^{-1}.

The preparation of α-Fmoc-amino acid pentafluorophenyl esters was carried out as described elsewhere [35].

Functionalization of monolithic material. Before converting epoxy groups, the disks were washed for 30 min with ethanol, 30 min with 50% ethanol solution in water and 30 min with distilled water. To convert the native epoxy groups into amino groups, the disks were immersed in 25% ammonia solution for 5 h at 40 °C [12]. To obtain hydroxyl groups, the disks were held in 0.1 M H_2SO_4 for 6 h at 80 °C or overnight in 0.1 M $HClO_4$ at room temperature. The functionalized disks were washed with water, 50% ethanol and DMF. The attachment of the linker Fmoc-β-Ala was carried out in two ways: (1) a reaction of OH-groups with a solution containing 0.2 M Fmoc-β-Ala-OH, 0.4 M NMI and 0.25 DIC 2 × 45 min; and (2) a reaction of NH_2-groups with a solution of 0.2 M Fmoc-β-Ala-OBt in DMF 2 × 45 min. The Fmoc-β-Ala-OBt was prepared immediately before use. Non-reacted amino groups were quenched by acetylation with a 20% acetic anhydride/DMF with a catalytic portion of DMAP.

Loading with β-Ala linker was determined by measuring the optical density ($\lambda = 301$ nm, $\varepsilon = 8100$) of the fulvene-piperidine adduct which was formed after removal of the Fmoc-protecting groups.

General procedure of peptide synthesis on GMA-EDMA monoliths. A functionalized disk was installed inside a standard cartridge. The solutions were pushed through the support by a syringe. Because of the porous peculiarities of the monolith, no stirring or shaking was applied during the reactions. The washing steps were carried out at the flow conditions for installing the cartridge into the chromatographic system. In comparison with solid phase synthesis on Merrifield resins, CIM® monoliths do not swell markedly in organic solvents [2,12].

0.3 M solutions of Fmoc-amino acid pentafluorophenyl esters in DMF were injected into the unit. Side-chain protected groups were tert-butoxycarbonyl (Boc) for lysine and arginine and acetamidomethyl (Acm) for cysteine. Fmoc-deprotection was carried out using 20% piperidine. BOC-side-chain groups were cleaved with 20% TosOH/AcOH, whereby the Acm-groups were not removed. The general scheme of synthesis was as follows: (1) acylation 2 × 15 min; (2) Fmoc — deprotection 1 × 5 min, 1 × 20 min; (3) coupling 2 × 15 min. Finally, the disks were washed with ethanol and stored in 20% ethanol at 4 °C.

Synthesis of peptides for covalent immobilization on monoliths. All peptides used as markers of affinity binding to t-PA were obtained by conventional SPPS using Boc-strategy according to the protocol: (a) 10% TEA/DMF, 1 × 1 min; (b) 10% TFA/DMF, 1 × 5 min; (c) DMF, 3 × 1 min; (d) Boc-AA-OBt, 3 eqv 1 × 120 min, (e) DMF, 3 × 1 min; (f) DCM, 3 × 1 min; (g) ninhydrin test; (h) 30% TFA/DCM, 2 × 15 min; (i) DCM, 3 × 1 min; (j) DMF, 3 × 1 min. The peptide

cleavage and side-chain deprotection procedure was performed with TFMSA/TFA as described elsewhere [36].

Quality and quantity control. The peptides synthesized by Boc-strategy and cleaved from the solid phase were purified by RP-HPLC. Conditions: eluent A — 0.1% TFA in water, B — 50% acetonitrile with 0.1% TFA, the flow rate for the analytical separations was 1 ml/min and for the preparative ones was 10 ml/min. For KCPGRVVGGC and KCPGRV separations a linear gradient from 5% to 25% B in 15 min was applied (t_R 6.70 and 5.95 min, respectively). For RVVGGC separation a linear gradient from 0% to 15% B in 15 min was used (t_R 11.22 min). The degree of purity of the products isolated by HPLC was 90%–95%. According to the results of the amino acid analysis, the yield of purified decapeptide was 47%, KCPGRV 52% and RVVGGC 42%. The amino acid analysis was carried out after treatment of the sample with 6 M HCl at 110 °C for 24 h.

In the case of Fmoc peptide synthesis on monoliths, control of the coupling reaction was performed by measuring the UV absorbance of the released fulvene/piperidine adducts at 301 nm. Some of the products were examined by amino acid analysis. In this case, the samples were prepared by acidic hydrolysis of the removed and non-removed from the sorbent peptides. In the latter case, the hydrolysis was carried out using a mixture of concentrated acids $HCl:CH_3CH_2COOH$ (1 : 1) at 140 °C for 8 h.

The quality of the obtained peptides was also investigated by ES-MS. Lyophilized samples of the synthesized peptides were dissolved in 50% acetonitrile with 1% AcOH to reach a concentration of 5–10 pmol/μl (sample volume 10 μl).

The RP-HPLC analysis of the peptides synthesized on monoliths was performed at a flow rate 1 ml/min; 0.1% H_3PO_4/H_2O and 0.1% H_3PO_4/AcN were used as buffers A and B, respectively. Linear gradients from 5% to 30% B in 25 min for CGGVVRGPCK-β-A, and from 1% to 21% B in 20 min for VRGPCK-β-A were applied.

Immobilization procedure. To immobilize the previously synthesized and purified peptides a recently developed procedure was used [37]. The amount of covalently bound ligand was calculated from the change in absorbance at 229 nm of the peptide solution before and after the solid phase reaction. The standard Lowry test [38] was used to determine peptide concentrations.

Determination of affinity parameters by means of HPMDAC. The affinity mode of high performance monolithic disk chromatography (HPMDC) represents a combination of adsorption and desorption steps using a stepwise gradient of mobile phase composition. For this purpose, solutions of t-PA standard with concentrations ranging from 0.01 to 0.3 mg/ml were passed through the corresponding disk. Unbound t-PA was removed with PBS buffer (pH 7.0) as well as a probable non-specifically bound part of t-PA — with 2 M NaCl. Affinity bound t-PA was eluted with 0.01 M HCl, pH 2.0. The flow rate for both adsorption and desorption steps was 2 ml/min.

The affinity characteristics of the prepared affinity CIM® disks, such as the maximum adsorption capacity (q_{max}) and the dynamic dissociation constants of affinity complexes (K_{diss}), were evaluated on the basis of the mathematical treatment of experimental adsorption isotherms (using software Origin 6.0) resulting from frontal analysis [37]. The results represent the average values calculated from linearized forms of the Langmuir equation.

Isolation of t-PA from a crude CHO cell supernatant by affinity HPMDC. The supernatant containing 30 μg/ml of t-PA was used in these experiments. At the same time, the total protein amount in the supernatant determined by the Lowry test was averaged to 150 μg/ml. 2 ml of a CHO-cell supernatant in PBS buffer was loaded on the disks modified by peptide ligands and adsorption at dynamic conditions was carried out. After removal of the ballast proteins by washing the disk with 2 M NaCl, the adsorbed t-PA was eluted using 0.01 M HCl, pH 2.0. The flow rate was 2.5 ml/min.

SDS-PAGE. For SDS-PAGE, lyophilized samples of affinity isolated t-PA were dissolved in a solvent containing 20 mM Tris-HCl (pH 8.0), 2 mM EDTA, 5.0% SDS and 0.02% bromophenol blue and heated for 5 min at 100 °C. After centrifugation, the samples were applied, in parallel with protein standard markers, to a plate covered with 12.5% polyacrylamide gel. Staining of protein zones after PAGE-SDS was done using 0.5% Coomassie solution.

RESULTS AND DISCUSSION

Peptide Synthesis

The original epoxy groups of the GMA-EDMA monolithic sorbent allow the formation of two

different anchoring functional groups (Figure 1). The epoxy groups can be converted easily to hydroxy functions by acidic hydrolysis, or to amino groups by reaction with ammonia or ethylene diamine. Thus, the attachment of a peptide can be performed by formation of ester or amide bond. In the first case, similar to conventional SPPS on polystyrene resin, the synthesized peptide can be removed from the matrix, whereas in the second, the attachment of a peptide is irreversible.

Theoretically, the total capacity of the GMA-EDMA monolithic disks is defined by the amount of epoxy groups that is about 3–4.5 mmol/ml sorbent (or 1.0–1.5 mmol/disk). The disappearance of specific IR absorption bands corresponding to epoxy rings and the appearance of those corresponding to hydroxy and amino groups confirmed a conversion process. The yield of these reactions depended significantly on the applied conditions. The best result of acidic hydrolysis (80%) was achieved when the disks were incubated in 0.1 M H_2SO_4 at 80 °C for 6 h, whereas the reaction running overnight at room temperature led to only 60% conversion. According to the elemental analysis data, the reaction of the epoxy groups with 25% ammonia aqueous solution (overnight, RT) led to 20% conversion, whereas incubating the same solution at 40 °C for 5 h gave a 45% yield.

Since the original concentration of the functional groups of GMA-EDMA matrix seems to be too high for total conversion and following use in affinity chromatography, it was reasonable to introduce some anchoring amino acid to allow the capacity of synthesized ligands to be controlled. β-Ala was used as a linker.

β-Ala was introduced in two ways: (1) carbodiimide method with using NMI as a catalyst; and (2) method of activated esters. The concentration of activated amino acid was varied from 0.1 to 0.4 M. Quantity control of β-Ala bound to the sorbent was carried out using amino acid analysis as well as by measuring absorbance of the fulvene-piperidine adduct (Table 1). The data obtained by both analytical approaches appeared to be in very good agreement. It was obvious that the value of the β-Ala concentration (3.5–7.8 μmol/ml range) satisfied the demands of the ligand's density recommended for affinity interactions (not more than 20 μmol/ml sorbent [2,18]). In the case of hydroxy-functionalized sorbent, the use of the carbodiimide method (Table 1) led to an increase in β-Ala content. In contrast, the β-Ala content of amino-functionalized monoliths was higher if the method of activated esters was used. Furthermore, the total amount of β-Ala was not directly proportional to the initial concentration of activating reagent.

The time required for the loading procedure can be reduced by operating at a higher temperature or by increasing the β-Ala concentration in the acylating mixture (Table 2). A rise of temperature from 20 °C to 40 °C (at a constant concentration of the initial acylating mixture) led to a 1.5–2.5 times higher β-Ala amount. A two-fold increase of the concentration of the acylating mixture also allowed a reduction of the reaction time. Repetition of the acylation step increased the β-Ala content only to some extent.

The procedure of SPPS on macroporous GMA-EDMA monolith includes: (a) coupling with N-α Fmoc-protected pentafluorophenyl ester of amino acids; (b) acetylation of excess of hydroxy or amino

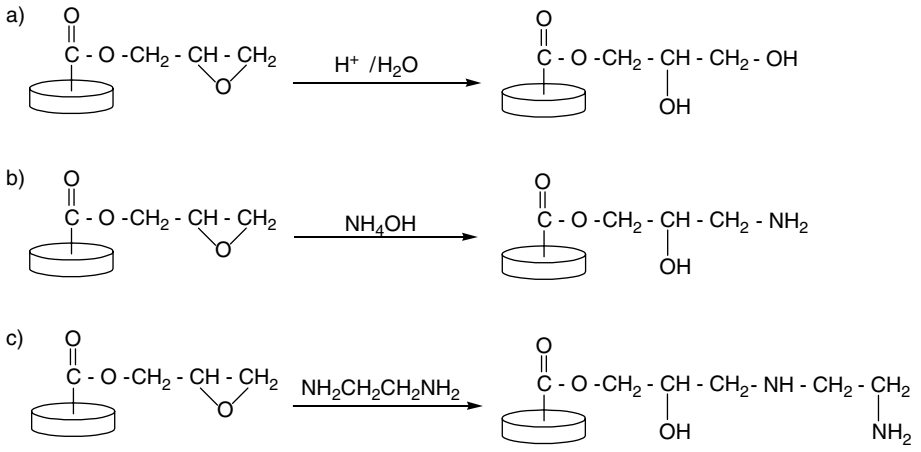

Figure 1 Three methods for functionalization of the CIM®-monoliths.

Table 1 Introduction of β-Ala

Concentration of activated amino acid, *M*	Carbodiimide method		Method of activated esters	
	1	2	1	2
	μmol/ml of sorbent		μmol/ml of sorbent	
OH—functionalized sorbent				
0.1	4.44	4.62	3.52	3.70
0.2	6.06	6.41	4.65	4.85
0.4	7.79	7.70	5.67	5.88
NH_2—functionalized sorbent				
0.2	3.88	3.94	6.35	6.44

Conditions: acylation 2×45 min, volume of acylating mixture 0.4 ml, RT, analytical methods: 1, amino acid analysis; 2, measuring of absorbance of fulvene–piperidine adduct forming during Fmoc-deprotection.

Table 2 The Dependence of the Amount of β-Ala Introduced into the Sorbent on Reaction Time and Temperature for OH — Disks

Concentration of activated amino acid (*M*)	Reaction time (min)	Concentration of β-Ala, μmol/ml of sorbent			
		One acylation		Two acylation	
		20 °C	40 °C	20 °C	40 °C
0.2	180	5.9	14.4	8.4	19.5
0.2	90	3.9	8.4	6.2	11.3
0.2	45	2.4	4.0	4.2	7.3
0.4	45	4.1	8.4	6.0	13.1

groups; (c) deprotection of Fmoc-groups by 20% piperidine in DMF; (d) side-chain deprotection.

In order to develop the procedure of synthesis, five model peptides consisting of 4–10 amino acids were synthesized on NH_2-functionalized GMA-EDMA disks and investigated with regard to the ratio of the attached amino acids. The results showed a decrease of coupling yields after the 7th –8th amino acid. The best coupling yields were achieved by double repetition of a reaction using 0.3 M solutions of pentafluorophenyl amino acid esters.

Three chosen peptides complementary to human tissue plasminogen activator (t-PA), e.g. KCPGRV, KCPGRVVGGC and RVVGGC, represent the parts of the t-PA binding site on the plasminogen molecule and correspond to its 557–562, 557–566 and 561–566 sequence positions. To evaluate the quality of the affinity sorbent prepared by direct stepwise synthesis on monolithic matrix, it seemed necessary to compare them with that modified by the same but separately synthesized and purified peptide. However, while the direct peptide synthesis on the monolith provided attachment to the matrix via the *C*-terminus, the method of immobilization of previously synthesized ligands takes place via the *N*-terminus. Therefore, synthesis by both methods but unbound to the surface peptides represent the retro forms.

Each of the three chosen peptides was synthesized in parallel four times: twice on OH-disks and twice on NH_2-supports. One disk of the series was used for analytical procedures and the other was intended for affinity testing. According to the data of amino acid analysis, the formation of the Val-Val bond represented a problem. Indeed, when the decapeptide was synthesized using a standard SPPS procedure, the analysis indicated only 1.3 Val residues (in comparison to a theoretical value 2) per peptide. To avoid this problem, triple (not double) couplings were used and after 7th amino acid, the reaction time was increased up to 20 min.

Figure 3 illustrates the RP-HPLC profiles of VRGPCK-β-A (**a**) and CGGVVRGPCK-β-A (**b**) peptides synthesized directly on GMA-EDMA disks, released from the monolith and desalted on Sephadex G-10. In both cases, one major peak of the product and a few minor peaks of byproducts were eluted from the column. The yields of target peptides were 83% and 86%, respectively, being sufficient for our further purposes (affinity chromatography).

According to MS data, all peptides synthesized on GMA-EDMA disks had molecular masses corresponding to the theoretical values. The theoretical molecular masses of peptides obtained on GMA-EDMA disks were: 799 for VRGPCK-β-A, 730 for CGGVVR-β-A and 1186 for CGGVVRGPCK-β-A (Acm-protection of Cys was not removed). The spectrum of VRGPCK-β-A (Figure 2) has two main peaks which belong to the one- and two-charged forms of the target peptide. The third one corresponding to M = 728 can be connected with the loss of β-Ala residue. A shorter sequence can be built up because of non-complete blocking of free groups (OH, NH_2) after β-Ala attachment. The spectrum of CGGVVRGPCK-β-A (Figure 2) indicates minor amounts of peptides with molecular masses equal to 1044, 1056 and 1115. The presence of a compound with M = 1115 is explained again by loading β-Ala,

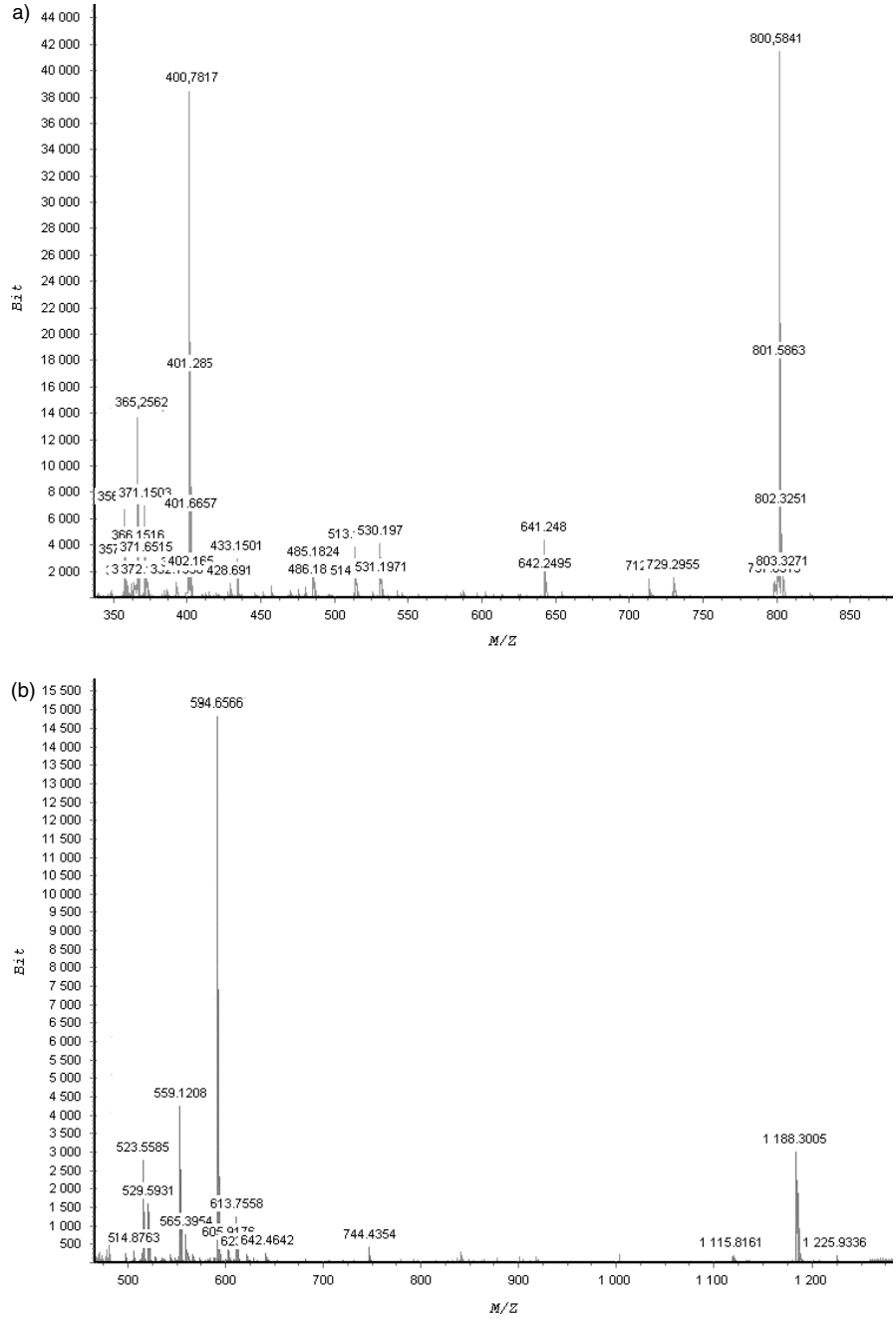

Figure 2 Electro-spray mass–spectra of peptides synthesized on flowing monoliths: (a) VRGPCK-β-A, (b) CGGVVRGPC-K-β-A.

and M = 1056 corresponds to the absence of one Val residue and the presence of an acetyl group bound to the previous amino acid (1186 − 99 + 43 = 1056). The 1044 value is a result of a missing Lys residue.

With regard to the amino acid analysis (Table 3), SPPS on Merrifield resin and GMA-EDMA monolithic material demonstrated comparable results.

Peptide Immobilization on Monolithic Disks

Due to the high chemical reactivity of epoxy groups on GMA-EDMA macroporous polymers, the immobilization procedure was carried out as a one-step process at static conditions [38]. No intermediate spacers were inserted in the cases discussed.

a)

Peak number	Retention time	Area percent	High percent
1	3.20	1.07	1.18
2	3.55	0.20	0.25
3	4.20	0.54	0.61
4	4.52	2.38	3.06
5	4.93	0.10	0.14
6	5.68	0.39	0.42
7	6.03	0.13	0.25
8	6.13	0.23	0.32
9	6.71	83.04	81.28
10	7.15	0.16	0.31
11	7.71	2.68	3.39
12	8.44	8.25	7.47
13	8.81	0.01	0.33
14	9.32	0.12	0.20
15	10.74	0.22	0.28
16	11.24	0.27	0.34
17	11.37	0.01	0.01
18	12.46	0.10	0.15

b)

Peak number	Retention time	Area percent	High percent
1	5.33	0.96	1.00
2	5.82	2.27	1.76
3	6.75	86.16	85.01
4	7.12	0.53	1.30
5	7.45	3.64	3.93
6	8.04	0.63	0.78
7	8.30	4.54	4.72
8	9.50	0.52	0.52
9	9.84	0.43	0.53
10	11.54	0.31	0.45

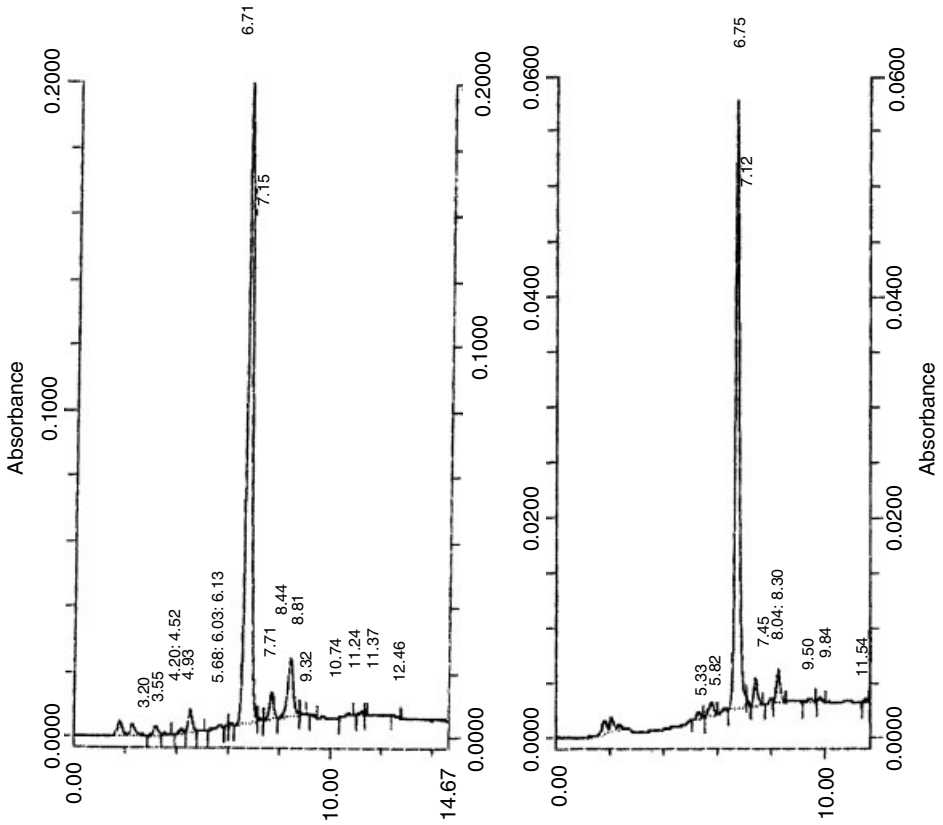

Figure 3 HPLC profiles of peptides of peptides synthesized on flowing monolithic disks: (a) VRGPCK-β-A; (b) CGGVVRGPCK-β-A. Conditions: flow rate 1 ml/min; eluent A 0.1% H_3PO_4/H_2O, B 0.1% H_3PO_4/AcN; a linear gradient (a) from 5% to 25% B; (b) from 1% to 21% B.

The results of functionalization of monolithic GMA-EDMA supports by the ligands of choice are presented in Table 4. The data demonstrate a similar molar surface concentration of all peptides, whereas the molar amount of immobilized plasminogen was much less.

Affinity Chromatography

The biospecific properties of monolithic peptidyl sorbents obtained by two procedures (direct SPPS and the immobilization of preliminary synthesized and purified ligands) were compared using affinity

Table 3 Comparative Amino Acid Analysis of Peptide obtained on the GMA-EDMA Monoliths and on the Conventional Polystyrene Resin

	Peptide[a]	Amino acid					
		β-Ala	Lys	Pro	Arg	Val	Gly
	β-AKCPGRV	1.00	0.95	1.00	0.93	0.82	0.85
→	β-AKCPGRVVGGC	1.00	1.00	0.92	0.80	1.65	2.70
	β-ARVVGGC	1.00	—	—	0.90	1.75	1.80
	KCPGRV	—	1.20	0.98	0.93	0.97	1.00
←	KCPGRVVGGC	—	1.00	1.15	0.93	1.90	3.20
	RVVGGC	—	—	—	0.98	1.88	2.00

[a] → Directly synthesized peptide.
← Immobilized peptide.

Table 4 Affinity Parameters of the t-PA Interactions with Immobilized and Directly Synthesized on GMA-EDMA Disk Peptides

←	Ligand[a]	Molecular mass	Q_{ligand} (μmol × 10^2 / ml of sorbent)	K_{diss} (μM)	Q_{adsorb} (μmol × 10^2 / ml of sorbent)
	Plasminogen	90 000	5.5	0.9 ± 0.2	0.6
	KCPGRV	728	260.0	1.5 ± 0.5	1.5
	KCPGRVVGGC	1 115	270.0	2.1 ± 0.2	1.8
→	RVVGGC	659	270.0	3.2 ± 0.3	2.5
	β-AKCPGRV	799	290.0	1.2 ± 0.4	1.6
	β-AKCPGRVVGGC	1 186	310.0	3.3 ± 0.7	1.8
	β-ARVVGGC	730	320.0	3.6 ± 0.6	3.4

[a] → Directly synthesized peptide.
← Immobilized peptide.

chromatography. The quantitative parameters of affinity binding were evaluated using the frontal elution approach [39]. In this case, the Langmuir-type adsorption isotherms were built and their linearized forms were used for a calculation of the constants of dissociation of affinity complexes, K_{diss}, as well as the maximum adsorption capacity of the affinity sorbent, q_{max}. Experiments were carried out to exclude any non-specific interactions.

It was established that both types of peptidyl support exhibited similar constants of affinity complex dissociation which were very close to that obtained for the t-PA natural substrate — plasminogen (Table 4). It is worth noticing that in spite of the similar affinity between the natural and synthetic ligands, the adsorption capacity is 3–4 times higher in the latter case.

The important difference between the stepwise built-up peptidyl disks and the attached peptides on disks is that the direct growth of a ligand on polymeric support ensures single-point binding with a surface, whereas the covalent immobilization may involve more than one group of a peptide. This branching is increased with increasing peptide length and the content of lysine residues.

However, the differences between the investigated affinity pairs were not significant. The most interesting result is the independence of affinity specificity on the mode of the peptidyl ligand attachment (via *C*- or *N*-terminus) to the surface.

All the investigated peptidyl ligands demonstrated a high affinity to t-PA (Table 4). The same sorbents were found to be inert to ovalbumin, bovine serum albumin, lysozyme, myoglobin and carbonic

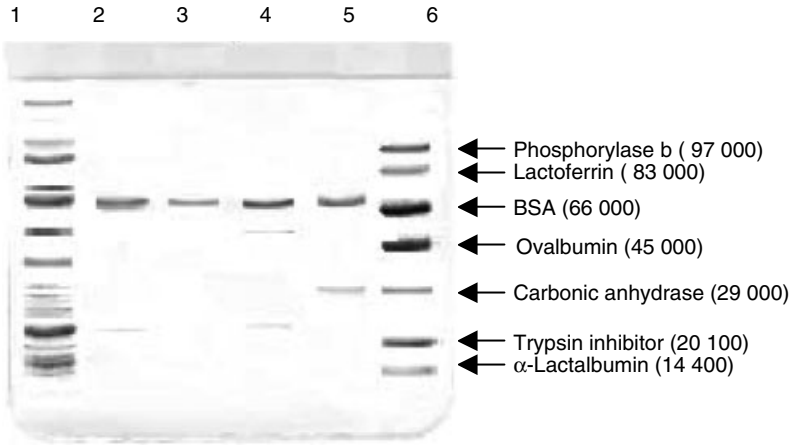

Figure 4 12.5% SDS-PAGE quality control of t-PA isolated from CHO-cell supernatant. Lane: 1, crude CHO-cell supernatant; 2, 4, t-PA isolated with GMA-EDMA-β-ARVVGGC and GMA-EDMA-β-AKCPGRVVGGC disks (directly stepwise synthesized ligands); 3, t-PA isolated with GMA-EDMA-KCPGRVVGGC (preliminary synthesized, purified and immobilized ligand); 5, t-PA standard; 6, protein markers.

anhydrase. The non-specific adsorption of foreign proteins from the model mixture did not exceed 5%. The material used in the monoliths containing epoxy, hydroxy and amino groups did not display any interactions with t-PA or other proteins under the same conditions. To prove the affinity of the chosen peptide ligands, two random sequences (KCLFVP and CGNLSTQY) were applied for t-PA isolation. No specific adsorption was observed in these cases.

The obtained affinity sorbents were used for t-PA separation from the crude CHO-supernatant. The ligands grown on the surface provoked considerable non-specific interactions which, however, were easily eliminated by washing with 2 M NaCl. This fact can be probably related to the minor failure sequences obtained. In contrast, in the case of preliminary synthesized, purified and immobilized ligands no non-specific adsorption was observed.

The quality control of the eluate was carried out by SDS PAGE (Figure 4) confirming the specificity to t-PA of both types of investigated sorbents. As expected, only t-PA was isolated using the disk with immobilized substrate — plasminogen. In general, preliminarily synthesized, purified and immobilized peptide ligands did not display any adsorption of foreign proteins. The exception was an insignificant amount of a protein (20 kDA) that was found only in the eluate obtained by using the RVVGGC ligand. The analogous situation was observed in the case of the same but directly synthesized peptide. The directly synthesized decapeptide revealed some minor non-specific adsorption of the two foreign proteins of molecular mass about 50 and 20 kDA. The total amounts of t-PA isolated from the supernatant using peptidylated sorbents were found to be about 45–55 μg corresponding to 80%–95% of loaded t-PA and coinciding with the t-PA isolated with the plasminogen disk (85%).

CONCLUSIONS

The data obtained in the presented research allow the follow conclusions:

- Macroporous monolithic sorbents (CIM® Disks) can be successfully used for peptide synthesis and affinity chromatography because of their excellent mass transfer properties, high content of reactive epoxy groups and high compatibility with organic media.
- The support functionalization procedures were investigated; it was shown that the affinity adsorption capacity defined by the concentration of affinity ligands can be carefully controlled.
- Several peptidyl GMA-EDMA monolithic disks were obtained and their quality was tested.
- The peptide ligands formed highly specific complexes with tissue plasminogen activator (t-PA); the affinity to t-PA of directly grown ligands and those immobilized after preliminary synthesis did not vary significantly.
- The affinity of t-PA to peptide ligands was similar to that of its natural substrate plasminogen,

however, the amount of t-PA separated by HPMDAC was three–four times higher in the case of peptidyl sorbents. Therefore, peptidyl solid phases seem to be very attractive for scaling up the isolation and down stream production of recombinant proteins.

- The affinity and specificity did not depend on the mode of surface attachment of peptide ligands (via *C*-terminal or *N*-terminal residue).
- The preparation of affinity matrices using direct SPPS is time-consuming, requires lower amounts of chemicals that, respectively, make the whole procedure much cheaper.

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